Universidade do Minho
Escola de Engenharia

João Manuel Teixeira de Oliveira

New 3D Scaffolds and Adequate Mesenchymal Stem Cells Culture Methodologies for Engineering an Articular Cartilage Transplant

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Tese de Doutoramento em Engenharia Biomédica

Trabalho efectuado sob a orientação do
Professor Rui Luís Gonçalves dos Reis
e co-orientação do
Professor Paul Vincent Hatton

Janeiro de 2009

TO MY PARENTS

“**Learning never exhausts the mind**”

Leonardo da Vinci
(1452-1519)

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New 3D Scaffolds and Adequate Mesenchymal Stem Cells Culture Methodologies for Engineering an Articular Cartilage Transplant

ABSTRACT

Cartilage is a type of dense connective tissue that possesses interesting features in terms of organization and functionality. It has only one type of cells – the chondrocytes – which are embedded in an extensive network constituted mostly of collagens and proteoglycans. It is also avascular, exhibiting a low metabolic rate and a subsequent low regenerative potential. Articular cartilage is a particular type of hyaline cartilage that has an important structural function in the skeletal system as a weight bearing tissue, creating smooth gliding areas that can absorb both shocks and loads in an efficient way. Due to its nature, articular cartilage function may be severely affected by trauma events, aging related degeneration such as osteoarthritis, or developmental disorders, since no efficient regeneration of the damaged tissue is performed. A direct result of this is chronic pain and disability conditions that seriously limit normal everyday life. Different possible solutions have been experimented to solve these conditions, such as prosthetic joint replacement, arthroplasty, and drilling, but the outcomes are still not satisfactory which imposed the need for alternative approaches. Tissue engineering has been proposed as a new method to address these problems, being widely studied as a promising therapeutic tool. The tissue engineering strategy usually implies the use of a 3D structure that is able to support cells growth and differentiation in an adequate environment towards the development of a functional tissue engineered system. The support systems fabricated for these purposes can be of natural or synthetic origin, or either a combination of both. Different materials have been produced and processed in various ways with the aim of conferring specific properties that are expected to render the best performances.

The main goal of the work described in this thesis was to develop a system that can be easily, efficiently, and successfully applied in the treatment of articular cartilage lesions. Due to its potential, the concept of tissue engineering was applied by using natural origin based structures combined with different cell types (cell lines, primary culture differentiated and undifferentiated cells) in adequate culturing environments. Among the several support structures studied, the emphasis of the work was put on the development and application of a minimally invasive

injectable hydrogel system which testing started from its ability to be used as a cell supporting material to *in vivo* functional studies in an articular cartilage rabbit knee defect model.

In the initial part of the work presented in this thesis, the first tested structures consisted of scaffolds of chitosan and polybutylene succinate processed by compression moulding with salt leaching that were used to support the growth and chondrogenic differentiation of BMC9 cells – a mouse bone marrow derived mesenchymal progenitor cell line. The cells were able to proliferate and colonize the scaffolds structure, remaining viable during the time of the experiments. Immunological analyses further indicated that the BMC9 cells were being differentiated towards the chondrogenic pathway.

A different structure of the scaffolds was experimented using the same blend of chitosan and polybutylene succinate by processing it in the form of fibres and producing a 3D fibre scaffold that was at this time used to culture primary culture chondrocytes from bovine origin. The chondrocytes exhibited a normal and typical morphology, colonising both the surface and inner pores of the scaffolds. Cartilage-like extracellular matrix formation was observed by the deposition of collagen type II and proteoglycans, indicating that chitosan and polybutylene succinate fibre based scaffolds had good potential as matrices for the regeneration of cartilage.

A different natural fibre based scaffold consisting of a blend of starch and polycaprolactone was also investigated regarding its ability to support growth and extracellular matrix formation by bovine articular chondrocytes seeded *in vitro*. The chondrocytes presented normal morphological features with extensive cells presence at the surface of the support structures, and penetrating the scaffolds pores. Qualitative and quantitative analyses showed that typical cartilage extracellular matrix components were being deposited during the course of the experiments thereby showing the potential of these systems for future applications in the field.

Another type of support, and the more extensively studied within the work described in this thesis, was a natural origin hydrogel – gellan gum - that was firstly tested to be used as a cell encapsulating agent. Gellan gum was shown to be versatile in terms of applications with the possibility of being used as a cell/drug delivery vehicle in different situations. In terms of cartilage regenerative approaches, its material properties and non cytotoxic nature were shown suitable for the proposed application. A final stage of this part involved the encapsulation and *in vitro* culturing

of human nasal chondrocytes in gellan gum hydrogels which remained viable showing the potential of these new systems as cell supports for cartilage regeneration.

In the following work, gellan gum hydrogels were tested for their ability to be used as injectable systems for delivering and maintaining human chondrocytes by *in situ* gelation, as well as for supporting cell viability and production of extracellular matrix. The characterization of some of the materials properties showed their injectability under physiological conditions and with the encapsulation and culturing of human articular chondrocytes it was possible to observe that cells were viable and actively depositing cartilage-like extracellular matrix. In a final stage, the *in vivo* performance of the gellan gum hydrogels, in terms of induced inflammatory reaction and integration into the host tissue, was performed upon subcutaneous implantation in mice. The results showed a residual response from the organisms and maintenance of the mechanical stability of the gels throughout the implantation periods.

These results prompted the study of the gellan gum with human chondrocytes systems *in vivo* to test for cartilage-like tissue formation. Gellan gum hydrogels were combined with human articular chondrocytes and were subcutaneously implanted in the back of nude mice. The results showed a homogeneous cell distribution and the typical round shape morphology of the chondrocytes within the matrix upon implantation. Proteoglycans synthesis was detected in the histological sections and a statistically significant increase of proteoglycans content in gellan gum-human articular chondrocytes tissue engineered constructs was measured with the GAG assay from 1 to 4 weeks of implantation. Real-time PCR analyses showed a statistically significant upregulation of collagen type II and aggrecan levels in the same periods, while the immunological assays suggested deposition of collagen type II along with some collagen type I. The overall data reinforced the previously observed potential of gellan gum hydrogels in the generation of a fully functional cartilage tissue engineered construct.

Finally, the last study performed under the scope of this thesis looked into the therapeutic effect of gellan gum hydrogels when combined with adipose tissue derived progenitor cells and injected in rabbit full-thickness articular cartilage defects in an autologous approach. Adipose tissue derived progenitor cells (chondrogenic pre-differentiated and non pre-differentiated) where compared with articular chondrocytes, gellan gum alone, and empty defects. The cell loaded hydrogels showed the best macroscopic appearance and integration with the native tissue. Histological scoring and staining, along with real-time semiquantitative PCR analyses, provided

results that taken together showed that gellan gum hydrogels in combination with adipose tissue derived progenitor cells constitute a promising approach for the treatment of articular cartilage defects, being a possible candidate for future clinical applications in this field.

As a concluding remark, it can be stated that the work performed in this thesis tested several supports for application in the cartilage tissue engineering field, with the main emphasis being put in gellan gum hydrogels. Gellan gum was originally suggested and tested as a new support to aid in cartilage tissue regeneration. Gellan gum was shown to be a promising biomaterial for these purposes as evidenced by its materials properties, *in vitro*, and *in vivo* results. Such evidences suggest that the herein described gellan gum systems combined with different cells types, namely adipose tissue derived progenitor cells, may have potential clinical application in the treatment of cartilage defects.

Novos Suportes Poliméricos 3D e Metodologias Adequadas de Cultura de Células Estaminais Mesenquimais para a Engenharia de Transplantes de Cartilagem Articular

RESUMO

A cartilagem é um tipo de tecido conectivo denso que possui características interessantes em termos da sua organização e funcionalidade. Tem apenas um tipo de células - os condrócitos - que estão incluídos numa matriz extracelular constituída maioritariamente de colagéneo e proteoglicanos. É igualmente avascular, exibindo uma baixa taxa metabólica e um baixo potencial regenerativo. A cartilagem articular é um tipo particular de cartilagem hialina que tem uma função estrutural importante no sistema esquelético já que pode ser vista como um sistema de suporte de cargas capaz de criar áreas de deslizamento suaves que podem absorver choques e cargas de uma forma eficaz. Devido a sua natureza, a função articular da cartilagem pode ser severamente afectada por eventos traumáticos, desgaste progressivo do tecido e mecanismos degenerativos tal como a osteoartrite, dado que uma regeneração eficiente do tecido danificado não acontece na maior parte dos casos. Uma consequência directa destes acontecimentos são condições crónicas de dor e incapacidade que limitam seriamente a vida quotidiana normal. Foram testadas diferentes soluções para resolver estes problemas, tais como a utilização de próteses, artroplastia, entre outros procedimentos cirúrgicos, mas a falta de resultados satisfatórios impõe a necessidade de aproximações alternativas. A engenharia de tecidos é um método inovador proposto para tratar estas situações, estando a ser extensamente estudado como uma ferramenta terapêutica promissora. A estratégia de engenharia de tecidos implica geralmente o uso de uma estrutura 3D que possa suportar o crescimento e diferenciação de células num ambiente adequado para o desenvolvimento de um tecido funcional. As estruturas de suporte fabricadas para este fim podem ser de origem natural ou sintética, ou alternativamente uma combinação de ambos. Têm sido produzidos e processados materiais com diferentes características tendo em vista a obtenção de propriedades específicas que permitam obter melhores desempenhos.

O objectivo principal do trabalho descrito nesta tese foi o desenvolvimento de um sistema que pudesse para ser aplicado facilmente, eficientemente, e com sucesso no tratamento de lesões articulares da cartilagem. Tendo em conta o seu potencial, o conceito de engenharia de tecidos

foi aplicado usando estruturas de origem natural combinadas com tipos diferentes de células (linhas celulares, culturas primárias de células diferenciadas e não diferenciadas) em ambientes de cultura adequados. Entre as diversas estruturas de suporte estudadas, o enfoque do trabalho recaiu fundamentalmente sobre o desenvolvimento e a aplicação de um sistema injectável de invasão reduzida. Os testes com o material, a goma gelana, e o sistema em si, foram iniciados com a avaliação das capacidades deste para ser utilizado nestas aplicações e desenrolaram-se até à realização de estudos funcionais *in vivo* num modelo animal (coelho) onde foram criados defeitos na cartilagem articular e avaliado o potencial regenerativo dos sistemas. articular do defeito do joelho do coelho da cartilagem.

Na parte inicial do trabalho apresentado nesta tese, os primeiros estudos foram realizados com estruturas de suporte 3D à base de polibutileno sucinato e quitosano processado através de moldação por compressão com lixiviação de sal. Estes suportes foram usados para suportar o crescimento e a diferenciação condrogénica de células BMC9 – uma linha celular mesenquimal progenitora obtida a partir de medula de rato. As células proliferaram e colonizaram as estruturas de suporte, permanecendo viáveis durante a o tempo das experiências. Análises morfológicas e imunológicas indicaram que as células BMC9 estavam a seguir a via de diferenciação condrogénica.

Uma outra estrutura de suporte foi criada usando a mesma mistura à base de polibutileno sucinato e quitosano mas processada desta vez sob a forma de fibras por microextrusão e formando o suporte 3D através de pontos de ligação entre as fibras. A estrutura foi utilizada como suporte ao cultivo de condrócitos articulares bovinos de cultura primária. Os condrócitos exibiram uma morfologia normal e típica, colonisando os poros de superfície e internos das estruturas de suporte. A formação de matriz extracellular cartilagínea foi observada pela deposição de colagénio tipo II e proteoglicanos, indicando que estes suportes à base de polibutileno sucinato e quitosano têm potencial para a regeneração de cartilagem.

Um suporte natural diferente, também à base de fibras, constituiu o objecto de estudo seguinte. Este foi fabricado a partir da mistura de amido de milho e policaprolactona e foi investigado igualmente acerca da sua capacidade para suportar o crescimento e a formação de matriz extracellular cartilagínea por condrócitos articulares bovinos de cultura primária. Os ccondrócitos apresentaram características morfológicas normais com presença extensiva de células na superfície e aréas internas das estruturas da sustentação. As análises qualitativas e quantitativas

mostraram existir deposição de componentes típicos da matriz extracellular da cartilagem, evidenciando desse modo o potencial destes sistemas para aplicações futuras nesta área

Um outro tipo de suporte, e o mais extensivamente estudado no âmbito do trabalho descrito nesta tese, foi um hidrogel de origem natural - a goma gelana. A capacidade da goma gelana ser utilizada com agente de encapsulamento de células foi inicialmente testada. Foi demonstrado que a goma gelana é versátil em termos de aplicações e pode ser utlizada como um veículo de entrega de células/fármacos em diferentes cenários. Em termos de regeneração de cartilagem, as suas propriedades materiais e natureza não citotóxica revelaram-se apropriadas para as aplicações propostas. A parte final deste primero estudo envolveu o encapsulamento e cultivo *in vitro* de condrócitos nasais humanos nos hidrogéis de goma gelana. Estes permaneceram viáveis confirmando novamente o potencial destes novos sistemas no suporte de células tendo como objectivo a regenração de cartilagem.

No trabalho seguinte, os hidrogéis de goma gelana foram optimisados para poderem ser utilizados como sistemas injectáveis capazes de entregar e manter condrócitos humanos no local do defeito através da gelificação *in situ*. Subsequentemente, estes deveriam manter a viabilidade das células encapsuladas e sustentaram a produção de matriz extracelular. A caracterização de algumas das propriedades destes materiais demonstrou o seu carácter injectável em condições fisiológicas, assim como foi possível constatar que as células encapsuladas e cultivadas se mantiveram viáveis sintetisando activamente matriz extracelular cartilagínea. Na parte final, o desempenho dos hidrogéis de goma gelana *in vivo* , no que se refere à reacção inflamatória e integração pelo tecido do anfitrião, foi testado através de implantação subcutânea em ratinhos. Os resultados mostraram uma resposta residual dos organismos e uma manutenção da estabilidade mecânica dos geis ao longo dos períodos de implantação.

A obtenção destes resultados suscitou o interesse de estudar a formação de tecido cartilagíneo pela goma gelana quando utilizada com condrócitos humanos *in vivo* . Os hidrogeis foram então combinados com os condrócitos articulares humanos e implantados subcutaneamente no dorso de ratinhos atímicos. Os resultados mostraram uma distribuição homogénea das células que apresentaram a morfologia típica dos condrócitos. A síntese de proteogicanos foi detectada nas secções histológicas e um aumento estatisiticamente significativo das quantidades de proteoglicanos foi medido com o teste de quantificação de glicosaminoglicanos durante a

primeira e quarta semanas de implantação. As análises de PCR em tempo real revelaram um aumento estatisiticamente significativo do colagénio tipo II e dos níveis de agrecano nos mesmos períodos, enquanto que os ensaios imunológicos sugeriram o depósito de colagénio tipo II, assim como algum colagénio tipo I. Estes dados reforçaram o potencial previamente observado dos hidrogeis de goma gelana na geração de um tecido cartilagíneo funcional.

Finalmente, o último estudo experimental realizado no âmbito desta tese teve como objectivo avaliar o efeito terapêutico dos hidrogeis de goma gelana combinados com células progenitoras do tecido adiposo quando injectados em defeitos totais da cartilagem articular de coelhos, num modelo de aproximação autólogo. Células progenitoras do tecido adiposo (sujeitas a pré-diferenciação condrogénica e não pré-differenciadas) foram comparadas com condrócitos articulares, a goma gelana apenas, e com os defeitos vazios. Os hidrogeis conjugados com células apresentaram os melhores resultados em termos macroscópicos e de integração com o tecido nativo. Os resultados da avaliação histológica quantitativa e qualitativa, assim como as análises semiquantitativas de PCR em tempo real, demonstraram que a combinação de goma gelana células progenitoras do tecido adiposo constituem uma aproximação promissora no tratamento de defeitos da cartilagem articular, sendo estes sistemas fortes candidatos para aplicações clínicas futuras neste campo.

De um ponto de vista geral, pode referir-se que o trabalho executado nesta tese testou diversos suportes com potencial aplicação no campo da engenharia de tecidos de cartilagem, tendo sido a ênfase principal colocada nos hidrogeis de goma gelana. Foi aqui demonstrado que a goma gelana é um material promissor para este tipo de aplicações, como se constatou pelas suas propriedades materiais, assim como resultados de ensaios *in vitro* e *in vivo* . Tais evidências sugerem que os sistemas de goma gelana aqui descritos combinados com diferentes tipos de células, nomeadamente células progenitoras do tecido adiposo, poderão ter uma potencial aplicação clínica no tratamento de defeitos da cartilagem.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

#

2D	two dimensional
3D	three dimensional
Ø	diameter
21G	21 gauge
$-\Delta\Delta Ct$	delta delta critical threshold

A

A/B	antibiotic
Å	ångström
ASC + GF	chondrogenic pre-differentiated rabbit adipose tissue derived progenitor cells
ASC	rabbit adipose tissue derived progenitor cells

B

BMC9	mouse bone marrow derived mesenchymal progenitor cell line
Ba^{2+}	barium
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin

C

C-PBS	chitosan-polybutylene succinate
Col I	collagen type I
Col II	collagen type II
$CaCl_2$	calcium chloride
Ca^{2+}	calcium
CO_2	carbon dioxide
CA	cellulose acetate
CDMP-2	cartilage-derived morphogenetic protein 2
COOH	carboxylic groupc
cDNA	complementary deoxyribonucleic acid
Ct	critical threshold
cm	centimetre

D

DMB	1,9-dimethylmethylene blue
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DAB	3,3'-diaminobenzidine
DSC	differential scanning calorimetry
DMA	dynamic mechanical analysis
DNA	deoxyribonucleic acid

E

E'	storage modulus
E''	loss modulus
ECM	extracellular matrix
ECACC	European Collection of Cell Cultures
Esec	secant modulus
EVOH	ethyl vinyl alcohol

F

FBS	foetal bovine serum

G

GAG	glycosaminoglycans
g/L	gram per litre
xg	centrifugal force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GvHD	graft-versus-host disease

H

Hz	hertz
hBMP-2	human bone morphogenentic protein-2
H&E	hematoxylin-eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hAC	human articular chondrocytes

I

ITS	insulin, transferrin, and selenium solution
IGF-I	insulin-like growth factor type I
i.m.	intramuscular

K

kDa	kilodalton
kV	kilovolt
K^+	potassium
keV	kiloelectron volt
kg	kilogram

L

L929	rat lung fibroblasts cell line

M

MEM	minimum essential medium
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
µ-CT	micro-computed tomography
MPa	megapascal
ml	mililiter
M	molar
mM	milimolar
mA	miliampere
mg	miligram
mm	milimeter
Mg^{2+}	magnesium
min	minute
ms	milisecond
mm^3	cubic milimeter
MC615	chondrocytic cell line
mRNA	messenger ribonucleic acid

N

n	total number of data points
ng	nanogram
nm	nanometer
NaCl	sodium chloride
Na^+	sodium
NaOH	sodium hydroxide

O

OD	optical density
OCT	optimal cutting temperature

P

PCR	polymerase chain reaction
p	probability value
PBS	phosphate buffered saline
PGA	polyglycolic acid
PLGA	poly(lactic-co-glycolic)
PHEMA	poly-2-hydroxyethyl methacrylate
PEGT/PBT	polyethylene glycol terephthalate/polybutylene terephthalate
P1	cell passage one
PCL	polycaprolactone
Pa	pascal

R

RGD	arginine-glycine-aspartic acid
rpm	revolutions per minute

S

Sox9	SRY (sex determining region Y)-box 9
SEM	scanning electron microscopy
SPCL	starch-polycaprolactone
s	second
SDS	sodium dodecyl sulfate
saline-T	saline-Tris

T

TEMED	N,N,N',N' tetramethylethylenediamine
TGF-β	transforming growth factor beta
TGF-β1	transforming growth factor beta-1
Tm	melting temperature
TBS	tris-buffered saline
TEM	transmission electron microscopy
TCPS	tissue culture polystyrene

U

µm	micrometer
UDP-glucose	uridine diphosphate glucose
µA	microampere

V

v/v	volume per volume

W

wt	weight
wt/v	weight per volume

LIST OF FIGURES

Gellan Gum Injectable Hydrogels for Cartilage Tissue Engineering Applications: *In vitro* Studies and Preliminary *In vivo* Evaluation

Figure VII.1. Rheological measurements of gellan gum solutions showing the relation temperature-viscosity and time-viscosity. The sol-gel transition is noticeable by the change in viscosity present in the graph around 39-38ºC with an extent of time for gelation of approximately 11 seconds.

Figure VII.2. Mechanical properties evaluation of gellan gum discs using dynamic mechanical analysis. The tests were performed in intervals of 0.1-15 Hz in hydrated samples.

Figure VII.3. Calcein AM/DAPI viability stain of human articular chondrocytes encapsulated in gellan gum hydrogels at 14 days of culture.

Figure VII.4. Histological analysis of sections of gellan gum with human articular chondrocytes after culturing. Hematoxylin-eosin (A,B – after 2 and 56 days of culture, respectively) was used for general cell morphology and distribution while alcian blue (C), and safranin-O (D) were performed for proteoglycans (glycosaminoglycans) detection.

Figure VII.5. Graphical representation of the real-time semiquantitative PCR analysis for collagen type I, collagen type II, aggrecan and Sox9 based on the mRNA produced by the encapsulated human articular chondrocytes after 14 days and 56 days of culture.

Figure VII.6. Histological analysis showing two different magnifications of sections of gellan gum after 7, 14 and 21 days of implantation. The images evidenced a good integration with the surrounding tissue and the progressive reduction of the fibrotic capsule that was almost unnoticed after 21 days.

Figure VII.7. Storage and loss modulus at 1 Hz obtained from compression dynamic mechanical tests of gellan gum discs after subcutaneous implantation in Balb/c mice. Statistical analysis revealed no difference through the various time points ($p>0.05$) and were performed using a two-sample t-test assuming unequal variances for $n=5$, $\alpha=0.05$**.**

Figure VII.8. Weight measurements of gellan gum discs after subcutaneous implantation in Balb/c mice. Statistical analysis revealed a significant difference in gellan discs implanted for 21 days when compared to no implant and 14 days of implantation. No statistical analysis through the various time points ($p>0.05$) was performed using a two-sample t-test assuming unequal variances for $n=5$, $\alpha=0.05$.

SECTION 3

CHAPTER VIII

Performance of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes for Cartilage Regeneration When Subcutaneously Implanted In Nude Mice

LIST OF TABLES

SHORT *CURRICULUM VITAE*

João Teixeira de Oliveira was born in Porto, Portugal, in 1980. He is currently a PhD student at the 3B's Research Group (Biomaterials, Biodegradables and Biomimetics), at the University of Minho, Braga, Portugal. His work is supervised by Prof. Rui L. Reis, also the Director of the 3B's Research Group, and Prof. Paul V. Hatton, from the Centre for Biomaterials and Tissue Engineering, University of Sheffield, United Kingdom.

He has a degree in Biology (scientific branch) from the Faculty of Sciences of the University of Porto that he finished with a final graduation mark of 16 (0-20 scale). In the final stage of his degree, he was a research trainee at the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP) working in breast pathology under the supervision of Prof. Fernando Schmitt, University of Porto, Portugal, where he received a traineeship mark of 19 (0-20 scale).

In January 2004, he formally started his PhD at the 3B's Research Group with a main focus on cartilage tissue engineering. In October 2004, he received a grant from the Portuguese Foundation for Science and Technology (FCT) which enabled him to perform work at the University of Sheffield, UK. During the course of his PhD research he has received the following awards: Fundação Calouste Gulbenkian Travel Award for attendance at 20th European Society for Biomaterials (ESB) Conference 2006, Nantes, France; Best 40 abstract submitted to the 20th European Society for Biomaterials (ESB) Conference 2006, Nantes, France - Invited paper for the Special Issue of the Journal of Materials Science: Materials in Medicine; European Molecular Biology Association/European Science Foundation (EMBO/ESF) Award for attendance at the ESF/EMBO Symposium on Stem Cells in Tissue engineering: isolation, culture, characterisation and applications, Sant Feliu de Guixols, Spain. He has been an invited reviewer for Biotechnology Letters, and for the Journal of Tissue Engineering and Regenerative Medicine.

As a researcher in the 3B's Research Group, he has been involved in the writing, preparation, and supporting management of several grant proposals both at National and European levels. He was also involved in the development and writing of the business plan of STEMMATERS which is the biotechnological spin-off of the 3B's Research Group that has been awarded in 2007 with the

National Award for Entrepreneurship (START). He was a member of the organization committee of the 2008 Annual Meeting of TERMIS-EU (Tissue Engineering and Regenerative Medicine International Society – EU chapter). Along with these activities, he is a Credited Technician on Animal Experimentation: Accreditation as a Competent Professional for the Practice of Animal Experimentation issued by Direcção Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas on 5th February 2007, and has been the President of the Executive Group of the Research and Development Grantholders Association of the University of Minho (2005/2006) and Board Member of the Portuguese Association of Grant-holders Researchers (2005/2006).

As results of his research work, he attended several important international meetings in his field of research mostly with oral communications. Presently, he is author of 11 research papers (8 as first author; 4 published and 7 submitted), 1 invited review paper, 2 book chapters, 29 international communications (oral and poster), and 2 patents (patents pending-EPO submitted).

LIST OF PUBLICATIONS

The work performed during this PhD resulted in the following publications:

INTERNATIONAL JOURNAL WITH REFEREES

JT Oliveira, A Crawford, JM Mundy, AR Moreira, ME Gomes, PV Hatton and R. L. Reis. A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes, Journal of Materials Science: Materials in Medicine, Vol 18, 295-302 (2006).

JT Oliveira, VM Correlo, PC Sol, AR Costa-Pinto, PB Malafaya, AJ Salgado, M Bhattacharya, P Charbord, NM Neves, RL Reis. Assessment of the Suitability of Chitosan/PolyButylene Succinate Scaffolds Seeded with Mouse Mesenchymal Progenitor Cells for a Cartilage Tissue Engineering Approach. Tissue Engineering Part A. October 1, 2008, 14(10): 1651-1661.

JT Oliveira, A Crawford, JM Mundy, PC Sol, VM Correlo, M Bhattacharya, NM Neves, PV Hatton, RL Reis, Novel Melt-Processable Chitosan-Polybutylene Succinate Fibre Scaffolds for Cartilage Tissue Engineering, 2008 (*submitted*).

JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, RL Reis, Gellan Gum: A New Biomaterial for Cartilage Tissue Engineering Applications, 2008 (*submitted*).

JT Oliveira, TC Santos, L Martins, R Picciochi, AP Marques, AG Castro, NM Neves, JF Mano, RL Reis, Gellan Gum Injectable Hydrogels for Cartilage Tissue Engineering Applications: *In vitro* Studies and Preliminary *in vivo* Evaluation, 2008 (*submitted*).

JT Oliveira, TC Santos, L Martins, MA Silva, AP Marques, AG Castro, NM Neves, RL Reis, Performance of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes for Cartilage Regeneration When Subcutaneously Implanted In Nude Mice, 2008 (*submitted*).

JT Oliveira, L Gardel, T Rada, L Martins, ME Gomes, RL Reis, Rabbit Articular Cartilage Full-Thickness Size Defects Treated With Novel Gellan Gum Injectable Hydrogels and Autologous Adipose Stem Cells, 2008 (*submitted*).

JT Oliveira, RL Reis. Polysaccharide Based Materials for Cartilage Tissue Engineering Applications, 2008 (*submitted*).

BOOK CHAPTERS

JT Oliveira and RL Reis, Hydrogels from Polysaccharide-Based Materials: Fundamentals and Applications in Regenerative Medicine, In Natural-based polymers for biomedical applications, eds. RL Reis, J Mano, N Neves, H Azevedo , AP Marques, ME Gomes, Woodhead Publishing Limited, Cambridge, United Kingdom, 2007, Part 4, pgs 538-553.

INTERNATIONAL CONFERENCES

JT Oliveira, AJ Salgado, VM Correlo, AR Pinto, M Bhattacharya, P Charbord, NM Neves, RL Reis (2005). Preliminary Assessment of the Behaviour of Chitosan/Polyester Scaffolds Seeded with Mouse Mesenchymal Stem Cells for Cartilage and Bone Tissue Engineering. 1º Encontro da Sociedade Portuguesa de Células Estaminais e Terapias Celulares. Hotel Vila Galé Ópera, Lisboa, 7-8 Janeiro 2005 *(poster presentation)*.

JT Oliveira, AJ Salgado, VM Correlo, AR Pinto, M Bhattacharya, P Charbord, NM Neves, RL Reis (2005). Preliminary Assessment of the Potential of Chitosan/Polyester Based Scaffolds for Bone and Cartilage Tissue Engineering. SFB 2005 / 30th Annual Meeting and Exposition: New Applications and Technologies, April 27-30, 2005, Memphis, Tennessee, USA / Memphis Convention Center *(oral presentation)*.

JT Oliveira, VM Correlo, A Crawford, JM Mundy, M Bhattacharya, NM Neves, PV Hatton, RL Reis. Chitosan-Poly(Butylene Succinate) Fiber Scaffolds Are Suitable For Cartilage Tissue Enginnering Applications. UKSB 2005 / United Kingdom Society for Biomaterials, 21-22nd June, 2005, Nottingham, England, UK *(poster presentation)*.

JT Oliveira, AJ Salgado, VM Correlo, AR Pinto, M Bhattacharya, P Charbord, NM Neves, RL Reis (2005). Preliminary Assessment of the Behaviour of Chitosan/Poly(Butylene Succinate) Scaffolds Seeded with Mouse Mesenchymal Stem Cells for Cartilage Tissue Engineering. ESB 2005 / 19th European Conference on Biomaterials, September 11-15, Sorrento, Italy *(oral presentation)*.

JT Oliveira, VM Correlo, A Crawford, JM Mundy, M Bhattacharya, NM Neves, PV Hatton, RL Reis. Chitosan-Polyester Scaffolds Seeded with Bovine Articular Chondrocytes for Cartilage Tissue Enginnering Applications. (P127) Artificial Organs, pg. 774, vol.29, nº9, 2005. European Society for Artificial Organs (ESAO), 5-8th October, 2005, Bologne, Italy *(poster presentation)*.

JT Oliveira, A Crawford, JM Mundy, PV Hatton, RL Reis. Bovine Articular Chondrocytes Cultured in a 3D Environment Using a Natural-Based Hydrogel Support. School of Engineering Day, 6-8th October, 2005, Guimarães, Portugal *(poster presentation)*.

JT Oliveira, ML Silva, VM Correlo, M Bhattacharya, P Charbord, RL Reis, NM Neves. Chondrogenic Differentiation of a Mouse Bone Marrow Derived Mesenchymal Progenitor Cell Line (BMC9) and a Primary Culture of Human Mesenchymal Stem Cells (hMSCs) onto Chitosan/Polyester Based Scaffolds. Genostem 2nd Workshop on Mesenchymal Stem Cells Engineering Connective Tissues Disorders, Porto, Portugal, January 18-21, 2006 *(poster presentation)*.

JT Oliveira, A Crawford, JM Mundy, AR Moreira, ME Gomes, PV Hatton, RL Reis. Starch-PolyCaprolactone Fiber Mesh Scaffolds Are Suitable For Cartilage Tissue Engineering Approaches. Society for Biomaterials (SFB) 2006, Pittsburgh, Pennsylvania *(poster presentation)*.

JT Oliveira, VM Correlo, A Crawford, JM Mundy, M Bhattacharya, NM Neves, PV Hatton, RL Reis. Novel Chitosan-Polyester Scaffolds Seeded With Bovine Articular Chondrocytes For Cartilage Tissue Engineering, 2006 Regenerate World Congress on Tissue Engineering and Regenerative Medicine, Pittsburgh, Pennsylvania *(poster presentation)*.

JT Oliveira, A Crawford, JM Mundy, AR Moreira, ME Gomes, PV Hatton, RL Reis. A Cartilage Tissue Engineering Approach Using Starch-PolyCaprolactone Fiber Mesh Scaffolds Seeded With

Bovine Articular Chondrocytes. 20th European Society for Biomaterials (ESB) conference 2006, 27th September-1st October, Nantes, France *(oral presentation).*

JT Oliveira, L Martins, R Picciochi, JF Mano, RL Reis. A new polysaccharide-based hydrogel supports human nasal chondrocytes development aiming at cartilage tissue engineering applications. Annual TERMIS-EU Meeting, October 8 - 11, 2006, Rotterdam, the Netherlands (*oral presentation*).

JT Oliveira, R Picciochi, AR Pinto, L Martins, PB Malafaya, RA Sousa, JF Mano, NM Neves, RL Reis. Gellan Gum Hydrogels as Supports for Human Articular Chondrocytes and Human Bone Marrow Cells for Cartilage Tissue Engineering Application, ESF/EMBO Symposium Stem Cells in Tissue engineering isolation, culture, characterisation and applications, 28th of October to 2nd of November 2006 Hotel Eden Roc, Sant Feliu de Guixols Spain, Spain (*oral presentation*).

JT Oliveira, R Picciochi, PB Malafaya, RA Sousa, TC Santos, AP Marques, L Martins, LG Pinto, R Fernandes, AG Castro, JF Mano, NM Neves, RL Reis. Gellan gum hydrogels as supports for cartilage tissue engineering approaches: *in vitro* and *in vivo* evaluation, 4th Marie Curie Cutting Edge Conference on biocompatibility evaluation and biological behaviour of polymeric biomaterials, 9-13th October 2007, Alvor, Algarve, Portugal (*oral presentation*).

JT Oliveira, RL Reis. Gellan gum hydrogels as supports for cartilage tissue engineering approaches: *in vitro* and *in vivo* evaluation, School of Engineering Day, University of Minho, Guimarães, Portugal, October 2007, (*poster presentation*).

JT Oliveira, L Gardel, L Martins, T Rada, ME Gomes, RL Reis. Injectable Gellan Gum Hydrogels With Encapsulated Adipose Derived Cells For Cartilage Tissue Engineering Applications: Performance In A Rabbit Knee Defect, 3rd International Meeting of the Portuguese Society for Stem Cells and Cell Therapies (SPCE-TC), April 23-24, 2008, University of Algarve, Faro, Portugal *(oral presentation).*

JT Oliveira, L Martins, R Picciochi, TC Santos, PB Malafaya, RA Sousa, AP Marques, AG Castro, JF Mano, NM Neves, RL Reis. Injectable Gellan Gum Hydrogels as Supports for Cartilage Tissue Engineering Applications: *In vitro* characterization and initial *In vivo* studies. 8th

World Biomaterials Congress, 28th May- 1st June 2008, Amsterdam, The Netherlands *(oral presentation).*

JT Oliveira, R Picciochi, TC Santos, L Martins, LG Pinto, PB Malafaya, RA Sousa, AP Marques, AG Castro, JF Mano, NM Neves, RL Reis. Injectable Gellan Gum Hydrogels as Supports for Cartilage Tissue Engineering Applications, Tissue Engineering Part A, 14(5): 748

PATENTS:

New Dynamic Cell Culturing Systems in 3D Ssupports / Novos Sistemas Dinâmicos de Cultura De Células em Suportes Tridimensionais, **JT Oliveira**, AJ Pedro, RL Reis – INPI, EPO submitted

Gellan Gum Based Hydrogels for Regenerative Medicine and Tissue Engineering Applications, Its System, and Processing Devices / Hidrogéis À Base De Goma Gelana Para Utilização Em Medicina Regenerativa e Engenharia De Tecidos, Seu Sistema E Dispositivos De Processamento, **JT Oliveira**, RA Sousa, RL Reis – INPI, EPO submitted

The collaborative work within the 3B's generated the following publications:

INTERNATIONAL JOURNAL WITH REFEREES

J Paredes, A Albergaria, **JT Oliveira**, C Jeronimo, F Milanezi, FC Schmitt (2005). P-Cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. Clinical Cancer Research. 2005 Aug 15;11(16):5869-77.

PB Malafaya, **JT Oliveira** and RL Reis, 2008, The effect of insulin-loaded chitosan particle aggregated scaffolds in chondrogenic differentiation, (*submitted*).

AJ Salgado, **JT Oliveira**, AJ Pedro and RL Reis. Adult Stem Cells in Bone and Cartilage Tissue Engineering, Current Stem Cell Research & Therapy, 345-364(20 (2006), Volume 1 *(invited review paper)*.

BOOK CHAPTERS

ME Gomes, **JT Oliveira**, MT Rodrigues, MI Santos, K Tuzlakoglu, CA Viegas, IR Dias and RL Reis, 2007, Starch-polycaprolactone based scaffolds in bone and cartilage tissue engineering approaches, In Natural-based polymers for biomedical applications, eds. RL Reis, JF Mano, NM Neves, HS Azevedo, AP Marques, ME Gomes, Woodhead Publishing Limited, Cambridge, United Kingdom, 2007, Part 2, pgs 248-261.

INTERNATIONAL CONFERENCES

ET Baran, I Pashkuleva, S Costa, **JT Oliveira**, RL Reis (2004). Peptide synthesis on carboxymethyl chitin. TESI-ETES 2004 Meeting, Lausanne, Switzerland *(oral presentation).*

AR Pinto, AJ Salgado, **JT Oliveira,** VM Correlo, M Bhattacharya, P Charbord, RL Reis, NM Neves (2005). Evaluation of the Adhesion, Proliferation and Differentiation of a Mouse Mesenchymal Stem Cell line on Novel Chitosan/Polyester Based Scaffolds. ESB 2005 / 19th European Conference on Biomaterials, September 11-15, Sorrento, Italy *(oral presentation)*.

RMP da Silva, AJ Pedro, **JT Oliveira**, C Elvira, JF Mano, J San Román, RL Reis (2005). Poly(N-isopropylacrylamide) surface grafted chitosan membranes as a new substrate for cell sheet manipulation. ESB 2005 / 19th European Conference on Biomaterials, September 11-15, Sorrento, Italy *(oral presentation)*.

AJ Salgado, **JT Oliveira**, RA Sousa, NM Neves, RL Reis, N Sousa, Development of a Surface Functional Multi-Supportive Structure for Spinal Cord Injury Repair, 1st Annual International Meeting of the Portuguese Society for Stem Cells and Cellular Therapies (SPCE-TC), 30-31 May, 2006, Madeira, Portugal *(poster presentation)*.

AJ Pedro, **JT Oliveira**, RA Sousa, RL Reis, Dynamic mechanical stimulation of tissue engineering constructs by hydrostatic pressure: preliminary evaluation of cell response, 1st Marie Curie Cutting Edge Conference / New developments on polymers for tissue engineering: replacement and regeneration, 1-5 June, 2006, Madeira, Portugal *(poster presentation)*.

PB Malafaya, AJ Pedro, **JT Oliveira**, A Peterbauer, C Gabriel, H Redl, RL Reis. Strategies for osteochondral tissue engineering applications tailoring chitosan-based bilayered scaffolds and surface interactions. 2nd Marie Curie Cutting Edge InVENTS Conference on Recent advances on polymeric based systems for controlled delivery of bioactive agents: Applications in Tissue Engineering, Alvor, Algarve, Portugal (2006) O12, *(oral presentation)*

NA Silva, AJ Salgado, RA Sousa, **JT Oliveira**, NM Neves, JF Mano, N Sousa and RL Reis, Development Of A Novel Hybrid Tissue Engineering Based Therapy For Spinal Cord Injury Repair, Third International Meeting of the Portuguese Society for Stem Cells and Cell Therapies, Faro, Portugal, April 2008 *(oral presentation).*

AJ Salgado, RA Sousa, **JT Oliveira**, NA Silva, NM Neves, RL Reis and N Sousa, Novel Tissue Engineering 3D Scaffolds for Spinal Cord Injury based on Starch/Polycaprolactone Blends: Development and Preliminary Assessment of Their Biological Performance, TERMIS-EU 2007, London, United Kingdom, September 2007 *(oral presentation).*

PB Malafaya, **JT Oliveira**, TC Santos, M van Griensven and RL Reis, 2008, Morphometric and mechanical characterization, insulin loading and in-vivo biocompatibility of chitosan particles aggregated scaffolds for tissue engineering, Tissue Engineering: Part A, 14(5) : 757

PB Malafaya, **JT Oliveira** and RL Reis, 2008, Development and characterization of natural-origin bilayered scaffolds for osteochondral tissue engineering under the scope of HIPPOCRATES project, Tissue Engineering: Part A, 14(5) : 719

NA Silva, AJ Salgado, RA Sousa, **JT Oliveira**, JF Mano, NM Neves, N Sousa and RL Reis, 2008, Starch/Gellan Gum Hybrid 3D Guidance Systems for Spinal Cord Injury Regeneration: Scaffolds Processing, Characterization and Biological Evaluation, Tissue Engineering: Part A 769, 14 : 769

JF Mano, **JT Oliveira**, CM Alves, PB Malafaya and RL Reis, 2008, Bone, cartilage and osteochondral tissue engineering strategies using natural origin polymers and ceramics, growth factors and progenitor cells, Tissue Engineering: Part A, 14(5) : 707

Correia C, Pedro AJ, **Oliveira JT** , Sousa RA and Reis RL, 2008, Hydrostatic pressure culture system for human chondrocyte cultivation and stimulation in gellan gum hydrogel disks, Tissue Engineering Part A, 14 (5) : 860-861.

Sangwon Chung, Martins A, Correlo V M , **Oliveira JT** , Marques AP, Neves NM, Mano JF, Sousa RA and Reis RL, 2008, Dependence of cell proliferation on the morphology of starch based scaffolds for tissue engineering, TISSUE ENGINEERING PART A, 14 : 718

INTRODUCTION TO THE THESIS FORMAT

This thesis is divided into four sections containing ten different chapters, with seven of them being experimental research. According to the 3B's Research Group internal policy, the thesis format is based on published or submitted papers, including the introduction section that consists of a review paper. The contents of each chapter are summarized below.

SECTION I (Chapter I)

Chapter I presents a comprehensive overview on the polysaccharide based materials used in cartilage tissue engineering applications. It includes an introduction to the cartilage tissue organization and associated diseases, followed by a brief description of the cartilage tissue engineering field. An introduction is provided on the types of polysaccharides used in cartilage tissue engineering and a detailed description on the most relevant is provided. For each, a description of the materials properties and characteristics is provided, along with the applications with cells in *in vitro* and *in vivo* scenarios within the cartilage tissue engineering field.

SECTION II (Chapter II)

Chapter II presents the materials and experimental methods used within the scope of this thesis. Although each part of the work is accompanied by its specific materials and methods section, this chapter intends to condensate and compile the relevant information on this matter.

SECTION III (Chapters III to IX)

These chapters describe the experimental work performed within the scope of this thesis.

Chapter III describes the testing of scaffolds of chitosan and polybutylene succinate processed by compression moulding with salt leaching that were used to support the growth and chondrogenic differentiation of cells from a mouse bone marrow derived mesenchymal progenitor cell line.

Chapter IV explains the work using the chitosan and polybutylene succinate blended material but processed in the form of a fibre scaffold that was at this time used to culture primary culture chondrocytes from bovine origin.

Chapter V describes the experiments conducted with a different natural fibre based scaffold consisting of a blend of starch and polycaprolactone. This 3D support was also investigated regarding its ability to support growth and extracellular matrix formation by bovine articular chondrocytes seeded *in vitro*.

Chapter VI presents the main material studied within the work described in this thesis. Gellan gum was initially tested in this part as a cell encapsulating agent, being characterised in terms of materials properties and combined with human nasal chondrocytes in preliminary *in vitro* studies.

Chapter VII describes the testing of gellan gum to be used as injectable systems for delivering and maintaining human chondrocytes by *in situ* gelation, as well as for supporting cell viability and production of extracellular matrix. The *in vivo* performance of the systems upon subcutaneous implantation in mice is also presented.

Chapter IX presents the final experimental study performed within the work of this thesis. This last study analysed the therapeutic effect of gellan gum hydrogels when combined with adipose tissue derived progenitor cells and injected in rabbit full-thickness articular cartilage defects in an autologous approach. A comparative analysis was conducted with articular chondrocytes, gellan gum alone, and empty defects.

SECTION IV (Chapter X)

Chapter X contains the general conclusions of the works carried out under the scope of this thesis. Some specific remarks and future prospects are also provided.

As a final note, it should be mentioned that the present thesis was also developed under the scope of the European Project HIPPOCRATES (NMP3-CT-2003-505758) and the European Network of Excellence EXPERTISSUES (NMP3-CT-2004-500283).

SECTION 1.

CHAPTER I.

Polysaccharide Based Materials for Cartilage Tissue Engineering Applications

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Polysaccharide Based Materials for Cartilage Tissue Engineering Applications*

ABSTRACT

Tissue engineering was proposed approximately 15 years ago as an alternative and innovative way to address tissue regeneration problems. During the development of this field, researchers have proposed a variety of ways of looking into the regeneration and engineering of tissues, using different types of materials coupled with a wide range of cells and bioactive agents. This trilogy is commonly considered the basis of a tissue engineering strategy, meaning by this the use of a support material, cells, and bioactive agents. Different researchers have been adding to these basic approaches other parameters able to improve the functionality of the tissue engineered construct, such as specific mechanical environments, conditioned gaseous atmospheres, among others. Nowadays, tissue engineering principles have been applied with different degrees of success to almost every tissue lacking efficient regeneration ability and the knowledge and intellectual property produced since then has experienced an immense growth. Materials for regenerating tissues, namely cartilage, have also been continuously increasing and most of the theoretical requirements for a tissue engineering support have been addressed by a single or a mixture of materials.

Due to their intrinsic features, polysaccharides are interesting for cartilage tissue engineering approaches and as a result their exploitation for this purpose has been increasing. The present paper intends to provide an overview of some of the most relevant polysaccharides used in cartilage tissue engineering research based on their proven claimed potential. Insights on basic aspects of their nature and structure, as well as their research status will be given. In addition, the evolution of the use of the selected materials in the cartilage tissue engineering field until the present time will be discussed. The gathered information is expected to provide the reader with a wider knowledge on polysaccharide based matrices with potential use in cartilage regeneration, as well as to increase awareness of these materials and maybe contribute for further applications of such systems.

*** This chapter is based on the following publication:**
JT Oliveira, RL Reis. Polysaccharide Based Materials for Cartilage Tissue Engineering Applications (*submitted*).

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1. INTRODUCTION

The progressive increase of life expectancy within the last century gave rise to new health problems, within which musculoskeletal conditions represent a considerable share. Examples of this are osteoarthritis, and rheumatoid arthritis that directly affect cartilage posing serious barriers to normal life quality and wellbeing.[1-3] Still within the musculoskeletal area, additional trauma injuries to the chondral and osteochondral regions turned the field into an increasing subject of attention among clinicians and scientists worldwide. Current therapies applied to treat cartilage related pathologies have relied mostly on the use of pharmaceutics, auto/allotransplant and prosthetic procedures.[4-7] Although beneficial in some cases, these solutions present disadvantages and are unable to assure a functional improvement and long-lasting recovery of the affected area to the patients, which demands for a constant search of new therapeutic solutions.[8,9] The concept of Tissue Engineering was proposed as a novel therapy able to provide the patients with renewed health and quality of life.[10] The common tissue engineering strategy makes use of a biomaterial support structure combined with a relevant cell population in specific culturing conditions in order to regenerate a functional tissue able to replace the affected one *in vivo*. Divergent opinions exist on various topics of the tissue engineering process such as whether the construct should be used in the early beginning of its development, or only after a certain degree of tissue formation is obtained. Nonetheless, it is consensual that several factors like the integration into the surrounding surface, biomechanical properties, and biocompatibility are key factors in the clinical success of the repaired tissue[11-13] For this, the support material, the cells, and culturing components should possess several features that assure their future clinical potential. The material should serve as a mechanical support for cell growth and allow cells differentiation into specific cell types. It should also be non cytotoxic in functional conditions, provide a non harsh environment to cells and adjacent tissues, and exhibit degradation profiles that can cope with the tissue formation extent. The cells should be functionally active, non immunogenic, easy to achieve and highly responsive to differentiation environmental cues. Tissue engineering approaches have been applied to cartilage using various materials and cells[14-17] in combination with culturing parameters (e.g. mechanical, chemical)[18,19] with the final goal of obtaining a cartilage tissue engineered construct with high performance *in vivo*. Polysaccharide based materials are among the class of natural materials and their potential role in future clinical applications of tissue engineered medical products has been increasing. Polysaccharides are widely distributed in nature being mainly regarded as sources of energy.[20,21] Their chemical behaviour and interesting structural similarities with biological molecules has grant

them great potential for future applications in the biomedical field, specially in cartilage regeneration.

2. CARTILAGE TISSUE ORGANIZATION

The human species is included in the vertebrates' branch of the phylogenetic tree, since it possesses a backbone or spinal column along with an internal skeletal structure that supports the organs and allows for their proper functioning. This evolutionary derivation that apparently started 530 million years ago provided a different way of exploring the Earth's resources, giving rise to the Amphibia, Reptilia, Aves and latest Mammalia classes.[22] The progressive development of a support structure – the skeleton – allowed for a new order of body organization to be settled and so bone and cartilage became an integral part of the skeleton providing mobility protection of vital organs, and housing of the bone marrow, reasons important enough to consider it vital for development and good quality of life.[23] Cartilage is a supporting connective tissue that comprises most of the temporary embryonic skeleton and is made of proteins, polyssacharides and a specific cell type, the chondrocyte. It consists of a matrix that is able to withstand physical deformation and facilitate tissue functionality with many spaces often termed lacunae which are occupied by the chondrocytes. The chondrocytes have the prime function of synthesizing and maintaining this extracellular matrix (ECM), and also of giving rise to cells mostly until adulthood, time after which these rates decrease.[12,24] Cartilage has an important role in embryonic development where it provides a model within which most bones develop. Its presence is decreased in adulthood persisting in a different number of areas, such as the joints, nose, and trachea, for example.[12] Three types of cartilage have been distinguished based on their histological and biomechanical properties: hyaline, elastic, and fibrous cartilage. Elastic and fibrous cartilages are less widely distributed and will not be further considered in this article. The most prevalent type is hyaline cartilage which is found in the skeleton of all vertebrates and possesses characteristic physical properties that are closely linked to the composition and organization of its extracellular matrix.[12,25,26] Articular cartilage, the most familiar hyaline cartilage, forms the smooth gliding surface of joints, such as knee and hip, as well as the *nucleus pulposus* of the invertebral discs, allowing these parts to articulate correctly and efficiently. The primary function of articular cartilage is physical, with water, ions, and aggrecan molecules within the collagenous meshwork playing key roles in the tissue load-bearing properties. The collagenous meshwork rich in type II collagen molecules gives the tissue tensile strength and hinders the

expansion of the aggrecan molecules that provide compressive stiffness. The highly sulfated aggrecan molecules interact noncovalently with a single strand of hyaluronan and link protein molecules to form aggregates of large size that become entrapped within the collagenous meshwork. Aggrecan molecules strongly oppose to fluid loss and water dispersion, thereby conferring cartilage a high compliance upon loading with the tissue rapidly recovering its elasticity when the load is removed.[27,28] Unlike most hyaline cartilage, the articular does not possess a superficial layer, called perichondrium that is a source for cell renewal. The extracellular matrix is differently distributed and organised in the cell surroundings and 3 zones can be identified: pericellular, territorial and interterritorial zones, going from near to further away from the cells, respectively. The pericellular matrix, has very little collagen but abundant proteoglycans; the territorial matrix, exhibits a high concentration of sulphated proteoglycans and a web of thin collagen fibrils that protect the cells; the interterritorial matrix constitutes the largest portion of articular cartilage.[29] Also when going from the surface to the subchondral bone, stratification occurs and the superficial, middle, deep, and calcified cartilage zones can be observed with chondrocytes from each part differing in size, shape and metabolic activity. The superficial zone is the thinnest, and forms the gliding surface of the joint being composed of collagen fibrils aligned parallel to the joint surface, with spindle shaped inactive chondrocytes. The middle zone is thicker and exhibits more spherical cells with larger collagen fibrils that are not oriented in a parallel fashion. In the deep zone, the cells are spheroidal, arranged in a columnar orientation and the collagen fibres are also oriented in a parallel fashion, but vertical to the joint surface. In the calcified cartilage part, collagen fibrils insert into the calcified cartilage, providing both a mechanical transition from the cartilage to bone, as well as fixation between these two tissues.[27,29] Cartilage is not a very dynamic tissue, exhibiting a low metabolic rate. It is characterized by low turnover and subsequent long half-lives of the constituent structural proteins. It is also avascular and therefore chondrocyte viability is dependent upon diffusion of nutrients, wastes, ions, and gases through the intercellular substance from adjacent capillaries.[29-31]

3. CARTILAGE ASSOCIATED DISEASES

Cartilage intrinsic features make the occurrence of a single cartilage lesion to have significant negative effects on normal mobility and movement. In fact, trauma and disease of bone and joints, frequently damaging both the articular cartilage and the subchondral bone, result in severe

pain and disability for millions of people worldwide posing a major health problem.[32,33] In 2003, the bulletin of the World Health Organization informed that self-reported persistent pain related to the musculoskeletal system is thought to affect up to 20% of adults.[1] In surveys carried out in Canada, the USA, and Western Europe, the prevalence of physical disabilities caused by a musculoskeletal condition repeatedly has been estimated at 4–5% of the adult population.[34] The major degenerative pathology associated with cartilage, mainly with articular cartilage, is arthritis that can be either inflammatory (rheumatoid arthritis) or non-inflammatory (osteoarthritis).[14,32,35,36] In both pathologies the erosion of the cartilage matrix through various actions on different extracellular matrix (ECM) components is present.[24] A joint replacement prosthesis is a widely accepted solution for these cases, but its limited lifespan makes critical the reconstruction of damaged cartilage to restore joint function.[11] Other approaches have been employed with some success, although their outcomes are dependent on several factors that can limit their widen application.[37,38] One example is the autologous chondrocyte implantation that has shown interesting results,[38] but limitations still exist, opening therefore the way for innovative technologies and alternative approaches to these conditions.

4. CARTILAGE TISSUE ENGINEERING

A good alternative to conventional treatments for damaged cartilage seems to be the combination of cells (such as chondrocytes or chondrogenic induced cells) with a biocompatible matrix that can be supplemented with bioactive agents of interest in order to promote the formation of a functional tissue engineered construct.[39] The field of Tissue Engineering has therefore emerged as a promising approach to treat cartilage loss or malfunction without the limitations of current therapies.[40-42] It can for example provide a valid alternative to organ and tissue transplantation, both of which are affected from a limitation of supply. Also, cells transplantation cultured in a biodegradable matrix that provides a temporary scaffolding to guide new tissue growth and organization, along with specific signals intended to retain tissue-specific gene expression offers the possibility of creating a completely natural tissue that will replace the damaged one. These support structures should fulfil some requirements in order to comply with a potential clinical application. They should be biocompatible, be able to aid in cell development and differentiation, allow for efficient nutrition and gas exchange, and have a degradation rate able to cope with the formation of the new tissue in order to maintain the mechanical stability of the system. The size and shape of the tissue to be regenerated, the nature and type of the cartilage defect, and the

conditions of the host should also be considered in the material selection.[42-45] Different types of natural and synthetic materials have been used to fabricate supports for cartilage tissue engineering.[16,46-53] Examples of the first type are fibrin,[48,54] collagen,[16] alginate,[42,49] chitosan,[50,55] and of the latter polyglycolic acid,[51,56] polylactic acid,[52] and polyethylene glycol.[53] Natural and synthetic materials present both advantages and disadvantages indeed. Natural materials may potentially interact with cell-surface receptors and most of them are biocompatible in various situations. Possible disadvantages may relate to difficult processing, concerns of disease transfer, and varied degradation rate from host to host. Synthetic materials can be manufactured reproducibly on a large scale, and exhibit tailored mechanical properties and degradation times. However, most of them lack cell-recognition signals, which is of extreme importance for integration with the surrounding tissues.[11,40] Several processing technologies can be applied with the expectation of improved performance of the systems in their final application. Depending on their role, they can be processed in ways that range from melt-based technologies, such as fibres extrusion coupled with fibre bonding to generate 3D scaffolds, to solvent based technologies.[46,57-59] These are expected to create a particular advantage, being it a better fitting ability, or a higher porosity for cells to penetrate and generate a functional engineered tissue. The processing of a material in a hydrogel is also quite appealing and interesting in cartilage tissue engineering approaches.[60-63]

An hydrogel may be defined as a network of polymer chains with great water absorbance ability. This implies that once put in an aqueous medium it is able absorb water and swell retaining the volume of water absorbed entrapped in the polymeric network.[64] Hydrogels may somehow mimic the hydrated environment of the cartilaginous tissue while also complying with mechanical properties suitable for the progressive generation of an adequate tissue engineered construct. They can be engineered for selective permeability, and exhibit dynamic molecular interactions by altering chemical signals, such as pH and ionic factors, and physical stimuli, such as temperature.[65] The associated biocompatibility, often a result of their hydrophilicity, is also a commonly referred advantage for their use in biomedical and pharmaceutical applications.[60,64,66] To this adds the good transport of nutrient to cells and products from cells that is normally assured, as well as the ability to be used as minimally invasive injectable systems. Commonly referred disadvantages are associated with difficult handling and inadequate mechanical properties.[67]

It is widely accepted by most authors that cells along with the support structure are key elements in the success of a tissue engineering product. Although some strategies involve the use of biomaterials, cells, and bioactive agents alone, most of the works reported make use of these

three agents in the pursuit of the best tissue substitute. In cartilage tissue engineering, most of the published studies refer the use of primary culture chondrocytes and bone marrow or adipose tissue derived cells. Primary culture chondrocytes can be collected from a relevant cartilage area, isolated by enzymatic digestion, and expanded *in vitro* for further use. Primary culture chondrocytes, and specially those in early passages, are considered good candidate cells to be applied in cartilage regeneration approaches since they maintain the phenotypic profile of differentiated chondrocytes.[12,68] Nonetheless, obtaining primary culture chondrocytes is frequently an added problem to the already existing pathological condition. When in fact a healthy cartilage part is located, the biopsy from the joint to obtain differentiated chondrocytes poses an additional injury to the cartilage surface, with possible harmful consequences to the surrounding articular cartilage. Such interventions frequently leave another regenerative problem at the donor site even when it is not located in a load bearing area.[69] Moreover, when chondrocytes are cultured on a tissue culture treated surface, they de-differentiate.[12,70] The process of de-differentiation involves a change in morphology and production of cartilage specific molecules. Cells loose their round shape and adopt a more fibroblastic like morphology, while decreasing also the production of collagen type II and aggrecan and increasing the production of type I collagen. When returned to a 3D environment, de-differentiated cells re-acquire the chondrocyte phenotype, re-expressing collagen type II and chondroitin sulphate proteoglycans.[12,71] This ability is however conditioned by the expansion and *in vitro* passaging of chondrocytes, since that they exhibit a finite capacity to form stable cartilage afterwards.[72] Due to these constraints, wide attention has also been given to alternative cells sources, such as stem cells, which can create functional cartilaginous tissues without having the limitations of primary culture chondrocytes isolation and culturing procedures.[73] A stem cell is a cell from the embryo, fetus or adult that, under certain conditions, can reproduce for long periods, and give rise to specialized cells of body tissues and organs.[69] The essential characteristics of all stem cells are prolonged self-renewal and the long term potential to form one or more differentiated cell types.[74-76] Adult stem cells from bone marrow and adipose tissue are frequently considered top candidates for tissue engineering and their application is widely described in the literature in combination with several supports and bioactive agents.[77,78] Adult mesenchymal stem cells (MSCs) are present in the bone marrow in which they reside as supportive cells for haematopoiesis and appear to function as a reservoir for various mesenchymal tissues. Bone marrow MSCs exist in a quantity of about 1 out of every 10^5 cells, and are able to differentiate into multiple mesenchymal lineages under defined culture conditions.[79] They can be distinguished through the screening of several surface markers and can be used as chondrocyte precursors in the development of differentiated chondrocytes when

supplied with the correct differentiation signals from the surrounding matrix and soluble mediators.[80-84] Adult mesenchymal stem cells can also be isolated from adipose tissue which is abundant and easily accessible after surgical procedures such as liposuction.[79,85] Adipose tissue MSCs acquire a fibroblast like morphology *in vitro* expansion, similar to the one observed for bone marrow MSCs and also express a serious of cell-specific proteins and CD markers that enable their isolation.[79,86] When comparing both stem cell sources with primary culture chondrocytes, some advantages may be clearly identified. A marrow aspirate and liposuction are easier to obtain than a cartilage biopsy. The collection procedures can be performed in parallel to another surgery, as in the case of liposuction. In bone marrow isolation, although the surgical procedure is more invasive, painful, and not frequently a parallel result of another intervention, the collection may also be achieved safely and easily under local anaesthesia. Moreover, these types of stem cells are quickly amplified in monolayers and de-differentiation is not an issue. Also, while chondrocytes proliferate slowly and tend to de-differentiate, stem cells mitotic potential remains high enabling an increased cell yield for cellular interventions that have such requisites.[69,87]

5. POLYSACCHARIDES IN CARTILAGE TISSUE ENGINEERING

5.1. INTRODUCTION

Polysaccharides are among the various types of natural biomaterials used in tissue engineering applications. Examples of these are alginate,[42,49] chitosan,[50,55] chondroitin sulphate,[88,89] hyaluronic acid,[90,91] starch,[46,92] and cellulose.[93,94]

Polysaccharides are widely distributed in nature in various forms and their typical application is normally associated with industrial processes in food, and cosmetic areas. Starch is widely used in the human diet, and alginate and xanthan gum are used as a cosmetic for skin treatments.[95-98] Polysaccharides can be described as polymers built of monosaccharides joined together by glycosidic bonds. They are frequently large and often branched macromolecules, being commonly amorphous, and insoluble in water.[99,100] Polysaccharides are usually regarded as sources of energy, starch being an example, but their molecular and physicochemical properties variety has recruited the interest of the scientific community for uses on different research areas. In fact, most polysaccharides can form hydrogels due to their physicochemical behaviour, while also presenting interesting structural similarities with biological molecules that are expected to place them in the frontline of future biomedical technology. In the regenerative medicine and

tissue engineering field, polysaccharides potential has been exploited for a wide range of tissues besides cartilage. Biodegradable porous scaffolds made of starch and polycaprolactone were able to maintain a normal expression of endothelial cells specific genes and proteins, indicating a potential application of these scaffolds in vascularization processes associated with bone tissue engineering.[101] For cardiac muscle tissue regeneration, cardiomyocytes were seeded within porous alginate scaffolds to achieve 3D functional cardiac constructs.[102] As a final example, alginate sponges were investigated for spinal cord tissue engineering applications, serving as adequate scaffolds for the outgrowth of regenerating axons and elongation of astrocytic processes.[103]

In the following parts of the manuscript, some insights on basic aspects of nature and structure of polysaccharides commonly used in cartilage tissue engineering will be given. Polysaccharides are known for their hydrogel forming ability and most are applied in such way, as in the case of minimally invasive injectable systems. Moreover, ongoing applications with relevant clinical potential in the cartilage tissue engineering area will be described. The gathered information is expected to provide a wider knowledge on polysaccharide based matrices used in cartilage regeneration and increase the interest of applying these materials to other fields.

5.2. AGAROSE

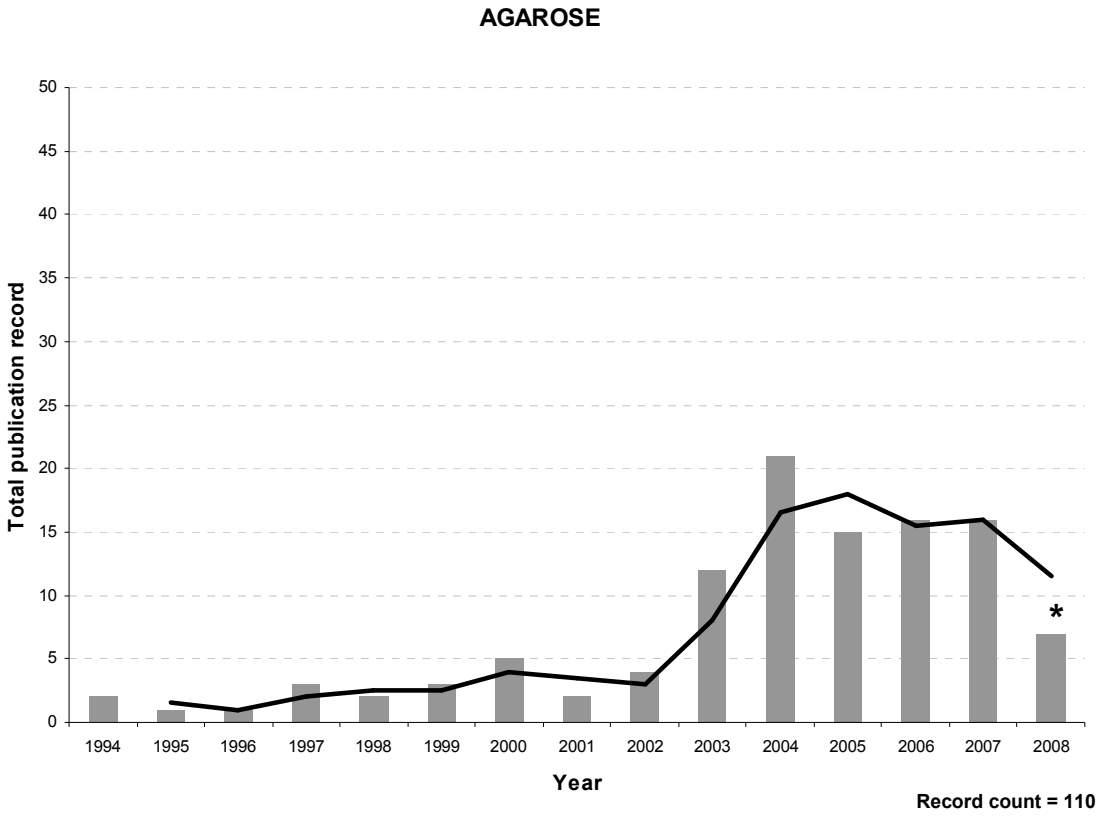

Figure I.1. Graphical representation of the search results performed with Web of Science® web platform using the keywords agarose and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Agarose is a linear polymer extracted from marine red algae constituted by (1→3)-β-D-galactopyranose-(1→4)-3,6-anhydro-α-L-galactopyranose units, being one of the two components that form agar.[104] Agarose forms a gel upon cooling of an agarose homogeneous solution below the coil-helix transition temperature when a 3D network of agarose fibers formed by helices develop.[105] This mechanism was previously suggested by different authors,[106,107] which suggested that the occurrence of double helices during cooling were responsible for the aggregation that produces the 3D hydrogel network due to hydrogen bonding and hydrophobic interactions. Nevertheless, other works have suggested that single chain formation would enable gel formation,[108,109] or the assembly of ternary complexes consisting of agarose-water-co solvent would lead to the same structure[110] The melting of agarose gels can occur at higher temperatures, normally around 85ºC.[105] Agarose can be processed without the use of harsh reagents in a relatively clean and simple process, and is non-toxic.[111] It is a neutral

polysaccharide which may bring some advantages in terms of non prejudicial interference with other materials or living tissues with which it may have contact.[104] Its gelling kinetics allow homogeneous distribution patterns in cell/drug encapsulation technologies[112] Some drawbacks include its lack of shapable stability, and poorly investigated biodegradability. Some concerns also relate with infectious security and lack of biocompatibility[112] Common uses of agarose are in biochemistry and molecular biology techniques, such as gel electrophoresis or chemotaxis studies.[113] The application of this material in cartilage regeneration is widely present in the literature. For instances, *in vitro* studies by Benya et al[114] showed that rabbit articular chondrocytes cultured in agarose gels were able to re-express the differentiated phenotype lost due to de-differentiation in monolayer culture. Agarose gels have also been used *in vivo* with allograft chondrocytes for repairing articular cartilage defects in rabbit knees. Control implants consisting of either empty defects or agarose with no cells produced fibrous tissue, insufficient healing and incomplete filling of the cartilage defects, while allograft chondrocytes in agarose formed superior repair cartilage in terms of type II collagen and proteoglycan content.[115] Agarose initial application in cartilage tissue engineering reports to 1994[116] and its use has slowly increased until 2002, time after which this tendency was much more pronounced, stabilising after 2004 until the present time (Figure 1).

5.3. ALGINATE

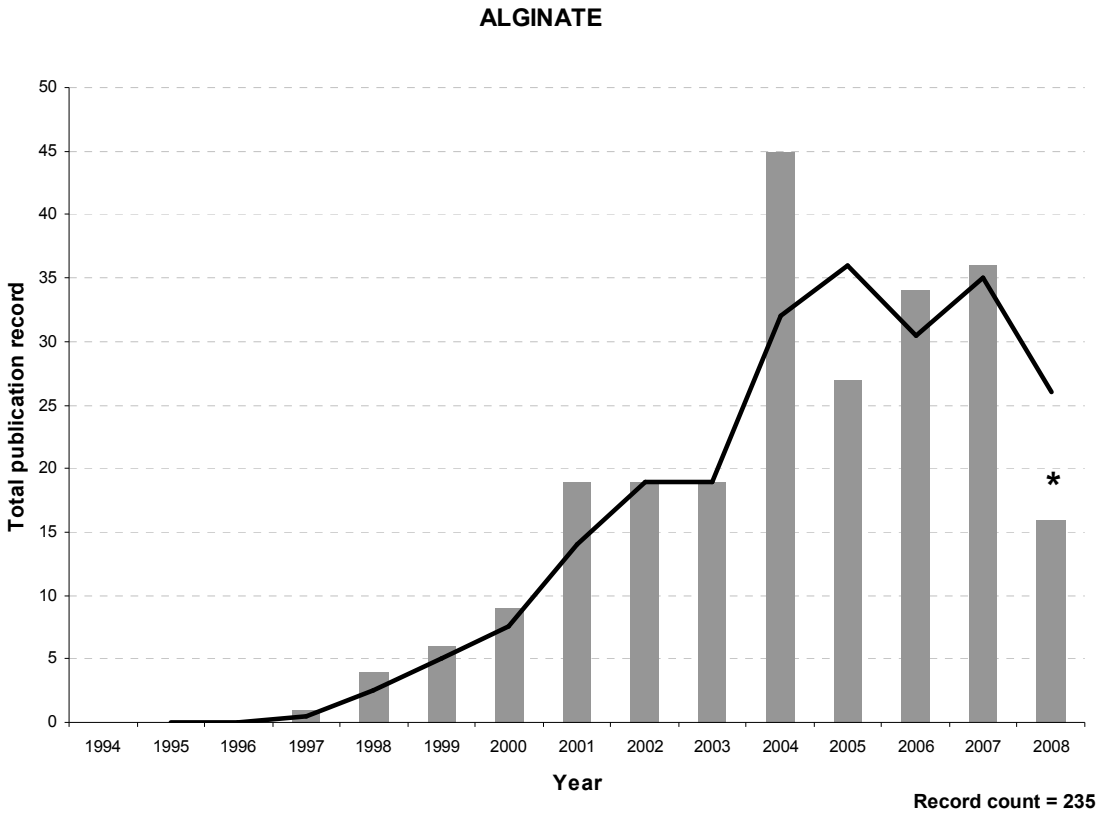

Figure I.2. Graphical representation of the search results performed with Web of Science® web platform using the keywords alginate and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Alginate is a natural anionic polysaccharide found in seaweed, which is composed of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid units. The alginate molecule is constituted by regions of sequential mannuronic acid units, guluronic acid units, or by a combination of both. The nature of the alginate dictates the amount and distribution profile of these units.[117,118] This material can forms gels in the presence of a small quantity of divalent cations (like Ca^{2+} or Ba^{2+}) that interact with the carboxylic groups present in the alginate backbone. These groups are present in the guluronic acid residues that when in contact with those ions form an "egg-box"-shaped structure giving rise to the hydrogel.[118] The formed hydrogel can be easily disrupted using a chelating agent (e.g. sodium citrate) which captures the cations that maintain the structural integrity of the network.[119] Alginate has a well characterized structure which allows for a range of comparative studies to be performed, and allows chemical modification through the carboxylic groups in its guluronic acid residues.[117] This last feature enabled that its lack of cell recognition

signals be overcome by chemically binding RGD peptides, which are extremely important regarding cell-material interactions.[117] On the other hand, associated disadvantages to the use of this material include the manufacturing process for the extraction of this polysaccharide from contaminated seaweed which leads to the presence of mitogenic, cytotoxic, and apoptosis inducing impurities in the final processed material. Even though such molecules can be removed by further purification steps, it is a time and money consuming process.[120] Moreover, variations in the mannuronic and guluronic acid composition in each individual sample confer variability to the processed samples.[121,122] This is an important issue since it has been shown that alginates with a high guluronic acid content develop a great inflammatory response.[123] Other drawbacks are common to other polysaccharide hydrogels and are related with inadequate mechanical properties and uncontrollable degradation profile.[118] General applications of alginate include its use as a mold-making material in dentistry, prosthetics, textiles, and in the food industry, for thickening soups and jellies.[124] In the tissue engineering field, the use of alginate for cartilage regeneration is well known. It has been used *in vitro* to encapsulate human articular chondrocytes and cultured in the presence of recombinant human BMP-2, which revealed to have positive effects on collagen type II expression.[125] *In vivo* tests have been conducted using alginate as a support for different relevant cell types that include primary culture chondrocytes and stem cells implanted in various animal models.[126-128] Bovine chondrocytes were mixed with a sodium alginate solution to create disks that were afterwards subcutaneously implanted in nude mice. Cartilage formation was observed and the histoarchitecture of the new cartilage resembled that of native cartilage.[126] Erickson et al[127] evaluated the chondrogenic potential of human adipose tissue-derived stromal cells when cultured in alginate gels and implanted subcutaneously in nude mice. Immunohistochemical analyses showed significant production of cartilage matrix molecules suggesting the potential of these systems in cartilage tissue engineering. Using the rabbit model, full-thickness defects were created and filled with alginate beads seeded with rabbit stromal cells. Histologic analysis showed viable, phenotypically chondrogenic cells in the defects embedded in a positively stained matrix for proteoglycans.[128] The application of alginate in cartilage tissue engineering dates back to 1997[129] and has since then revealed a constant increasing tendency until the present time (Figure 2).

5.4. CELLULOSE

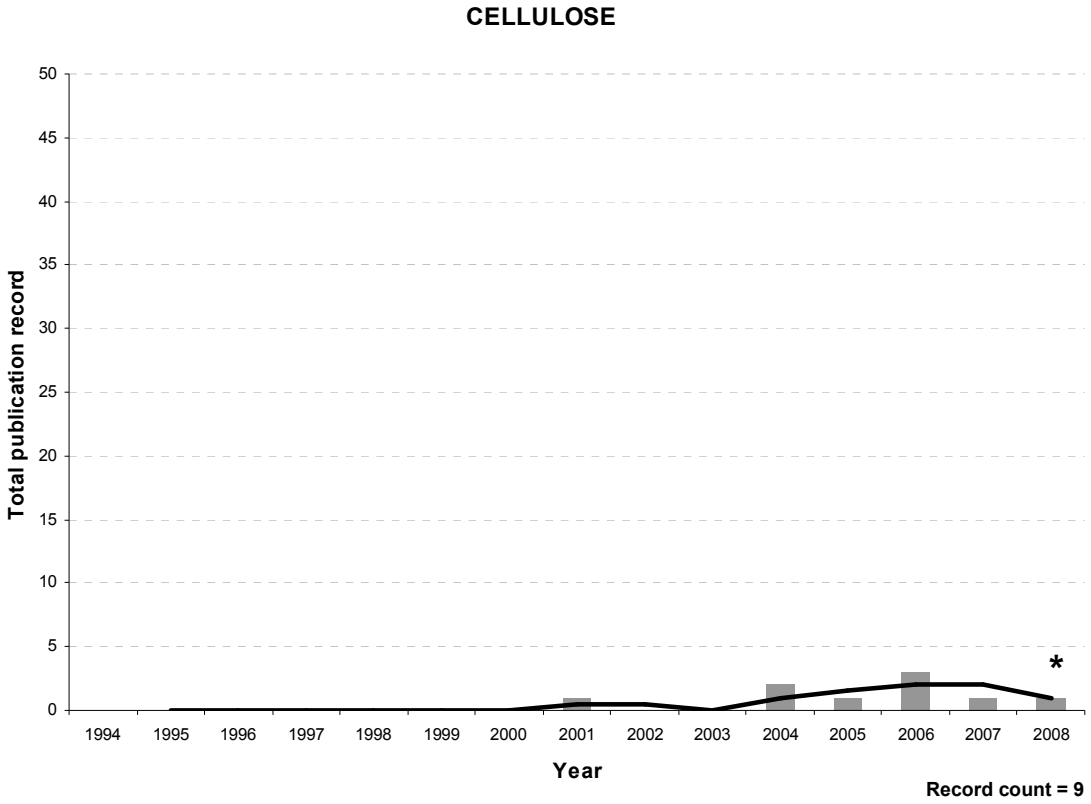

Figure I.3. Graphical representation of the search results performed with Web of Science® web platform using the keywords cellulose and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Cellulose is the most widespread polymeric material in nature. The most common is a fibrous, tough, water-insoluble material that can be found in cell walls of plants, mainly in stalks, stems, or trunks. Cellulose is composed of β-D-glucan units linked by (1→4) glycosidic bonds that are formed by a simple polymerization of glucose residues from a substrate such as UDP-glucose.[130,131] The stereochemistry conferred by the glycosidic linkage creates a linear extended glucan chain that enables a precise and ordered interaction between different chains. This material exists as a combination of various chains strongly linked by hydrogen bonding, named microfibrils, instead of a single chain, which contributes to its rigid structure.[131] The hydroxyl groups that hold the cellulose chains together account for the high degree of crystallinity, low solubility, and poor degradation *in vivo*. Cellulose possesses high strength in the wet state, and has been shown to be biocompatible.[132] In addition, it is not biodegradable due to lack of digestive enzymes in the human organism.[133,134]

Cellulose can also be found in microorganisms and the gram-negative bacterium *Acetobacter xylinum* has been used for this purpose.[135-137] This cellulose is structurally similar to the cellulose produced by plants and can be highly purified resembling in cristallinity and average microfibrillar width that from many plants and algae.[138] It constitutes a good alternative to plant and algae cellulose being also applied in different fields.[94,139,140] Bacterial cellulose contains approximately 90% water as prepared, but although this water can be easily squeezed out, the recovery in the swelling property is complicated by the hydrogen bonds.[141] Bacterial cellulose exhibits a rapid growth and the ability to be maintained under controlled conditions, which is another advantage.[138] On the other hand, the inherent low water solubility may compromise its degradation profiles both *in vitro* and *in vivo*.[138]

Cellulose has its major applications in the paper and textile industries.[142] Concerning the biomedical field, frequently cellulose derivatives, such as methylcellulose, hydroxypropylcellulose, and carboxymethylcellulose, are used as starting materials due to the new functionalities gained upon these modifications.[143,144] Applications of cellulose based materials in cartilage tissue engineering have been reported.[94,145] *In vitro* experiments performed by Svensson et al[94] revealed that bacterial cellulose supported bovine chondrocytes proliferation to some extent, while providing significant advantages in terms of mechanical properties. Most of the tests described using cellulose based supports for cartilage regeneration are performed *in vitro* and no significant *in vivo* data exist on this matter. *In vivo* studies have been however performed to assess the biocompatibility of a bacterial cellulose scaffold by subcutaneously implanting them in rats. There were no macroscopic or microscopic signs of inflammation around the implants, and no fibrotic capsule or giant cells were observed, being this a potential scaffold candidate for further *in vivo* screenings.[145]

Cellulose, independently of the source of origin, has a recent history in the cartilage regeneration field, starting in 2001[146] and exhibiting a mild increase until the present time (Figure 3).

5.5. CHITOSAN

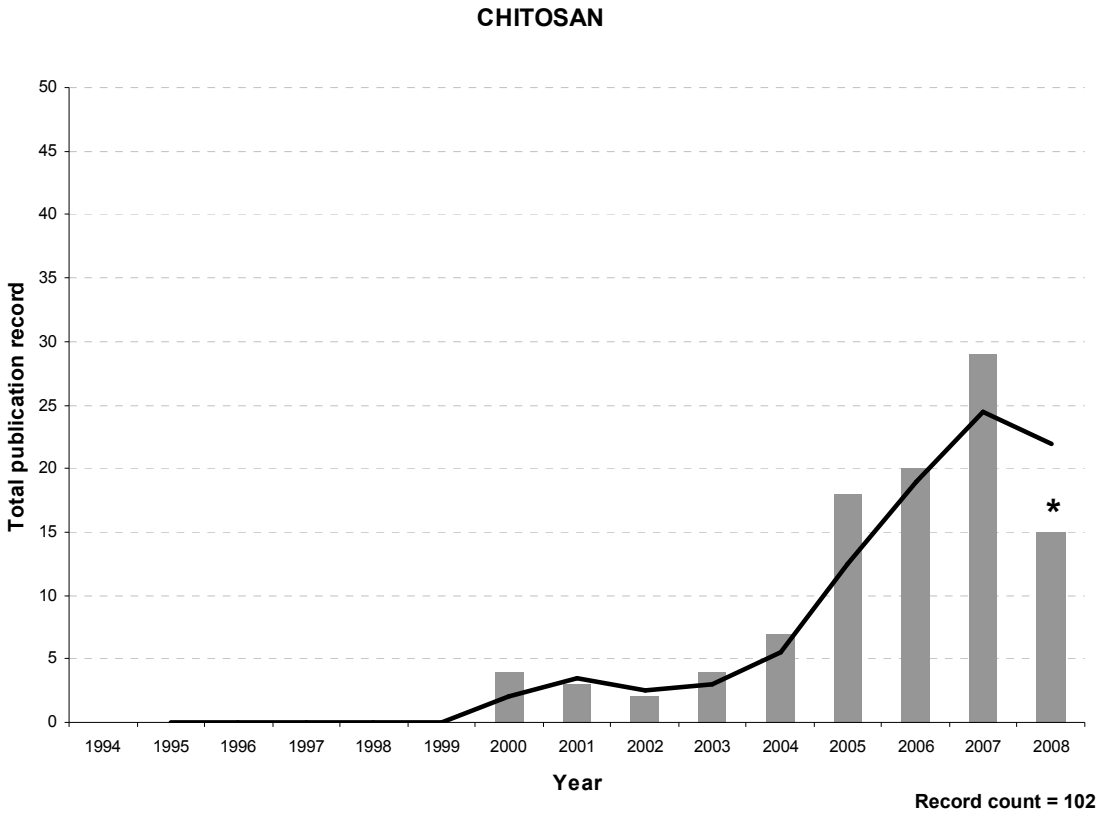

Figure I.4. Graphical representation of the search results performed with Web of Science® web platform using the keywords chitosan and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Chitosan is a semi-crystalline cationic polysaccharide that results from the partial alkaline deacetylation of chitin. It is constituted by β-1,4-linked 2-amino-2-deoxy-D-glucose and is normally insoluble in aqueous solutions above pH 7.[147] However, in dilute acids (pH 6), the free amine groups are protonated and the molecule becomes soluble. This pH-dependent solubility provides a convenient mechanism for processing under mild conditions.[148] Chitosan is widely suggested for biomedical applications[149-151] due to its intrinsic features such as hydrophilicity, or ready solubility in dilute acids which renders it more accessible for chemical reactions. This polymer can be easily produced due to the high annual production and great accessibility of chitin. It has been described to be non-toxic, biodegradable and have antibacterial activity.[152] It is also biocompatible, while possessing structural similarities to glycosaminoglycans, which are structural components of the cartilage extracellular matrix.[153-155] Associated disadvantages include the inadequate mechanical properties often exhibited by chitosan, as well as neutrophiles

recruitment ability, a feature normally linked with acute inflammation.[156] Even so, this appears not to influence its biocompatibility.[157] Chitosan serves different applications and its use ranges from the food industry[158] to the biomedical[159] and pharmaceutical fields.[160,161] It has been specially used for cartilage regeneration and the reported studies with this material have been increasing in the last years. *In vitro* studies performed by Malafaya et al[162] seeded human adipose derived mesenchymal stem cells onto chitosan particles agglomerated scaffolds, where these were shown to differentiate towards the chondrogenic lineage. Chenite et al[163] evaluated the *in vivo* performance of chitosan-glycerophosphate gels by mixing them with primary culture bovine chondrocytes and implanting subcutaneously in athymic mice. The implant area revealed several areas of remodeling chondrocytes secreting a matrix characteristic of normal cartilage. Other *in vivo* studies performed in nude mice and rabbits have employed chitosan developed in the form of an arthroscopically injectable vehicle for cell-assisted cartilage repair. The chitosan self-gelling solution was able to preserve chondrocyte viability and phenotype after injection, and to reside in the created articular defects *in vivo*.[55] Chitosan initial reports for use in cartilage tissue engineering precede cellulose.[89] Nonetheless, its increase has revealed substantial after 2004, a tendency that is still observed nowadays (Figure 4).

5.6. CHONDROITIN SULPHATE

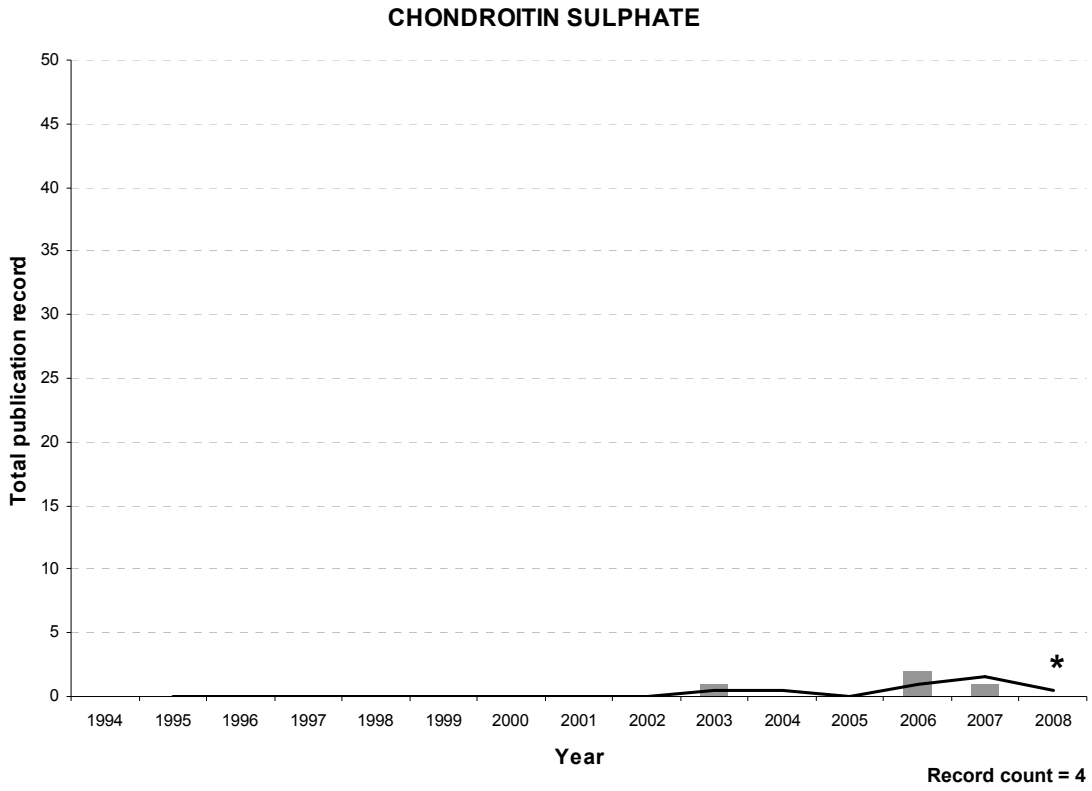

Figure I.5. Graphical representation of the search results performed with Web of Science® web platform using the keywords chondroitin sulphate and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of repeating dissacharide units of D-glucuronic acid and N-acetylgalactosamine.[20,164] These sugars can exist in wide extensions in a chondroitin chain and be sulfated in different positions. This molecule is an important structural component of the extracellular matrix of cartilaginous tissues, and by binding core protein gives rise to aggrecan, the most important proteoglycan in cartilage. The tightly packed and highly charged sulfate groups of chondroitin sulfate generate electrostatic repulsion that provides much of the resistance of cartilage to compression.[165] This cooperates in the functioning of aggrecan as a shock absorbing molecule.[20] Sources for chondroitin sulphate include extracts of cow trachea and pig ear and nose cartilage, although shark cartilage may also be used.[166]

Chondroitin sulphate is quite water soluble, which limits its use alone in the solid state for biomedical applications, being frequently combined with other polymers.[167-169] In fact, its anionic nature enables efficient interaction with cationic molecules to form interesting structures.[170] Its overall ability to function as a cell interacting molecule has widespread its use in a variety of biomedical applications. Chondroitin sulphate is currently used as an ingredient in dietary supplements,[171,172] with the ultimate goal of relieving some of the pain and disability of patients with musculoskeletal pathologies, namely osteoarthritis. Even so, none of its benefitial effect when compared to control groups has been proved, according to Clegg et al[173] Due to its nature, chondroitin sulfate has been used in the development of supports for cartilage tissue engineering applications. Sechriest et al[170] fabricated a chondroitin sulfate-chitosan support for chondrogenesis onto which bovine articular chondrocytes were seeded and were shown to form focal adhesions while maintaining a phenotype of differentiated chondrocytes with collagen type II and proteoglycan production. Although this is material is a component of natural hyaline cartilage, not so many works are found beyond the *in vitro* testing and even this is normally accompanied by coupling chondroitin sulphate with other materials.[169] The reasons for such occurrences probably lie in the high water solubility degree of chondroitin sulphate, which may pose important

hurdles to its use alone. In fact, the use of chondroitin sulphate is somehow recent with the first reports starting in 2003[174] and keeping a stable pattern until the present years (Figure 5).

5.7. GELLAN GUM

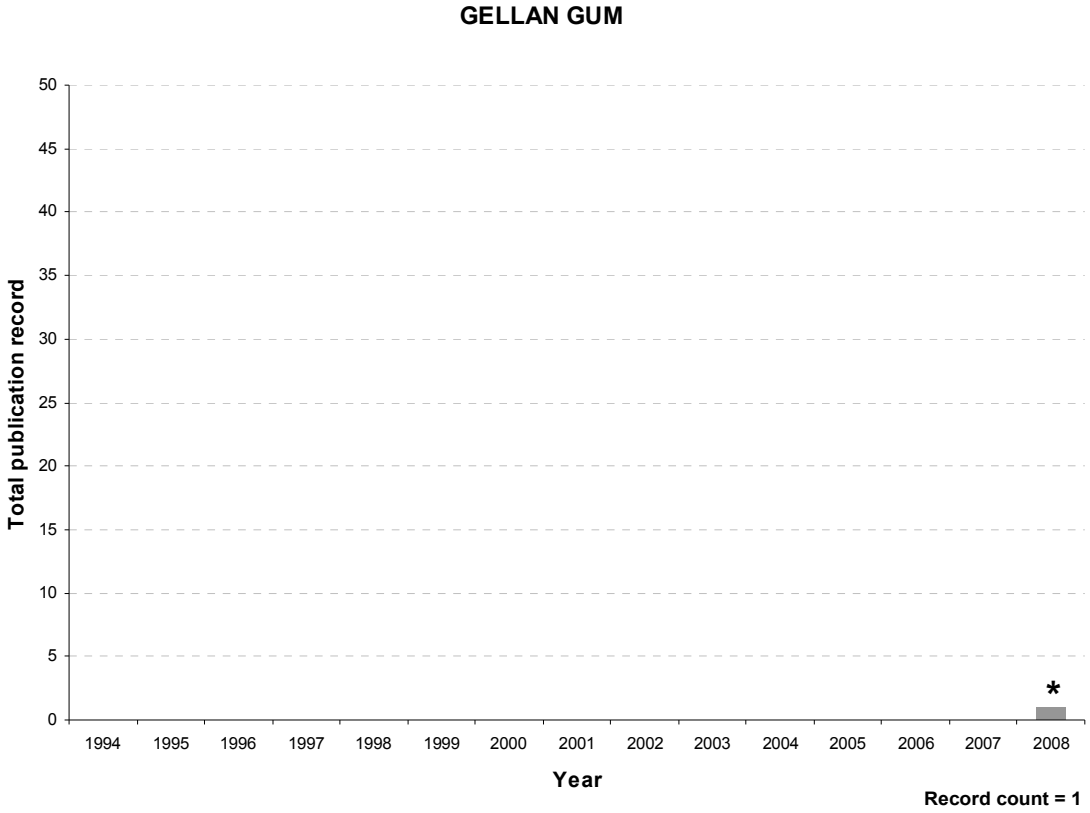

Figure I.6. Graphical representation of the search results performed with Web of Science® web platform using the keywords gellan gum and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Gellan gum is a linear anionic polysaccharide composed of tetrasaccharide repeating units of 1,3-β-D-glucose, 1,4-β–D-glucuronic acid, 1,4-β-D-glucose, 1,4-α-L-rhamnose, containing one carboxyl side group, and was initially described by Moorhouse et al[175,176] Gellan gum exists in two forms, acetylated - which is the initial product produced by *Sphingomonas paucimobilis*, and deacetylated - the processed and most common form. They form thermoreversible gels with differences in mechanical properties from soft and elastic for the acetylated form, to hard and brittle for the fully deacetylated polysaccharide.[177,178] Gellan gum has an ionotropic gelation,

similar to other polysaccharides, like alginate or carrageenan.[179,180] At high temperatures gellan gum is in the coil form, but upon temperature decrease transits to a double-helix. These helices self assemble to form oriented bundles, called junction zones. Afterwards, untwined regions of polysaccharide chains link the junction zones leading to the formation of a stable gel.[181] The gelation of gellan gum solutions is strongly influenced by the chemical nature and quantity of cations present in the solution. Divalent cations promote the gelation much more strongly than monovalent cations. In monovalent cations, the gelation is mainly a result of the screening of the electrostatic repulsion between the ionized carboxylate groups on the gellan gum chains. In the case of divalent cations, the gelation and aggregation of gellan occurs via a chemical bonding between divalent cations and two carboxylate groups belonging to glucuronic acid molecules in the Gellan chains, in adittion to the screening effect.[182] Gellan gum can be easily processed into transparent gels that are resistant to heat and acid stress without the use of harsh reagents.[183] It has been shown to be not cytotoxic[184] and can be used as an injectable system.[185] It has been previously used *in vivo* in human patients as an ocular drug delivery vehicle.[186,187] One major drawback includes its inadequate mechanical properties. Gellan gum gels are commonly used in the food industry as thickening agents or stabilizers[188] and in the biomedical field most applications are suggested for drug delivery approaches.[189,190] In tissue engineering, these materials have been suggested mostly for cartilage regeneration. Smith et al[191] performed an initial evaluation of gellan gum as a material for tissue engineering. Studies performed by Oliveira et al,[185,192] involved the encapsulation and *in vitro* culturing of human nasal chondrocytes and human articular chondrocytes in gellan gum hydrogels. In the first, gellan gum hydrogels were able to support nasal chondrocytes development; in the second, injectable gellan gum hydrogels were efficient in the encapsulation and support of human articular chondrocytes, while also enabling active extracellular matrix components synthesis as determined by realtime PCR and histological analysis. *In vivo* evaluation of gellan gum systems has been performed in nude mice and rabbit models. In the first, human adipose tissue derived cells subjected to different culturing conditions were encapsulated and subcutaneously implanted in the back of nude mice. Results evidenced the cells were viable and depositing extracellular matrix components when using human articular chondrocytes as a standard control, although a better performance was obtained with encapsulated human chondrocytes.[193] In the latter, adipose tissue derived cells were also applied mixed with gellan gum and injected in critical size defects using an autologous approach. Results were promising in terms of formation of a functional cartilage tissue engineered substitute when using specific combinations of adipose tissue derived cells and injectable gellan gum hydrogels.[194] Gellan gum is a new material in cartilage tissue engineering, and also in other

tissue engineering areas. So far, only one report is found in 2008[195] on its use as an injectable system for cartilage regeneration (Figure 6), but there is a probable tendency for increase due to other works in tissue engineering or related fields.[191,196]

5.8. HYALURONIC ACID

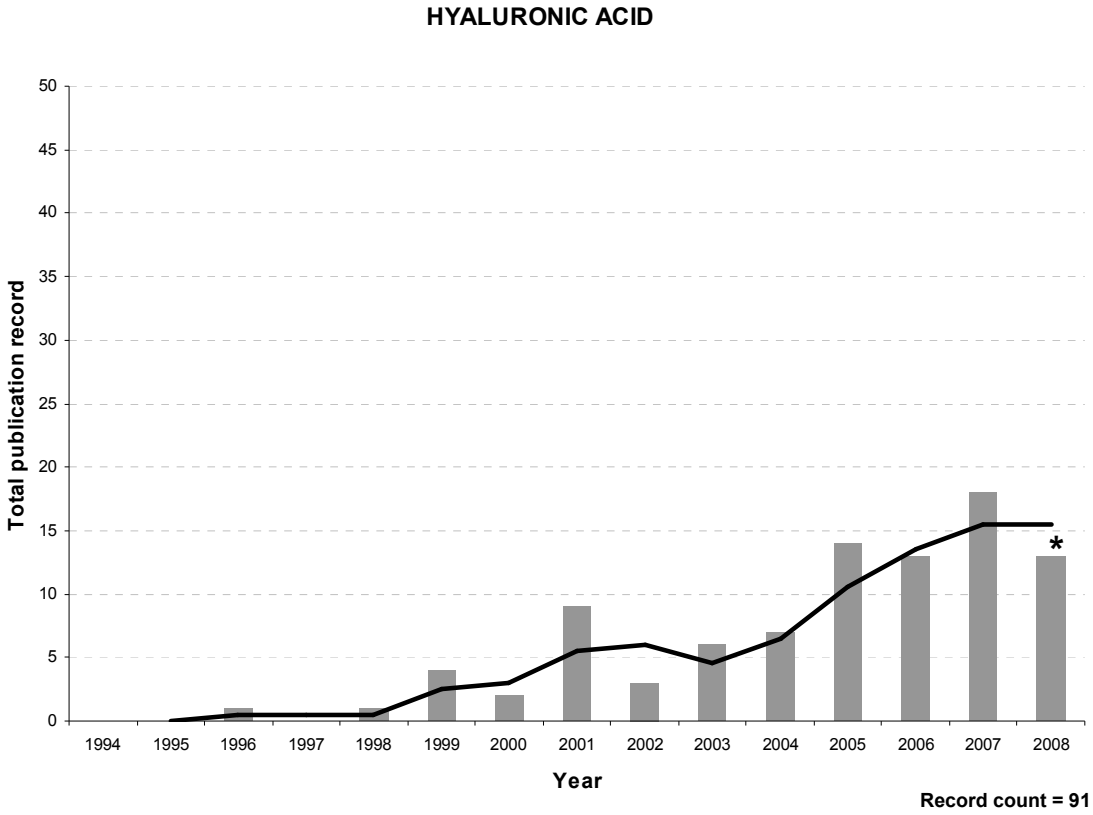

Figure I.7. Graphical representation of the search results performed with Web of Science® web platform using the keywords hyaluronic acid and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Hyaluronan is a non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. Hyaluronic acid is a linear, negatively charged polysaccharide constituted by a mixture of two sugars, glucuronic acid and N-acetyl glucosamine. It is linked together via alternating β-1,4 and β-1,3 glycosidic bonds constituting large molecules. Bulky groups on each sugar molecule are in sterically favored positions while the smaller hydrogens assume the less favorable axial positions, which confers them energetic stability.[197] Hyaluronic

acid may derive from different sources, being the most prevalent rooster combs[198] and by bacterial recombinant production.[199] They possess different qualities such as variations in rheological properties.[200] Hyaluronic acid is highly viscous in solution, but its shear dependent viscosity degree allows them to be injected through a small gauge needle.[201] This molecule is one of the chief components of the extracellular matrix, contributing significantly to cell proliferation and migration. Hyaluronic acid is soluble in water and is not resistant to enzymatic degradation. The modification of its chemical structure has granted specific attention to this material, mainly the formation of esters of hyaluronic acid through the esterification of its carboxylic groups.[202-204] This change enhances their processability and resistance to a range of conditions, enabling their processing into membranes, spheres, and different porous structures.[203-205] Hyaluronic acid has the possibility of being administered as an injectable system and is typically biocompatible *in vivo*.[206] As with other systems, inadequate mechanical properties are associated, and connection with malignant tumours progression and use as a tumour marker as been described.[207,208] Although hyaluronic acid has been used in the cosmetic industry, it is most widely applied for tissue engineering and related approaches, namely cartilage.[203,209] One example is the culturing of human nasoseptal chondrocytes on hyaluronic acid benzyl ester derived scaffolds (HYAFF®11) which evidenced the expression of collagen type II and indicated the ability of these cells to maintain a chondrocytic phenotype[210] In an *in vivo* scenario, benzyl ester hyaluronic acid derivatives (HYAFF® 11) were seeded with human cells and subcutaneously implanted *in vivo* in athymic nude mice. The results after 1 month revealed the development of tissue similar to hyaline cartilage.[210] In another study, autologous chondrocytes seeded on a hyaluronan derivative (Hyaff-11) were applied in the repair of full thickness defects created in rabbits, with the results evidencing efficacy on the repair of these lesions.[210] Hyaluronic acid is part of the pioneer group of polysaccharides proposed for cartilage tissue engineering applications. It is currently the most advanced polysaccharide in terms of clinical application studies.[211,212] It has been initially reported in 1996[213] and has since then revealed a constant increasing tendency until the present time (Figure 7).

5.9. STARCH

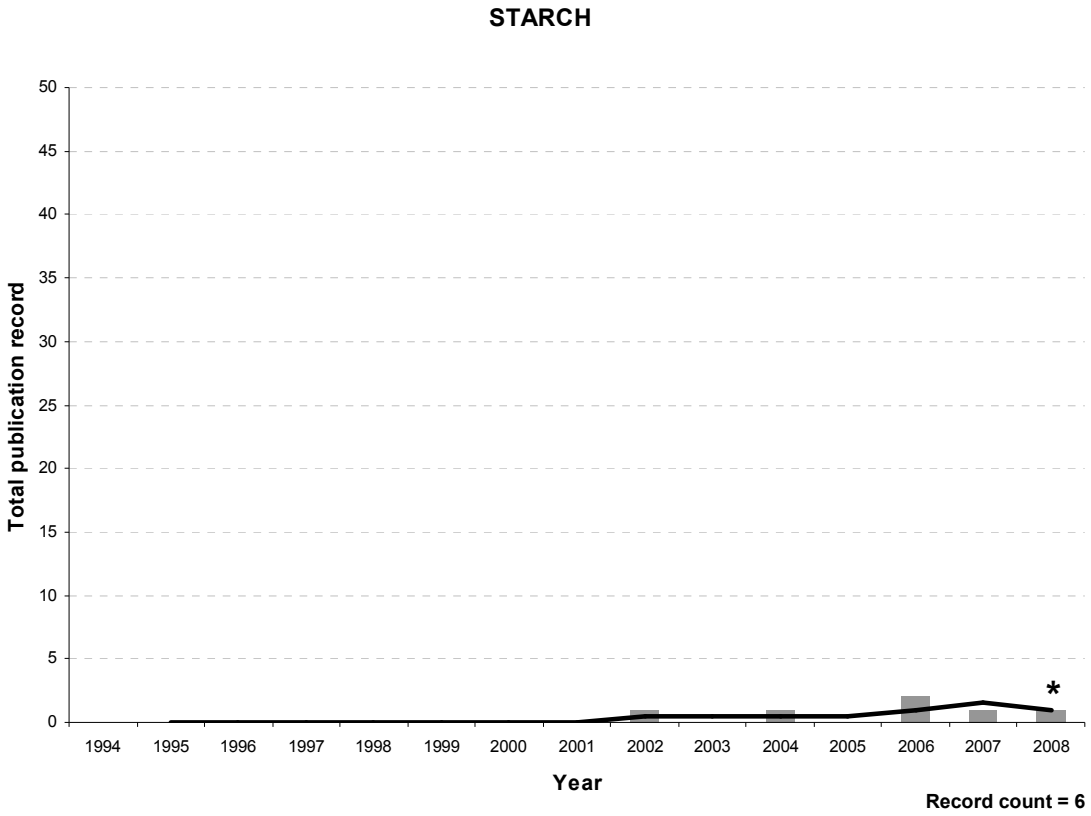

Figure I.8. Graphical representation of the search results performed with Web of Science® web platform using the keywords starch and cartilage tissue engineering. The variation throughout the years of the association of these two topics is presented. The data represents a search for the publications on all available years of search *until 12th July 2008 with a minimum threshold of 1.

Starch is a natural polymer made of a combination of two polymeric carbohydrates, amylose and amylopectin.[214,215] In terms of relative weight percentages, the amount of amylopectin is higher than amylose in common types of cereal endosperm starches.[216] Amylopectin is the highly branched component of starch, being formed of chains of α-D-glucopyranosyl units linked mainly by (1→ 4) linkages but with 5-6% of (1→ 6) bonds at the branch points. The overall composition has hundreds of short (1→ 4)-α-glucan chains interlinked by (1→ 6)-α-linkages.[216,217] Amylose is a linear molecule of (1→ 4) linked α-D-glucopyranosyl units, even though some molecules are slightly branched by (1→ 6)-α-linkages. Evidences suggest that the branched linkages are frequently located rather near the reducing terminal end and/or they have multiple branched chains.[218] Water soluble starches can be dispersed in water and upon heating form a paste. When a cooling regime is imposed, the starch paste increases in viscosity giving rise to a hydrogel caused by the physical crosslinking of hydrogen bonds.[219] Starch-based polymers are

degradable and biocompatible.[220] They exhibit distinct structural forms and properties that can be tailored by the other component of the starch-based blend. Moreover, they are an abundant and low cost product.[221] Some drawbacks associated with this material include its processability (namely by melt-based routes) that often implies the use of a synthetic polymer.[46,92,222] Starch is commonly used as a food thickening agent,[223,224] being also applied in the manufacturing of adhesives, paper, and textiles.[225] Cartilage regeneration approaches have been conducted using starch-based polymers.[226,227] Oliveira and Reis et al[226] seeded bovine articular chondrocytes onto starch-polycaprolactone fiber mesh scaffolds under dynamic conditions and observed active cells proliferation, as well as deposition of specific extracellular matrix components, such as collagen type II. Their use *in vivo* is not so widespread but some studies have been put forward for studying starch based blends in vivo behaviour.[228,229] One example is the work by Salgado et al[229] that implanted starch based blends in rats in order to evaluate the in vivo endosseous response of three different structures. Starch has a recent history in the cartilage regeneration field, starting in 2002[230] during the same period of cellulose and chitosan, exhibiting a consistent increase until the present time (Figure 8).

6. FINAL REMARKS

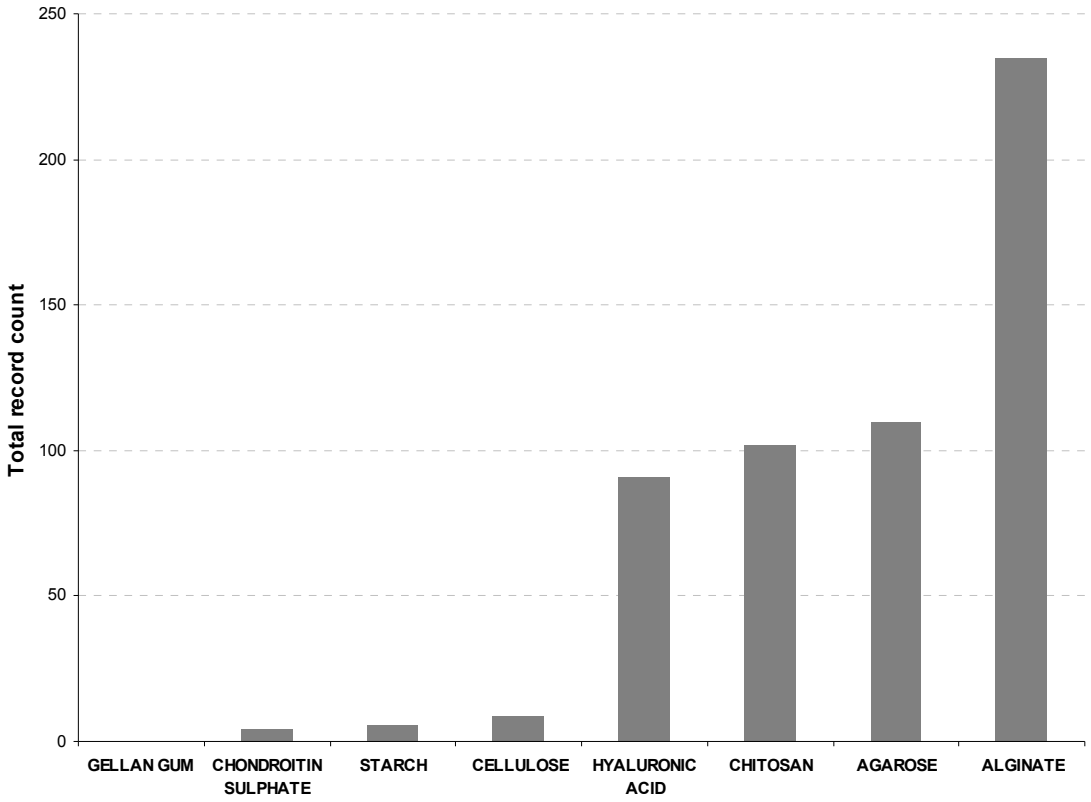

Figure I.9. Graphical representation of the search results performed with Web of Science® web platform using the total record count for each search presented in figures 1-8.

Materials used in tissue engineering approaches should allow proper conditions for cells viability and function, and once implanted in a living organism must not elicit a severe inflammatory and immune response from the host contributing to the formation of a well integrated functional tissue.[231,232] The use of polysaccharides has been increasing in the biomedical field, mainly in cartilage tissue engineering. The analyses performed using Web of Science® web platform reveal an increasing tendency for the application of these materials in cartilage regeneration as presented on Figures 1-9. Two subsets can be clearly identified regard their prevalence in the cartilage tissue engineering field, being the first led by alginate and followed by agarose, chitosan, and hyaluronic acid, and the second gathering the other polysaccharides referred, namely cellulose, starch, chondroitin sulphate, and gellan gum. Some of the latter have only recently been proposed which reflects the few records present in the literature. According to the data collected from the literature, the reasons for using polysaccharides in cartilage regeneration applications lie in their intrinsic features such as chemical similarity with native tissue components, non harsh processing, variable hydrophilic degrees, and biocompatibility, for

example. Hyaluronic acid and chondroitin sulphate exist naturally in cartilage extracellular matrix, and chitosan and gellan gum possess glucosamine and glucuronic acid, respectively, which are present in the extracellular matrix glycosaminoglycans structure. The extended variety of existing polysaccharides and their inherent features opens a wide range of opportunities for synergistic fabrications of new and multifunctional materials. Combinations of anionic and cationic polymers may form an interesting new structure, confer a determined function through the binding of bioactive agents of interest, or generate support systems with enhanced abilities in the regeneration of a chosen tissue. Not every characteristic comes as an advantage though and some problems still exist in some polysaccharides, as for example easy solubility in water that may jeopardise the formation of a stable hydrogel, or in some cases the low mechanical stiffness, which are common trends among these materials. Nonetheless, research on polysaccharide based materials for cartilage tissue engineering applications is continuously increasing and improving and alternative strategies for some of these problems encompass the fabrication of multimaterial systems or modification of the original structures. Chitosan has been combined with chondroitin sulphate in order to promote an improved support for chondrogenesis.[89] Another example is alginate that was coupled with PLGA and tested *in vitro* and *in vivo* with human adipose derived stem cells for cartilage regeneration.[233] The addition of bioactive agents of interest is another way to confer a better outcome performance to the overall tissue engineered constructs.[206,233]

This paper has described a range of polysaccharides used in cartilage tissue engineering research using relevant cells types in *in vitro* and *in vivo* scenarios and showed their evolution in the field throughout the years. Studies performed so far have ultimately been taken through *in vivo* testing of polysaccharide based materials in simulated clinical scenarios. Some of the herein described polysaccharides have been extensively evaluated in different research units worldwide and should be put forward to clinical trials in order to maximise the generated knowledge and reach the fabrication of medical products and technologies that make use of the developed systems and concepts.

REFERENCES

[1.] Brundtland, G. H., A WHO Scientific Group on the Burden of Musculoskeletal Conditions at the Start of the New Millennium met in Geneva from 13 to 15 January 2000., *Burden of Musculoskeletal Conditions at the Start of the New Millennium* 919, 1-218, 2003.

[2.] Altman, R., Asch, E., Bloch, D., Bole, G., Borenstein, D., Brandt, K., Christy, W., Cooke, T. D., Greenwald, R., Hochberg, M., Howell, D., Kaplan, D., Koopman, W., Longley, S., Mankin, H., McShane, D. J., Medsger, T., Meenan, R., Mikkelsen, W., Moskowitz, R., Murphy, W., Rothschild, B., Segal, M., Sokoloff, L., and Wolfe, F., Development of criteria for the classification and reporting of osteoarthritis-classification of osteoarthritis of the knee *Arthritis and Rheumatism* 29 (8), 1039-1049, 1986.

[3.] Lawrence, R. C., Helmick, C. G., Arnett, F. C., Deyo, R. A., Felson, D. T., Giannini, E. H., Heyse, S. P., Hirsch, R., Hochberg, M. C., Hunder, G. G., Liang, M. H., Pillemer, S. R., Steen, V. D., and Wolfe, F., Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States, *Arthritis and Rheumatism* 41 (5), 778-799, 1998.

[4.] Laurencin, C. T., Ambrosio, A. M. A., Borden, M. D., and Cooper, J. A., Tissue Engineering: Orthopedic Applications, *Annual Review of Biomedical Engineering* 1 (1), 19-46, 1999.

[5.] Steadman, J. R., Rodkey, W. G., and Rodrigo, J. J., Microfracture: surgical technique and rehabilitation to treat chondral defects, *Clin Orthop Relat Res* (391 Suppl), S362-9, 2001.

[6.] Boyd, A. D., Ewald, F. C., Thomas, W. H., Poss, R., and Sledge, C. B., Long-term complications after total knee arthroplasty with or without resurfacing of the patella *Journal of Bone and Joint Surgery-American Volume* 75A (5), 674-681, 1993.

[7.] Holland, T. A. and Mikos, A. G., Advances in drug delivery for articular cartilage, *J Control Release* 86 (1), 1-14, 2003.

[8.] Anderson, A. F., Richards, D. B., Pagnani, M. J., and Hovis, W. D., Antegrade drilling for osteochondritis dissecans of the knee, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 13 (3), 319-324, 1997.

[9.] Howard A. Breinan, S. D. M. H.-P. H. M. S., Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes, *Journal of Orthopaedic Research* 18 (5), 781-789, 2000.

[10.] Langer, R. and Vacanti, J. P., Tissue engineering, *Science* 260 (5110), 920-6, 1993.

[11.] Reinholz, G. G., Lu, L., Saris, D. B. F., Yaszemski, M. J., and O'Driscoll, S. W., Animal models for cartilage reconstruction, *Biomaterials* 25 (9), 1511-1521, 2004.

[12.] Cancedda, R., Descalzi Cancedda, F., and Castagnola, P., Chondrocyte differentiation, *Int Rev Cytol* 159, 265-358, 1995.

[13.] Griffith, L. G. and Naughton, G., Tissue Engineering--Current Challenges and Expanding Opportunities, *Science* 295 (5557), 1009-1014, 2002.

[14.] Risbud, M. V. and Sittinger, M., Tissue engineering: advances in in vitro cartilage generation, *Trends Biotechnol* 20 (8), 351-6, 2002.

[15.] Tuzlakoglu, K., Alves, C. M., Mano, J. F., and Reis, R. L., Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications, *Macromolecular Bioscience* 4 (8), 811-819, 2004.

[16.] Lee, C. R., Breinan, H. A., Nehrer, S., and Spector, M., Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior in vitro, *Tissue Eng* 6 (5), 555-65, 2000.

[17.] Hutmacher, D. W., Scaffolds in tissue engineering bone and cartilage, *Biomaterials* 21 (24), 2529-2543, 2000.

[18.] Freed, L. E., Langer, R., Martin, I., Pellis, N. R., and Vunjak-Novakovic, G., Tissue engineering of cartilage in space, *Proc Natl Acad Sci U S A* 94 (25), 13885-90, 1997.

[19.] Gomes, M. E., Sikavitsas, V. I., Behravesh, E., Reis, R. L., and Mikos, A. G., Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds, *Journal of Biomedical Materials Research Part A* 67A (1), 87-95, 2003.

[20.] Malafaya, P. B., Silva, G. A., and Reis, R. L., Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications, *Advanced Drug Delivery Reviews* 59 (4-5), 207-233, 2007.

[21.] Knudsen, K. E. B., Jensen, B. B., and Hansen, I., Digestion of polysaccharides and other major components in the small and large-intestine of pigs fed on diets consisting of oat fractions rich in beta-d-glucan, *British Journal of Nutrition* 70 (2), 537-556, 1993.

[22.] Zimmer, C., Fossil offers a glimpse into mammals' past, *Science* 283 (5410), 1989-90, 1999.

[23.] Zimmer, C., Evolution. In search of vertebrate origins: beyond brain and bone, *Science* 287 (5458), 1576-9, 2000.

[24.] Archer, C. W. and Francis-West, P., The chondrocyte, *Int J Biochem Cell Biol* 35 (4), 401-4, 2003.

[25.] Buckwalter, J. A., Martin, J., and Mankin, H. J., Synovial joint degeneration and the syndrome of osteoarthritis, *Instr Course Lect* 49, 481-9, 2000.

[26.] Reginato, A. M. and Olsen, B. R., The role of structural genes in the pathogenesis of osteoarthritic disorders, *Arthritis Res* 4 (6), 337-45, 2002.

[27.] Ge, Z., Hu, Y., Heng, B. C., Yang, Z., Ouyang, H., Lee, E. H., and Cao, T., Osteoarthritis and therapy, *Arthritis Rheum* 55 (3), 493-500, 2006.

[28.] Knudson, C. B. and Knudson, W., Cartilage proteoglycans, *Seminars in Cell & Developmental Biology* 12 (2), 69-78, 2001.

[29.] Newman, A. P., Articular cartilage repair, *Am J Sports Med* 26 (2), 309-24, 1998.

[30.] Christgau, S. and Cloos, P. A. C., Cartilage degradation products as markers for evaluation of patients with rheumatic disease, *Clinical and Applied Immunology Reviews* 4 (4), 277, 2004.

[31.] Arkill, K. P. and Winlove, C. P., Solute transport in the deep and calcified zones of articular cartilage, *Osteoarthritis and Cartilage* 16 (6), 708-714, 2008.

[32.] Rodan, G. A. and Martin, T. J., Therapeutic approaches to bone diseases, *Science* 289 (5484), 1508-14, 2000.

[33.] Goltzman, D., Discoveries, drugs and skeletal disorders, *Nat Rev Drug Discov* 1 (10), 784-96, 2002.

[34.] Reynolds, D. L., Chambers, L. W., Badley, E. M., Bennett, K. J., Goldsmith, C. H., Jamieson, E., Torrance, G. W., and Tugwell, P., Physical-Disability among Canadians Reporting Musculoskeletal Diseases, *Journal of Rheumatology* 19 (7), 1020-1030, 1992.

[35.] Firestein, G. S., Evolving concepts of rheumatoid arthritis, *Nature* 423 (6937), 356-61, 2003.

[36.] Hyllested, J. L., Veje, K., and Ostergaard, K., Histochemical studies of the extracellular matrix of human articular cartilage--a review, *Osteoarthritis Cartilage* 10 (5), 333-43, 2002.

[37.] Wakitani, S., Goto, T., Pineda, S. J., Young, R. G., Mansour, J. M., Caplan, A. I., and Goldberg, V. M., Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage, *J Bone Joint Surg Am* 76 (4), 579-92, 1994.

[38.] Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L., Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, *N Engl J Med* 331 (14), 889-95, 1994.

[39.] Perka, C., Schultz, O., Spitzer, R. S., and Lindenhayn, K., The influence of transforming growth factor beta1 on mesenchymal cell repair of full-thickness cartilage defects, *J Biomed Mater Res* 52 (3), 543-52, 2000.

[40.] Kim, B. S. and Mooney, D. J., Development of biocompatible synthetic extracellular matrices for tissue engineering, *Trends Biotechnol* 16 (5), 224-30, 1998.

[41.] Duda, G. N., Haisch, A., Endres, M., Gebert, C., Schroeder, D., Hoffmann, J. E., and Sittinger, M., Mechanical quality of tissue engineered cartilage: results after 6 and 12 weeks in vivo, *J Biomed Mater Res* 53 (6), 673-7, 2000.

[42.] Chang, S. C., Rowley, J. A., Tobias, G., Genes, N. G., Roy, A. K., Mooney, D. J., Vacanti, C. A., and Bonassar, L. J., Injection molding of chondrocyte/alginate constructs in the shape of facial implants, *J Biomed Mater Res* 55 (4), 503-11, 2001.

[43.] Vacanti, J. P., Langer, R., Upton, J., and Marler, J. J., Transplantation of cells in matrices for tissue regeneration, *Adv Drug Deliv Rev* 33 (1-2), 165-182, 1998.

[44.] Temenoff, J. S. and Mikos, A. G., Injectable biodegradable materials for orthopedic tissue engineering, *Biomaterials* 21 (23), 2405-12, 2000.

[45.] Hunziker, E. B., Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects, *Osteoarthritis Cartilage* 10 (6), 432-63, 2002.

[46.] Oliveira, J. T., Crawford, A., Mundy, J. M., Moreira, A. R., Gomes, M. E., Hatton, P. V., and Reis, R. L., A cartilage tissue engineering approach combining starch-polycaprolactone fibre

mesh scaffolds with bovine articular chondrocytes, *Journal of Materials Science-Materials in Medicine* 18 (2), 295-302, 2007.

[47.] Brandl, F., Sommer, F., and Goepferich, A., Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior, *Biomaterials* 28 (2), 134-146, 2007.

[48.] Perka, C., Spitzer, R. S., Lindenhayn, K., Sittinger, M., and Schultz, O., Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants, *J Biomed Mater Res* 49 (3), 305-11, 2000.

[49.] Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M., and Guilak, F., Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds, *Biomaterials* 25 (16), 3211-3222, 2004.

[50.] Malafaya, P. B., Pedro, A. J., Peterbauer, A., Gabriel, C., Redl, H., and Reis, R. L., Chitosan particles agglomerated scaffolds for cartilage and osteochondral tissue engineering approaches with adipose tissue derived stem cells, *Journal of Materials Science: Materials in Medicine* V16 (12), 1077-1085, 2005.

[51.] Dunkelman, N. S., Zimber, M.P., LeBaron, R.G., Pavelec, R., Kwan, M., Purchio, A.F., , Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system., *Biotechnology and Bioengineering* 46 (4), 299-305, 1995.

[52.] Gugala, Z. and Gogolewski, S., In vitro growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold, *Journal of Biomedical Materials Research* 49 (2), 183-191, 2000.

[53.] Bryant, S. J., Bender, R. J., Durand, K. L., and Anseth, K. S., Encapsulating chondrocytes in degrading PEG hydrogels with high modulus: engineering gel structural changes to facilitate cartilaginous tissue production, *Biotechnol Bioeng* 86 (7), 747-55, 2004.

[54.] Schuller, G. C., Tichy, B., Majdisova, Z., Jagersberger, T., van Griensven, M., Marlovits, S., and Redl, H., An in vivo mouse model for human cartilage regeneration, *J Tissue Eng Regen Med* 2 (4), 202-9, 2008.

[55.] Hoemann, C. D., Sun, J., Légaré, A., McKee, M. D., and Buschmann, M. D., Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle, *Osteoarthritis and Cartilage* 13 (4), 318-329, 2005.

[56.] Wang, Y.-C., Lin, M.-C., Wang, D.-M., and Hsieh, H.-J., Fabrication of a novel porous PGA-chitosan hybrid matrix for tissue engineering, *Biomaterials* 24 (6), 1047-1057, 2003.

[57.] Kubo, K. and Kuroyanagi, Y., Spongy matrix of hyaluronic acid and collagen as a cultured dermal substitute: evaluation in an animal test, *Journal of Artificial Organs* 6 (1), 64-70, 2003.

[58.] Gomes, M. E., Godinho, J. S., Tchalamov, D., Cunha, A. M., and Reis, R. L., Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties, *Materials Science and Engineering: C* 20 (1-2), 19-26, 2002.

[59.] Moroni, L., Schotel, R., Sohier, J., de Wijn, J. R., and van Blitterswijk, C. A., Polymer hollow fiber three-dimensional matrices with controllable cavity and shell thickness, *Biomaterials* 27 (35), 5918-5926, 2006.

[60.] Anna C. Jen, M. Conley Wake, and Antonios G. Mikos, Review: Hydrogels for cell immobilization, 1996, pp. 357-364.

[61.] Coviello, T., Matricardi, P., Alhaique, F, Drug delivery strategies using polysaccharidic gels, *Expert Opinion on Drug Delivery* 3 (3), 395-404, 2006.

[62.] Kisiday, J., Jin, M., Kurz, B., Hung, H., Semino, C., Zhang, S., and Grodzinsky, A. J., Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair, *PNAS* 99 (15), 9996-10001, 2002.

[63.] Passaretti, D., Silverman, R. P., Huang, W., Kirchhoff, C. H., Ashiku, S., Randolph, M. A., and Yaremchuk, M. J., Cultured Chondrocytes Produce Injectable Tissue-Engineered Cartilage in Hydrogel Polymer, *Tissue Engineering* 7 (6), 805-815, 2001.

[64.] Ratner, B. D., Hoffman, A.S., Schoen, F.J., Lemons, J.E., *Biomaterials Science: An Introduction to Materials in Medicine* Academic Press, Inc, New York, 1996.

[65.] Prabaharan, M. and Mano, J. F., Stimuli-responsive hydrogels based on polysaccharides incorporated with thermo-responsive polymers as novel biomaterials, *Macromolecular Bioscience* 6 (12), 991-1008, 2006.

[66.] Park, K., Shalaby, W.S., Park, H., *Biodegradable hydrogels for drug delivery* Technomic, Lancaster, 1993.

[67.] Hoffman, A. S., Hydrogels for Biomedical Applications, *Advanced Drug Delivery Reviews* 54 (1), 3-12, 2002.

[68.] Mauck, R. L., Yuan, X., and Tuan, R. S., Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture, *Osteoarthritis and Cartilage* 14 (2), 179-189, 2006.

[69.] Cancedda, R., Dozin, B., Giannoni, P., and Quarto, R., Tissue engineering and cell therapy of cartilage and bone, *Matrix Biol* 22 (1), 81-91, 2003.

[70.] Jakob, M., Demarteau, O., Schafer, D., Hintermann, B., Dick, W., Heberer, M., and Martin, I., Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro, *Journal of Cellular Biochemistry* 81 (2), 368-377, 2001.

[71.] Vunjak-Novakovic, G. and Freed, L. E., Culture of organized cell communities, *Adv Drug Deliv Rev* 33 (1-2), 15-30, 1998.

[72.] Francesco Dell'Accio, C. D. B. F. P. L., Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo, *Arthritis & Rheumatism* 44 (7), 1608-1619, 2001.

[73.] Stefan Bajada, I. M. J. B. R. N. A., Updates on stem cells and their applications in regenerative medicine, *Journal of Tissue Engineering and Regenerative Medicine* 2 (4), 169-183, 2008.

[74.] Bianco, P. and Robey, P. G., Stem cells in tissue engineering, *Nature* 414 (6859), 118-121, 2001.

[75.] Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M., Embryonic stem cell lines derived from human blastocysts, *Science* 282 (5391), 1145-1147, 1998.

[76.] Becker, A. J., Till, J. E., and McCulloch, E. A., Cytological demonstration of clonal nature of spleen colonies derived from transplanted mouse marrow cells, *Nature* 197 (486), 452-&, 1963.

[77.] Betre, H., Ong, S. R., Guilak, F., Chilkoti, A., Fermor, B., and Setton, L. A., Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide, *Biomaterials* 27 (1), 91-9, 2006.

[78.] Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T., Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold, *Biochemical and Biophysical Research Communications* 322 (1), 50, 2004.

[79.] Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H., Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng* 7 (2), 211-28, 2001.

[80.] van der Kraan, P. M., Buma, P., van Kuppevelt, T., and van den Berg, W. B., Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering, *Osteoarthritis Cartilage* 10 (8), 631-7, 2002.

[81.] Sekiya, I., Vuoristo, J. T., Larson, B. L., and Prockop, D. J., In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis, *Proc Natl Acad Sci U S A* 99 (7), 4397-402, 2002.

[82.] Deans, R. J. and Moseley, A. B., Mesenchymal stem cells: Biology and potential clinical uses, *Experimental Hematology* 28 (8), 875, 2000.

[83.] Manas K. Majumdar, V. B. D. P. P. E. A. M., Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells, *Journal of Cellular Physiology* 185 (1), 98-106, 2000.

[84.] Wakitani, S., Nawata, M., Tensho, K., Okabe, T., Machida, H., and Ohgushi, H., Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees, *J Tissue Eng Regen Med* 1 (1), 74-9, 2007.

[85.] Gimble, J. M., Guilak, F., and Schatten, G. P., Differentiation Potential of Adipose Derived Adult Stem (ADAS) Cells, in *Current Topics in Developmental Biology* Academic Press, 2003, pp. 137-160.

[86.] Peister, A., Mellad, J. A., Larson, B. L., Hall, B. M., Gibson, L. F., and Prockop, D. J., Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential, *Blood* 103, 1662 - 1668, 2004.

[87.] Martin, I., Padera, R. F., Vunjak-Novakovic, G., and Freed, L. E., In vitro differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues, *J Orthop Res* 16 (2), 181-9, 1998.

[88.] Chang, C.-H., Liu, H.-C., Lin, C.-C., Chou, C.-H., and Lin, F.-H., Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering, *Biomaterials* 24 (26), 4853-4858, 2003.

[89.] Sechriest, V. F., Miao, Y. J., Niyibizi, C., Westerhausen-Larson, A., Matthew, H. W., Evans, C. H., Fu, F. H., and Suh, J. K., GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis, *J Biomed Mater Res* 49 (4), 534-41, 2000.

[90.] Girotto, D., Urbani, S., Brun, P., Renier, D., Barbucci, R., and Abatangelo, G., Tissue-specific gene expression in chondrocytes grown on three-dimensional hyaluronic acid scaffolds, *Biomaterials* 24 (19), 3265-3275, 2003.

[91.] Campoccia, D., Doherty, P., Radice, M., Brun, P., Abatangelo, G., and Williams, D. F., Semisynthetic resorbable materials from hyaluronan esterification, *Biomaterials* 19 (23), 2101, 1998.

[92.] Gomes, M. E., Azevedo, H. S., Moreira, A. R., Ella, V., Kellomaki, M., and Reis, R. L., Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: structure, mechanical properties and degradation behaviour, *J Tissue Eng Regen Med* 2 (5), 243-52, 2008.

[93.] Müller, F. A., Müller, L., Hofmann, I., Greil, P., Wenzel, M. M., and Staudenmaier, R., Cellulose-based scaffold materials for cartilage tissue engineering, *Biomaterials* 27 (21), 3955-3963, 2006.

[94.] Svensson, A., Nicklasson, E., Harrah, T., Panilaitis, B., Kaplan, D. L., Brittberg, M., and Gatenholm, P., Bacterial cellulose as a potential scaffold for tissue engineering of cartilage, *Biomaterials* 26 (4), 419-431, 2005.

[95.] Funami, T., Noda, S., Hiroe, M., Asai, I., Ikeda, S., and Nishinari, K., Functions of iota-carrageenan on the gelatinization and retrogradation behaviors of corn starch in the presence or absence of various salts, *Food Hydrocolloids* 22 (7), 1273-1282, 2008.

[96.] Goñi, I., García-Diz, L., Mañas, E., and Saura-Calixto, F., Analysis of resistant starch: a method for foods and food products, *Food Chemistry* 56 (4), 445-449, 1996.

[97.] http://www.cargill.com/products/food/ps_food_starch.htm.

[98.] http://shopping.aol.com/estee-lauder-re-nutriv-intensive-lifting-serum-1floz30ml/65052635.

[99.] Vlachou, S., Politou, A., Dais, P., Mazeau, K., and Taravel, F. R., Structure and dynamics of the branched polysaccharide scleroglucan in dilute solutions studied by 1D and 2D NMR spectroscopy, *Carbohydrate Polymers* 46 (4), 349-363, 2001.

[100.] Amici, E., Clark, A. H., Normand, V., and Johnson, N. B., Interpenetrating network formation in agarose-sodium gellan gel composites, *Carbohydrate Polymers* 46 (4), 383-391, 2001.

[101.] Santos, M. I., Fuchs, S., Gomes, M. E., Unger, R. E., Reis, R. L., and Kirkpatrick, C. J., Response of micro- and macrovascular endothelial cells to starch-based fiber meshes for bone tissue engineering, *Biomaterials* 28 (2), 240-248, 2007.

[102.] Ayelet Dar, M. S. J. L. S. C., Cardiac tissue engineering Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds, *Biotechnology and Bioengineering* 80 (3), 305-312, 2002.

[103.] Kataoka, K., Suzuki, Y., Kitada, M., Hashimoto, T., Chou, H., Bai, H., Ohta, M., Wu, S., Suzuki, K., and Ide, C., Alginate Enhances Elongation of Early Regenerating Axons in Spinal Cord of Young Rats, *Tissue Engineering* 10 (3-4), 493-504, 2004.

[104.] Renn, D. W., Agar and Agarose - Indispensable Partners in Biotechnology, *Industrial & Engineering Chemistry Product Research and Development* 23 (1), 17-21, 1984.

[105.] Normand, V., Lootens, D. L., Amici, E., Plucknett, K. P., and Aymard, P., New Insight into Agarose Gel Mechanical Properties, 2000, pp. 730-738.

[106.] Anderson, N. S., Campbell, J. W., Harding, M. M., Rees, D. A., and Samuel, J. W. B., X-ray diffraction studies of polysaccharide sulphates: Double helix models for [kappa]- and [iota]-carrageenans, *Journal of Molecular Biology* 45 (1), 85-88, 1969.

[107.] Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C. M., Moorhouse, R., and Rees, D. A., The agarose double helix and its function in agarose gel structure, *Journal of Molecular Biology* 90 (2), 269-272, 1974.

[108.] Foord, S. A. and Atkins, E. D. T., New X-Ray-Diffraction Results from Agarose - Extended Single Helix Structures and Implications for Gelation Mechanism, *Biopolymers* 28 (8), 1345-1365, 1989.

[109.] Guenet, J. M., Brulet, A., and Rochas, C., Agarose Chain Conformation in the Sol State by Neutron-Scattering, *International Journal of Biological Macromolecules* 15 (2), 131-132, 1993.

[110.] Ramzi, M., Rochas, C., and Guenet, J. M., Phase behavior of agarose in binary solvents, *Macromolecules* 29 (13), 4668-4674, 1996.

[111.] Luo, Y. and Shoichet, M. S., A photolabile hydrogel for guided three-dimensional cell growth and migration, *Nat Mater* 3 (4), 249-53, 2004.

[112.] Haisch, A., Kläring, S., Gröger, A., Gebert, C., and Sittinger, M., A tissue-engineering model for the manufacture of auricular-shaped cartilage implants, *European Archives of Oto-Rhino-Laryngology* 259 (6), 316-321, 2002.

[113.] Nelson, R. D., Quie, P. G., and Simmons, R. L., Chemotaxis Under Agarose: A New and Simple Method for Measuring Chemotaxis and Spontaneous Migration of Human Polymorphonuclear Leukocytes and Monocytes, 1975, pp. 1650-1656.

[114.] Benya, P. D. and Shaffer, J. D., Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels, *Cell* 30 (1), 215-24, 1982.

[115.] Rahfoth, B., Weisser, J., Sternkopf, F., Aigner, T., von der Mark, K., and Bräuer, R., Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits, *Osteoarthritis and Cartilage* 6 (1), 50-65, 1998.

[116.] Sittinger, M., Bujia, J., Minuth, W. W., Hammer, C., and Burmester, G. R., Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture, *Biomaterials* 15 (6), 451-6, 1994.

[117.] Rowley, J. A., Madlambayan, G., and Mooney, D. J., Alginate hydrogels as synthetic extracellular matrix materials, *Biomaterials* 20 (1), 45-53, 1999.

[118.] Varghese, S., Elisseefff, J.H., Hydrogels for Musculoskeletal Tissue Engineering, *Advances in Polymer Science* 203 50, 2006.

[119.] van Osch, G. J. V. M., van den Berg, W. B., Hunziker, E. B., and Hausselmann, H. J., Differential effects of IGF-1 ans TGF[beta]-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes, *Osteoarthritis and Cartilage* 6 (3), 187-195, 1998.

[120.] Zimmermann, U., Klock, G., Federlin, K., Hannig, K., Kowalski, M., Bretzel, R. G., Horcher, A., Entenmann, H., Sieber, U., and Zekorn, T., Production of Mitogen-Contamination Free Alginates with Variable Ratios of Mannuronic Acid to Guluronic Acid by Free-Flow Electrophoresis, *Electrophoresis* 13 (5), 269-274, 1992.

[121.] Leinfelder, U., Brunnenmeier, F., Cramer, H., Schiller, J., Arnold, K., Vasquez, J. A., and Zimmermann, U., A highly sensitive cell assay for validation of purification regimes of alginates, *Biomaterials* 24 (23), 4161-4172, 2003.

[122.] Zimmermann, U., Mimietz, S., Zimmermann, H., Hillgartner, M., Schneider, H., Ludwig, J., Hasse, C., Haase, A., Rothmund, M., and Fuhr, G., Hydrogel-based non-autologous cell and tissue therapy, *Biotechniques* 29 (3), 564-+, 2000.

[123.] De Vos, P., Hoogmoed, C. G., and Busscher, H. J., Chemistry and biocompatibility of alginate-PLL capsules for immunoprotection of mammalian cells, *Journal of Biomedical Materials Research* 60 (2), 252-259, 2002.

[124.] Sharon-Buller, A. and Sela, M., An acrylic resin prosthesis for obturation of an orocutaneous fistula, *The Journal of Prosthetic Dentistry* 97 (3), 179-180, 2007.

[125.] Grunder, T., Gaissmaier, C., Fritz, J., Stoop, R., Hortschansky, P., Mollenhauer, J., and Aicher, W. K., Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads, *Osteoarthritis and Cartilage* 12 (7), 559-567, 2004.

[126.] Paige, K. T., Cima, L. G., Yaremchuk, M. J., Schloo, B. L., Vacanti, J. P., and Vacanti, C. A., De novo cartilage generation using calcium alginate-chondrocyte constructs, *Plast Reconstr Surg* 97 (1), 168-78; discussion 179-80, 1996.

[127.] Erickson, G. R., Gimble, J. M., Franklin, D. M., Rice, H. E., Awad, H., and Guilak, F., Chondrogenic Potential of Adipose Tissue-Derived Stromal Cells in Vitro and in Vivo, *Biochemical and Biophysical Research Communications* 290 (2), 763-769, 2002.

[128.] Diduch, D. R., Jordan, L. C. M., Mierisch, C. M., and Balian, G., Marrow stromal cells embedded in alginate for repair of osteochondral defects, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 16 (6), 571-577, 2000.

[129.] Ashiku, S. K., Randolph, M. A., and Vacanti, C. A., Tissue engineered cartilage, in *Porous Materials for Tissue Engineering* Transtec Publications Ltd, Zurich-Uetikon, 1997, pp. 129-150.

[130.] O'Sullivan, A., Cellulose: the structure slowly unravels, *Cellulose* 4 (3), 173-207, 1997.

[131.] Delmer, D. P. and Amor, Y., Cellulose Biosynthesis, 1995, pp. 987-1000.

[132.] Bosch, T., Schmidt, B., Samtleben, W., and Gurland, H. J., Biocompatibility and clinical performance of a new modified cellulose membrane, *Clin Nephrol* 26 Suppl 1, S22-9, 1986.

[133.] Takeaki Miyamoto, Shin-ichi Takahashi, Hiraku Ito, Hiroshi Inagaki, and Yasuhara Noishiki, Tissue biocompatibility of cellulose and its derivatives, 1989, pp. 125-133.

[134.] Hayashi, T., Biodegradable Polymers for Biomedical Uses, *Progress in Polymer Science* 19 (4), 663-702, 1994.

[135.] Hestrin, S., *Synthesis of polymeric homopolysaccharides* Academic Press, Inc, New York, 1962.

[136.] Colvin, J., *The biosynthesis of cellulose* Academic Press, Inc, New York, 1980.

[137.] Wong, H. C., Fear, A. L., Calhoon, R. D., Eichinger, G. H., Mayer, R., Amikam, D., Benziman, M., Gelfand, D. H., Measde, J. H., Emerick, A. W., Bruner, R., Ben-Bassat, A., and Tal, R., Genetic Organization of the Cellulose Synthase Operon in Acetobacter xylinum, 1990, pp. 8130-8134.

[138.] Ross, P., Mayer, R., and Benziman, M., Cellulose biosynthesis and function in bacteria, 1991, pp. 35-58.

[139.] Klemm, D., Schumann, D., Udhardt, U., and Marsch, S., Bacterial synthesized cellulose -- artificial blood vessels for microsurgery, *Progress in Polymer Science* 26 (9), 1561-1603, 2001.

[140.] Legeza, V. I., Galenko-Yaroshevskii, V. P., Zinov'ev, E. V., Paramonov, B. A., Kreichman, G. S., Turkovskii, I. I., Gumenyuk, E. S., Karnovich, A. G., and Khripunov, A. K., Effects of new wound dressings on healing of thermal burns of the skin in acute radiation disease, *Bulletin of Experimental Biology and Medicine* 138 (3), 311-315, 2004.

[141.] A. Nakayama, A. Kakugo, J. P. Gong, Y. Osada, M. Takai, T. Erata, and S. Kawano, High Mechanical Strength Double-Network Hydrogel with Bacterial Cellulose, 2004, pp. 1124-1128.

[142.] Princi, E., Vicini, S., Pedemonte, E., Gentile, G., Cocca, M., and Martuscelli, E., Synthesis and mechanical characterisation of cellulose based textiles grafted with acrylic monomers, *European Polymer Journal* 42 (1), 51-60, 2006.

[143.] Anna Gutowska, Byeongmoon Jeong, and Marek Jasionowski, Injectable gels for tissue engineering, 2001, pp. 342-349.

[144.] Aase Bodin, Sebastian Concaro, Mats Brittberg, and Paul Gatenholm, Bacterial cellulose as a potential meniscus implant, *Journal of Tissue Engineering and Regenerative Medicine* 1 (5), 406-408, 2007.

[145.] Gisela Helenius, Henrik Bäckdahl, Aase Bodin, Ulf Nannmark, Paul Gatenholm, and Bo Risberg, In vivo biocompatibility of bacterial cellulose, *Journal of Biomedical Materials Research Part A* 76A (2), 431-438, 2006.

[146.] Ko, I. K. and Iwata, H., An approach to constructing three-dimensional tissue, in *Conference on Bioartificial Organs III - Tissue Sourcing, Immunoisoloation, and Clinical Trials*, Hunkeler, D. C. A. P. A. R. R. New York Acad Sciences, Davos, Switzerland, 2000, pp. 443-455.

[147.] Freier, T., Koh, H. S., Kazazian, K., and Shoichet, M. S., Controlling cell adhesion and degradation of chitosan films by N-acetylation, *Biomaterials* 26 (29), 5872-8, 2005.

[148.] Madihally, S. V. and Matthew, H. W. T., Porous chitosan scaffolds for tissue engineering, *Biomaterials* 20 (12), 1133-1142, 1999.

[149.] Khor, E. and Lim, L. Y., Implantable applications of chitin and chitosan, *Biomaterials* 24 (13), 2339-2349, 2003.

[150.] Malafaya, P. B., Santos, T. C., van Griensven, M., and Reis, R. L., Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures, *Biomaterials* 29 (29), 3914-3926, 2008.

[151.] Correlo, V. M., Boesel, L. F., Pinho, E., Costa-Pinto, A. R., Alves da Silva, M. L., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties, *J Biomed Mater Res A*, 2008.

[152.] Sashiwa, H. and Aiba, S.-i., Chemically modified chitin and chitosan as biomaterials, *Progress in Polymer Science* 29 (9), 887-908, 2004.

[153.] Mahmoudifar, N. and Doran, P. M., Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors, *Biomaterials* In Press, Corrected Proof.

[154.] Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering A* In Press, Corrected Proof, 2005.

[155.] Drury, J. L. and Mooney, D. J., Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials* 24 (24), 4337-51, 2003.

[156.] Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N. H., Tanioka, S., and Shigemasa, Y., Migration of canine neutrophils to chitin and chitosan, *J Vet Med Sci* 56 (6), 1215-6, 1994.

[157.] Pamela J. VandeVord, H. W. T. M. S. P. D. L. M. B. W. P. H. W., Evaluation of the biocompatibility of a chitosan scaffold in mice, 2002, pp. 585-590.

[158.] Devlieghere, F., Vermeulen, A., and Debevere, J., Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables, *Food Microbiology* 21 (6), 703-714, 2004.

[159.] da Silva, R. M. P., Lopez-Perez, P. M., Elvira, C., Mano, J. F., Roman, J. S., and Reis, R. L., Poly(N-Isopropylacrylamide) Surface-Grafted Chitosan Membranes as a New Substrate for Cell Sheet Engineering and Manipulation, *Biotechnology and Bioengineering* 101 (6), 1321-1331, 2008.

[160.] Jayakumar, R., Reis, R. L., and Mano, J. F., Phosphorous containing chitosan beads for controlled oral drug delivery, *Journal of Bioactive and Compatible Polymers* 21 (4), 327-340, 2006.

[161.] Prabaharan, M., Reis, R. L., and Mano, J. F., Carboxymethyl chitosan-graft-phosphatidylethanolamine: Amphiphilic matrices for controlled drug delivery, *Reactive & Functional Polymers* 67 (1), 43-52, 2007.

[162.] Malafaya, P., Pedro, A., Peterbauer, A., Gabriel, C., Redl, H., and Reis, R., Chitosan particles agglomerated scaffolds for cartilage and osteochondral tissue engineering approaches with adipose tissue derived stem cells, *Journal of Materials Science: Materials in Medicine* 16 (12), 1077-1085, 2005.

[163.] Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C. D., Leroux, J. C., Atkinson, B. L., Binette, F., and Selmani, A., Novel injectable neutral solutions of chitosan form biodegradable gels in situ, *Biomaterials* 21 (21), 2155-2161, 2000.

[164.] Wang, S.-C., Chen, B.-H., Wang, L.-F., and Chen, J.-S., Characterization of chondroitin sulfate and its interpenetrating polymer network hydrogels for sustained-drug release, *International Journal of Pharmaceutics* 329 (1-2), 103-109, 2007.

[165.] Roughley, P. J., Alini, M., and Antoniou, J., The role of proteoglycans in aging, degeneration and repair of the intervertebral disc, 2002, pp. 869-874.

[166.] Mauck, R. L., Soltz, M. A., Wang, C. C. B., Wong, D. D., Chao, P.-H. G., Valhmu, W. B., Hung, C. T., and Ateshian, G. A., Functional Tissue Engineering of Articular Cartilage Through Dynamic Loading of Chondrocyte-Seeded Agarose Gels, *Journal of Biomechanical Engineering* 122 (3), 252-260, 2000.

[167.] Qiang Li, Christopher G. Williams, Danny D. N. Sun, Jun Wang, Kam Leong, and Jennifer H. Elisseeff, Photocrosslinkable polysaccharides based on chondroitin sulfate, 2004, pp. 28-33.

[168.] Lee, C. R., Breinan, H. A., Nehrer, S., and Spector, M., Articular Cartilage Chondrocytes in Type I and Type II Collagen-GAG Matrices Exhibit Contractile Behavior in Vitro, 2000, pp. 555-565.

[169.] Li, Q., Williams, C. G., Sun, D. D., Wang, J., Leong, K., and Elisseeff, J. H., Photocrosslinkable polysaccharides based on chondroitin sulfate, *J Biomed Mater Res A* 68 (1), 28-33, 2004.

[170.] V. Franklin Sechriest, Yi Jian Miao, Christopher Niyibizi, Andrea Westerhausen-Larson, Howard W. Matthew, Christopher H. Evans, Freddie H. Fu , and Jun-Kyo Suh, GAG-augmented polysaccharide hydrogel: A novel biocompatible and biodegradable material to support chondrogenesis, 2000, pp. 534-541.

[171.] Annett, P., WO2007144381-A1; EP1870104-A1, Composition useful for the relief of joint pain in athletes comprises food grade antiinflammatory substance and chondroitin sulfate or glucosamine. WO2007144381-A1 21 Dec 2007 A61K-031/715 200812.

[172.] Xu, L., WO200193838-A; WO200193838-A1; AU200175516-A, Composition useful as dietary supplement for reducing or preventing joint pain comprises chondroitin sulfate. WO200193838-A WO200193838-A1 13 Dec 2001 A61K-009/20 200221.

[173.] Clegg, D. O., Reda, D. J., Harris, C. L., Klein, M. A., O'Dell, J. R., Hooper, M. M., Bradley, J. D., Bingham, C. O., 3rd, Weisman, M. H., Jackson, C. G., Lane, N. E., Cush, J. J., Moreland, L. W., Schumacher, H. R., Jr., Oddis, C. V., Wolfe, F., Molitor, J. A., Yocum, D. E., Schnitzer, T. J., Furst, D. E., Sawitzke, A. D., Shi, H., Brandt, K. D., Moskowitz, R. W., and Williams, H. J., Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis, *N Engl J Med* 354 (8), 795-808, 2006.

[174.] Lee, D. A., Reisler, T., and Bader, D. L., Expansion of chondrocytes for tissue engineering in alginate beads enhances chondrocytic phenotype compared to conventional monolayer techniques, *Acta Orthopaedica Scandinavica* 74 (1), 6-15, 2003.

[175.] Jansson, P.-E., Lindberg, B., and Sandford, P. A., Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea, *Carbohydrate Research* 124 (1), 135-139, 1983.

[176.] Moorhouse R., Colegrove G.T., Sandford P.A., Baird J.K., and Kang K.S., *PS-60: a new gel-forming polysaccharide* D.A. Brandt, Washington DC, 1981.

[177.] Kang, K. S., Veeder, G. T., Mirrasoul, P. J., Kaneko, T., and Cottrell, I. W., Agar-Like Polysaccharide Produced by a Pseudomonas Species - Production and Basic Properties, *Applied and Environmental Microbiology* 43 (5), 1086-1091, 1982.

[178.] Grasdalen, H. and Smidsrod, O., Gelation of gellan gum, *Carbohydrate Polymers* 7 (5), 371-393, 1987.

[179.] Arnott, S., Scott, W. E., Rees, D. A., and McNab, C. G. A., i-Carrageenan: Molecular structure and packing of polysaccharide double helices in oriented fibres of divalent cation salts, *Journal of Molecular Biology* 90 (2), 253-256, 1974.

[180.] Drury, J. L. and Mooney, D. J., Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials* 24 (24), 4337-4351, 2003.

[181.] Quinn, F. X., Hatakeyama, T., Yoshida, H., Takahashi, M., and Hatakeyama, H., The conformational properties of gellan gum hydrogels, *Polymer Gels and Networks* 1 (2), 93-114, 1993.

[182.] Singh, B. N. and Kim, K. H., Effects of divalent cations on drug encapsulation efficiency of deacylated gellan gum, Informa Healthcare, 2005, pp. 761 - 771.

[183.] Ogawa, E., Temperature dependence of the conformational properties of sodium-type gellan gum in aqueous solutions, in *Physical Chemistry and Industrial Application of Gellan Gum*1999, pp. 8-14.

[184.] JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, and RL Reis, Gellan gum: A new biomaterial for cartilage tissue engineering applications *submitted*, 2008.

[185.] JT Oliveira , R Picciochi, AR Costa-Pinto, L Martins, PB Malafaya, RA Sousa, JF Mano, NM Neves, and RL Reis, Gellan Gum Hydrogels as Supports for Human Articular Chondrocytes and

Human Bone Marrow Cells for Cartilage Tissue Engineering Application, in *ESF/EMBO Symposium Stem Cells in Tissue engineering isolation, culture, characterisation and applications*Sant Feliu de Guixols, Spain, 2006.

[186.] Dickstein, K., HaPnes, R., and Aarsland, T., Comparison of aqueous and gellan ophthalmic timolol with placebo on the 24-hour heart rate response in patients on treatment for glaucoma, *American Journal of Ophthalmology* 132 (5), 626-632, 2001.

[187.] Shedden, A., Laurence, J., and Tipping, R., Efficacy and tolerability of timolol maleate ophthalmic gel-forming solution versus timolol ophthalmic solution in adults with open-angle glaucoma or ocular hypertension: a six-month, double-masked, multicenter study, *Clinical Therapeutics* 23 (3), 440-450, 2001.

[188.] Mao, R., Tang, J., and Swanson, B. G., Texture properties of high and low acyl mixed gellan gels, *Carbohydrate Polymers* 41 (4), 331-338, 2000.

[189.] Coviello, T., Dentini, M., Rambone, G., Desideri, P., Carafa, M., Murtas, E., Riccieri, F. M., and Alhaique, F., A novel co-crosslinked polysaccharide: studies for a controlled delivery matrix, *Journal of Controlled Release* 55 (1), 57-66, 1998.

[190.] Kubo, W., Miyazaki, S., and Attwood, D., Oral sustained delivery of paracetamol from in situ-gelling gellan and sodium alginate formulations, *International Journal of Pharmaceutics* 258 (1-2), 55-64, 2003.

[191.] Smith, A. M., Shelton, R. M., Perrie, Y., and Harris, J. J., An initial evaluation of gellan gum as a material for tissue engineering applications, *Journal of Biomaterials Applications* 22 (3), 241-254, 2007.

[192.] JT Oliveira , L. Martins, R. Picciochi, João F. Mano, and R. L. Reis, A new polysaccharide-based hydrogel supports human nasal chondrocytes development aiming at cartilage tissue engineering applications, in *Annual Tissue Engineering and Regenerative Medicine Society-European Chapter (TERMIS-EU)*Rotterdam, Netherlands, 2006.

[193.] J. T. Oliveira, R. P., T. C. Santos, L. Martins, L.G. Pinto, P. B. Malafaya, R. A. Sousa, A.P.Marques, A. G. Castro, J. F. Mano, N. M. Neves, R. L. Reis, 2008.

[194.] J. T. Oliveira, L. M., R. Picciochi, T. C. Santos, P. B. Malafaya, R. A. Sousa, A.P.Marques, A. G. Castro, J. F. Mano, N. M. Neves, R. L. Reis, 2008.

[195.] Oliveira, J. T., Picciochi, R., Santos, T. C., Martins, L., Pinto, L. G., Malafaya, P. B., Sousa, R. A., Marques, A. P., Castro, A. G., Mano, J. F., Neves, N. M., and Reis, R. L., Injectable gellan gum hydrogels as supports for cartilage tissue engineering applications, in *Annual Tissue-Engineering-and-Regenerative-Medicine-International-Society-Europ ean-Chapter Meeting* Mary Ann Liebert Inc, Porto, PORTUGAL, 2008, pp. OP165.

[196.] Wang, C. M., Gong, Y. H., Lin, Y. M., Shen, J. B., and Wang, D. A., A novel gellan gel-based microcarrier for anchorage-dependent cell delivery, *Acta Biomaterialia* 4 (5), 1226-1234, 2008.

[197.] Haxaire, K., Braccini, I., Milas, M., Rinaudo, M., and Perez, S., Conformational behavior of hyaluronan in relation to its physical properties as probed by molecular modeling, 2000, pp. 587-594.

[198.] Swann, D. A., Studies on hyaluronic acid: I. The preparation and properties of rooster comb hyaluronic acid, *Biochimica et Biophysica Acta (BBA) - General Subjects* 156 (1), 17-30, 1968.

[199.] Tlapak-Simmons, V. L., Baggenstoss, B. A., Kumari, K., Heldermon, C., and Weigel, P. H., Kinetic Characterization of the Recombinant Hyaluronan Synthases from Streptococcus pyogenes and Streptococcus equisimilis, *J. Biol. Chem.* 274 (7), 4246-4253, 1999.

[200.] Price, R. D., Berry M.G., Navsaria, H.A., Hyaluronic acid: the scientific and clinical evidence *Journal of Plastic, Reconstructive & Aesthetic Surgery*, 2007.

[201.] Mallapragada, S., Narasimhan, B., *Biodegradable Polymeric Materials and Their Applications* American Scientific Publishers, Stevenson Ranch, California, 2006.

[202.] Della Valle. F. and Romeo, A., Italy, 1987, Hyaluronic acid esters and their medical and cosmetic uses and formulations.

[203.] Cortivo, R., Brun, P., Rastrelli, A., and Abatangelo, G., In vitro studies on biocompatibility of hyaluronic acid esters, *Biomaterials* 12 (8), 727-730, 1991.

[204.] Barbucci, R., Rappuoli, R., Borzacchiello, A., and Ambrosio, L., Synthesis, chemical and rheological characterization of new hyaluronic acid-based hydrogels, *Journal of Biomaterials Science, Polymer Edition* 11 (4), 383-399, 2000.

[205.] Luis A. Solchaga, James E. Dennis, Victor M. Goldberg, and Arnold I. Caplan, Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage, 1999, pp. 205-213.

[206.] Na, K., Kim, S., Woo, D. G., Sun, B. K., Yang, H. N., Chung, H. M., and Park, K. H., Synergistic effect of TGFbeta-3 on chondrogenic differentiation of rabbit chondrocytes in thermo-reversible hydrogel constructs blended with hyaluronic acid by in vivo test, *J Biotechnol* 128 (2), 412-22, 2007.

[207.] Meyer, K. and Chaffee, E., Hyaluronic acid in the pleural fluid associated with a malignant tumor involving the pleura and peritoneum, 1940, pp. 83-91.

[208.] Naor, D., Sionov, R. V., and Ish-Shalom, D., CD44: structure, function, and association with the malignant process, *Adv Cancer Res* 71, 241-319, 1997.

[209.] Lowe, N. J., Maxwell, C. A., Lowe, P., Duick, M. G., and Shah, K., Hyaluronic acid skin fillers: Adverse reactions and skin testing, *Journal of the American Academy of Dermatology* 45 (6), 930-933, 2001.

[210.] J. Aigner, J. Tegeler , P. Hutzler, D. Campoccia, A. Pavesio, C. Hammer, E. Kastenbauer, and A. Naumann, Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester, *J Biomed Mater Res* 42 (2), 172-81, 1998.

[211.] Gobbi, A., Kon, E., Berruto, M., Francisco, R., Filardo, G., and Marcacci, M., Patellofemoral Full-Thickness Chondral Defects Treated With Hyalograft-C: A Clinical, Arthroscopic, and Histologic Review, *Am J Sports Med* 34 (11), 1763-1773, 2006.

[212.] Hollander, A. P., Dickinson, S. C., Sims, T. J., Brun, P., Cortivo, R., Kon, E., Marcacci, M., Zanasi, S., Borrione, A., Luca, C. D., Pavesio, A., Soranzo, C., and Abatangelo, G., Maturation

of Tissue Engineered Cartilage Implanted in Injured and Osteoarthritic Human Knees, *Tissue Engineering* 12 (7), 1787-1798, 2006.

[213.] Batchelor, A. W. and Stachowiak, G. W., Arthritis and the interacting mechanisms of synovial joint lubrication .2. Joint lubrication and its relation to arthritis, *Journal of Orthopaedic Rheumatology* 9 (1), 11-21, 1996.

[214.] Han, J.-A. and Lim, S.-T., Structural changes of corn starches by heating and stirring in DMSO measured by SEC-MALLS-RI system, *Carbohydrate Polymers* 55 (3), 265-272, 2004.

[215.] Funami, T., Kataoka, Y., Omoto, T., Goto, Y., Asai, I., and Nishinari, K., Food hydrocolloids control the gelatinization and retrogradation behavior of starch. 2b. Functions of guar gums with different molecular weights on the retrogradation behavior of corn starch, *Food Hydrocolloids* 19 (1), 25-36, 2005.

[216.] Buleon, A., Colonna, P., Planchot, V., and Ball, S., Starch granules: structure and biosynthesis, *International Journal of Biological Macromolecules* 23 (2), 85-112, 1998.

[217.] Reis, R. L., Cunha, A. M., Buschow, K. H. J., Robert, W. C., Merton, C. F., Bernard, I., Edward, J. K., Subhash, M., and Patrick, V., Starch Polymers, in *Encyclopedia of Materials: Science and Technology* Elsevier, Oxford, 2001, pp. 8810-8815.

[218.] Takeda, Y., Hizukuri, S., Takeda, C., and Suzuki, A., Structures of branched molecules of amyloses of various origins, and molar fractions of branched and unbranched molecules, *Carbohydrate Research* 165 (1), 139-145, 1987.

[219.] Xiudong You, Li Li, Jianping Gao, Jiugao Yu, and Zhuxuan Zhao, Biodegradable extruded starch blends, *Journal of Applied Polymer Science* 88 (3), 627-635, 2003.

[220.] Mendes, S. C., Reis, R. L., Bovell, Y. P., Cunha, A. M., van Blitterswijk, C. A., and de Bruijn, J. D., Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: a preliminary study, *Biomaterials* 22 (14), 2057-2064, 2001.

[221.] Manuela E. Gomes, Vassilios I. Sikavitsas, Esfandiar Behravesh, Rui L. Reis, and Antonios G. Mikos, Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds, 2003, pp. 87-95.

[222.] Salgado, A. J., Gomes, M. E., Chou, A., Coutinho, O. P., Reis, R. L., and Hutmacher, D. W., Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds, *Materials Science and Engineering: C* 20 (1-2), 27-33, 2002.

[223.] Fama, L., Gerschenson, L., and Goyanes, S., Starch-vegetable fibre composites to protect food products, *Carbohydrate Polymers* 75 (2), 230-235, 2009.

[224.] Kraak, A., Industrial applications of potato starch products, *Industrial Crops and Products* 1 (2-4), 107-112, 1992.

[225.] Chen, X., Chen, H., and Yang, G., CN101245568-A, Colored paper coating includes urea resin, oxidized starch, organic pigment, dispersant, firming agent, and water. CN101245568-A 20 Aug 2008 D21H-019/00 200881 Pages: 4.

[226.] Oliveira, J., Crawford, A., Mundy, J., Moreira, A., Gomes, M., Hatton, P., and Reis, R., A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes, *Journal of Materials Science: Materials in Medicine* 18 (2), 295-302, 2007.

[227.] J. F. Mano, R. L. R., Osteochondral defects: present situation and tissue engineering approaches, *Journal of Tissue Engineering and Regenerative Medicine* 1 (4), 261-273, 2007.

[228.] Marques, A. P., Reis, R. L., and Hunt, J. A., An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats, *Macromolecular Bioscience* 5 (8), 775-785, 2005.

[229.] Salgado, A. J., Coutinho, O. P., Reis, R. L., and Davies, J. E., In vivo response to starch-based scaffolds designed for bone tissue engineering applications, *J Biomed Mater Res A* 80 (4), 983-9, 2007.

[230.] Gomes, M. E., Godinho, J. S., Tchalamov, D., Cunha, A. M., and Reis, R. L., Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties, in *International Conference on Materials for Advanced Technologies (ICMAT2001)* Elsevier Science Bv, Singapore, Singapore, 2001, pp. 19-26.

[231.] Jen, A. C., Wake, M. C., and Mikos, A. G., Review: Hydrogels for cell immobilization, *Biotechnology and Bioengineering* 50 (4), 357-364, 1996.

[232.] Lee, K. Y. and Mooney, D. J., Hydrogels for tissue engineering, *Chemical Reviews* 101 (7), 1869-1879, 2001.

[233.] Jin, X. B., Sun, Y. S., Zhang, K., Wang, J., Shi, T. P., Ju, X. D., and Lou, S. Q., Tissue engineered cartilage from hTGF beta2 transduced human adipose derived stem cells seeded in PLGA/alginate compound in vitro and in vivo, *J Biomed Mater Res A*, 2007.

SECTION 2.

CHAPTER II.

Materials and Methods

CHAPTER II.

Materials and Methods

This chapter describes the materials and experimental methods used within the scope of this thesis. Although each part of the work is accompanied by its specific materials and methods section, this chapter intends to condensate and compile the relevant information on this matter. This exercise is expected to provide the reader with a more comprehensive view of the experimental and analytical tools used, as well as to facilitate their use by others.

1. MATERIALS

1.1. CHITOSAN/POLYBUTYLENE SUCCINATE (C-PBS)

A new material consisting of a 50/50 (%wt) blend of chitosan, a natural polymer derived from the deacetylation of chitin, and polybutylene succinate, a synthetic polymer, was produced (C-PBS). The rationale was to combine the biological properties of chitosan with the mechanical support conferred by polybutylene succinate in an attempt to confer a better performance to the tissue engineered construct. Chitosan is a partially deacetylated derivative of chitin, which is the primary structural polymer in arthropod exoskeletons, shells of crustaceans, and the cuticles of insects.[1] Chitosan is a semi-crystalline polysaccharide that is normally insoluble in aqueous solutions above pH 7. However, in dilute acids (pH 6), the free amine groups are protonated and the molecule becomes soluble. This pH-dependent solubility provides a convenient mechanism for processing under mild conditions.[2] Chitosan is reported to be non-toxic, biodegradable and biocompatible[3], and has structural similarities to glycosaminoglycans, which are structural components of the cartilage extracellular matrix.[4] It serves different applications and its use ranges from the food industry to the biomedical and pharmaceutical fields.[5,6] Polybutylene succinate (PBS) is one of the most accessible biodegradable polymers, and has been extensively studied for its potential use as a future conventional plastic, serving also as a support for different approaches in the medical field.[7,8] It is an aliphatic polyester presenting good processability and flexibility, and having degradation products that are non-toxic and can enter the metabolic cycles of bioorganisms. Chitosan was obtained from France Chitine, France, and polybutylene succinate was obtained from Showa Highpolymer Co. Ltd., Tokyo, Japan.

1.2. STARCH/POLYCAPROLACTONE (SPCL)

A blended material consisting of 30/70% (wt) corn starch and polycaprolactone was produced (SPCL). Following the strategy of the chitosan and polybutylene succinate blends, the objective of combining these materials is the improved performance they might confer ultimately to the tissue engineered construct in an *in vivo* scenario. Starch is a natural polymer made of a combination of two polymeric carbohydrates, amylose and amylopectin.[9,10] It has been put forward as a cell support material in combination with synthetic polymers such as polycaprolactone (PCL), polylactic acid (PLA), ethyl vinyl alcohol (EVOH), and cellulose acetate (CA). Several studies have been conducted with these materials, mainly in bone tissue engineering.[11-14] Polycaprolactone (PCL) is a synthetic semicrystalline biodegrabable polymer belonging to the family of poly-α-hydroxyl polyesters that has also been used for such approaches.[15,16] The SPCL blend used was purchased from Novamont S.p.A. Italy.

1.3. POLYGLYCOLIC ACID (PGA)

Polyglycolic acid, also known as polyglycolide, is a biodegradable, thermoplastic synthetic polymer. It can be prepared from glycolic acid by polycondensation or ring-opening polymerization and it has been one of the first materials used for tissue engineering applications.[17] The processing of polyglycolic acid was not performed in the scope of this thesis, and they were purchased in the form of non-woven fibre scaffolds from Albany international, Bury, Lancashire, UK and used as comparator in Chapter IV and V.

1.4. GELLAN GUM

Gellan gum is a linear anionic polysaccharide composed of tetrasaccharide (1,3-β-D-glucose, 1,4-β–D-glucuronic acid, 1,4-β-D-glucose, 1,4-α-L-rhamnose) repeating units, containing one carboxyl side group, and was initially described by Moorhouse et al.[18,19] This material has a broad use in the food industry and biomedical fields, mostly due to its processing into transparent gels that are resistant to heat and acid stress. Two Gellan gum forms exist, acetylated and deacetylated, being the latter the most common and commercially available form. Both form thermoreversible gels, varying in their mechanical properties from soft and elastic for the acetylated form to hard and brittle for the fully deacetylated polysaccharide.[20,21] Gellan gum was purchased from Sigma, St. Louis, MO, USA, Cat nº G1910.

1.5. AGAROSE

Agarose is a constituent of agar, which in turn consists of a heterogeneous mixture of agaropectin and agarose. Agarose has a neutral charge and lower degree of chemical complexity when compared to agaropectin which makes it less likely to interact with biomolecules, such as proteins. Agarose type VII was purchased from Sigma, St. Louis, MO, USA, Cat nº A6560 and was used as comparator in Chapter VI.

2. SCAFFOLDS PRODUCTION

2.1. CHITOSAN/POLYBUTYLENE SUCCINATE SCAFFOLDS (COMPRESSION MOULDING AND SALT LEACHING)

The chitosan/polybutylene succinate 50/50 (%wt) blend was compounded in a twin screw extruder. The details of the processing conditions are summarized elsewhere.[3] The methodology used for the scaffolds production was based on compression moulding followed by salt leaching. Before using it in the scaffolds processing, the salt was grinded and sieved to obtain particles with size between 63 $\mu m < d < 125\ \mu m$. The compounded polymeric blend was ground, mixed with salt and compression molded into discs. The salt content was 80% by weight. The discs were cut into 5x5x5 mm cubes. These cubes were then immersed in distilled water to leach out the salt, dried, and used for cell culture and proliferation studies after sterilization by ethylene oxide.

2.2. CHITOSAN/POLYBUTYLENE SUCCINATE FIBRE SCAFFOLDS

For the production of the chitosan/polybutylene succinate fibre scaffolds the raw materials (chitosan and PBS) were compounded on a twin screw extruder in a ratio of 50/50 wt%. The polymeric fibres were obtained by further re-extruding the C-PBS blend using a microextruder. Afterwards, the processed fibres were packed in an appropriate mould, compressed, and heated above the melting temperature (Tm) of the thermoplastic for a determined residence period, thereby allowing the fibres to bond and consequently to obtain a mechanically stable macroporous fibre mesh structure. The scaffolds were further cut in a cylindrical shape (Ø 7 mm x thickness 1.5 mm).

2.3. STARCH/POLYCAPROLACTONE FIBRE SCAFFOLDS

The methodology used to produce the scaffolds was melt spinning (to obtain the polymeric fibres) followed by fibre bonding.[22] This processing technique involves fibre packing in an appropriate

mould, with posterior heating below the melting temperature (Tm) for a determined residence period that will allow the fibres to form a stable fibre mesh structure. The material used was a 30/70% (wt) blend of corn starch with polycaprolactone (SPCL). These scaffolds have already been shown previously to be suitable for conducting a bone tissue engineering approach[12,23]. The scaffolds produced were cut in a cylindrical shape, with dimensions of 7mm diameter x 3mm thickness.

2.4. GELLAN GUM HYDROGELS

Gellan gum was processed in different ways giving rise to various structures, therefore evidencing the versatility of this natural biomaterial. The processing involved temperature-dependent and pH-dependent reactions.

2.4.1. GELLAN GUM HYDROGELS DISCS

Gellan gum discs were produced in the following way. Gellan gum powder was mixed with distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90ºC, under which complete and homogeneous dispersion of the material was obtained. The solution was kept at this temperature during 20-30 minutes. Afterwards, $CaCl_2$ (Merck, DE) was added to obtain a final concentration of 0.03% (w/v) in the gellan gum solution and the temperature was progressively decreased to 50ºC. Gellan discs were produced by casting the solution into cylindrical moulds and allowing it to rest at room temperature for 2-5 minutes and form a solid gel. The discs were then cut using a borer for final discs dimensions of Ø 6±0.01 mm x 5.5±0.46 mm height.

2.4.2. GELLAN GUM HYDROGELS MEMBRANES

Gellan gum membranes were produced using the same starting process applied to the discs. Gellan gum powder was mixed with distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90ºC, under which complete and homogeneous dispersion of the material was obtained. The solution was kept at this temperature during 20-30 minutes. Afterwards, $CaCl_2$ (Merck, DE) was added to obtain a final concentration of 0.03% (w/v) in the gellan gum solution and the temperature was progressively decreased to 50ºC. Gellan gum membranes were produced by casting the solution into Petri dishes and allowing it to stand at room temperature for 2-5 minutes and form a solid gel. The Petri dishes were kept in an oven at 37ºC for 90 minutes.

2.4.3. GELLAN GUM HYDROGELS FIBRES

For the production of gellan gum fibres the methodology was as follows. Gellan gum powder was mixed with a NaOH 0.10 M solution and stirred at room temperature with a final concentration of 4% (w/v). Gellan gum fibres were produced by extruding the gellan gum solution into a L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.2 ml/min, using a 21G needle. The gellan gum fibres formed were then washed in distilled water, pressed into cylindrical moulds, and dried overnight at 37ºC.

2.4.4. GELLAN GUM HYDROGELS PARTICLES

The production of gellan gum particles followed the same starting process applied to the fibres. Gellan gum powder was mixed with a NaOH 0.10 M solution and stirred at room temperature with a final concentration of 4% (w/v). Gellan gum particles were produced by extruding the Gellan gum 4% (w/v) solution dropwise to an L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.8 ml/min, using a 21G needle.

2.4.5. GELLAN GUM HYDROGELS SCAFFOLDS

Gellan gum scaffolds were produced by immersing gellan gum 0.7% (w/v) (Ø 6±0.01 mm x 5.5±0.46 mm height) discs in liquid nitrogen for 1-2 minutes and quickly transferring them to a lyophilizator (Telstar Cryodos-80, Telstar, Spain) where they were lyophilized during 2 days.

2.5. AGAROSE HYDROGELS

Agarose hydrogels were produced following standard procedures detailed elsewhere.[24] Briefly, a sterile agarose type VII low Tm 4% (w/v) solution prepared in sterile PBS was heated to 70ºC for 30 seconds, until complete dissolution. The solution was then allowed to rest room temperature, thereby decreasing the temperature and promoting gelation.

3. SCAFFOLDS CHARACTERIZATION

3.1. SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy analyses provide images of the surface of a given sample by scanning it with a high-energy beam of electrons. In chapters III and IV, SEM analyses of the scaffolds were performed using a Leica Cambridge S360 (Leica Cambridge, Cambridge, UK).

Prior to SEM analysis, sample surfaces were gold sputtered (Fisons Instruments, Sputter Coater SC502, UK).

3.2. TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscopy is a technique that provides information on the ultrastructural aspects of a given sample through the use of a beam of electrons that interacts with the specimen as it passes through it. In chapter VI, gellan gum discs were characterised and prepared for transmission microscopy analysis in the following way. Briefly, sections of $1mm^3$ were fixed in formalin-glutaraldehyde-osmium tetroxide for 2 h at room temperature and then washed three times in PBS. Semithin sections (1µm) were cut from epon-embedded blocks and stained with toluidine blue. Ultrathin sections (600 Å) were cut in a ultratome (Reichert Ultranova Leica), mounted onto copper grids, stained with uranyl acetate (7min) and lead citrate (5 min) and observed on a Zeiss 902A (50 Kv) electron microscope.

3.3. MICRO-COMPUTED TOMOGRAPHY (µ-CT)

Micro-computed tomography provides valuable information on the 3d morphology of a chosen sample. Some of its advantages include its non destructive character and the possibility to assess several parameters in a quantitative way. Micro-computed tomography equipment (SkyScan, Belgium) was used in chapters III and IV for a more detailed analysis of the morphology of the developed scaffolds and CT Analyser and CT Vol Realistic 3D Visualization were used as image processing softwares, both from SkyScan (Belgium). In Chapter V, the porosity of the scaffolds was determined by microcomputerized tomography (µCT) (ScanCo Medical µCT 80, Bassersdorf, Switzerland) at a resolution of 10 mm, and using at least 3 samples. In chapter VI, lyophilized gellan gum 0.7% discs were analysed under micro-computed tomography (µ-CT) using a high-resolution µ-CT Skyscan 1072 scanner (Skyscan, Kontich, Belgium) using a resolution of 6.76 µm pixel size and integration time of 1.7 ms. The x-ray source was set at 70keV of energy and 142 µA of current. Approximately 500 projections were acquired over a rotation range of 180° and a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was a 500 serial of 1024x1024 bitmap images. Representative data sets of 150 slices were segmented into binary images (CT Analyser, v1.5.1.5, SkyScan) with a dynamic threshold of 70-255 (grey values) that was applied to build the 3D models. 3D virtual models (height 1mm x Ø 3mm) of representative regions in the bulk of the hydrogels were created, visualized and

registered using image processing software (CT Analyser, v1.5.1.5 and ANT 3D creator, v2.4, both from SkyScan).

3.4. MECHANICAL TESTING

3.4.1. STATIC COMPRESSION TESTS

In Chapters III and IV, uniaxial compression tests were performed to assess the mechanical properties of the produced scaffolds in the dry state using a Universal tensile testing machine (Instron 4505 Universal Machine). Mechanical testing was performed under compression using a crosshead speed of 5mm/min and the results averaged from tests conducted in at least five specimens. The details regarding these methods are presented elsewhere.[25]

3.4.2. DYNAMIC MECHANICAL ANALYSIS (DMA)

Dynamic mechanical analysis is a technique used to study and characterize materials that is useful for observing the viscoelastic nature of polymers. In chapter VI and VII, dynamic mechanical analysis (DMA) was conducted to characterize the mechanical behaviour of Gellan gum hydrogel discs. Gellan gum discs were subjected to compression cycles of increasing frequencies ranging from 0.1-10 Hz with constant amplitude displacements of 0.1 mm using a Tritec 2000 DMA (Triton Technology, UK). Storage and loss modulus were measured and experiments were conducted at room temperature. The described values for the compression modulus were collected at a frequency of 1 Hz. Statistical analysis was performed using confidence intervals based on the experimental results, with a confidence level of 99%. In the work described on each chapter, the gellan gum concentrations and the number of samples had specific values.

3.5. RHEOLOGICAL STUDIES

Rheology, sometimes also synonymously referred to as rheometry, provides information on the relation of the flow/deformation behaviour of materials. In chapters VI and VII, cone-plate rheometry was conducted for gellan gum hydrogels in order to assess their rheological behaviour dependence of temperature and time. For this purpose, gellan gum powder was mixed at room temperature with distilled water under constant stirring. The solution was heated to 90ºC and kept at this temperature for 30 minutes. Afterwards, $CaCl_2$ was added to the Gellan gum solution and rheological measurements were performed using a controlled stress cone-plate rheometer (Reometer Reologica, StressTech, Sweden). For each measurement, a volume of 2 ml of the

Gellan gum solution was placed in the bottom plate of the rheometer and held at a constant temperature of 70ºC. The polymer solution was allowed to rest for 1 minute before starting the experiments. Measurements were performed by cooling each sample from 70ºC to 25ºC (at a cooling rate of -6ºC/min) applying a constant shear stress of 0.1 Pa. Temperature, time, shear rate and viscosity were constantly measured. The confidence intervals were estimated with a confidence level of 99%. In the work described on each chapter, the gellan gum concentrations and the number of samples had specific values.

3.6. *IN VITRO* CYTOTOXICITY TESTS

Cytotoxicity tests are usually quick and standardized methods to determine if a given material contains significant quantities of harmful leachables and their effect on cellular components. To assess the short-term cytotoxicity of the developed scaffolds, minimum essential medium (MEM) extraction (chapter III) and 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) tests (chapter III and VI), both within a 24 h extraction period, according to ISO/EN 10993 part 5 guidelines[26] were used, in order to establish the possible toxic effects of leachables released from the scaffolds during extraction. Latex rubber was used as positive control for cell death due to its high cytotoxicity to cells, and culture medium was used as a negative control representing the ideal situation for cell proliferation. The results are presented after normalisation with the negative control. The objectives of the MEM extraction test are to evaluate changes in cell morphology and growth inhibition, whereas the MTS test determines whether cells are metabolically active.

3.6.1. MEM EXTRACTION TEST

The ratio of material weight to extract fluid was constant and equal to 0.2g/ml for porous samples. For the positive control the ratio of material outer surface to extraction fluid was 2.5 cm^2/ml. Test material (n=6) and positive control were extracted for 24h at 37ºC, using complete culture medium as extraction fluid. Before the tests, culture medium was removed from the wells and an identical volume (2 ml) of extraction fluid was added. For the MEM extraction test, the cells were seeded in 24 well plates (n=3) at a density of $1.25x10^5$ cells/well. They were incubated for 24h at 37ºC, in a humidified atmosphere with 5% CO_2 after this. Cell response was evaluated after 24, 48, and 72h of incubation. Confluence of the monolayer, degree of floating cells, and changes in morphology were analyzed by visual observation. After 72h, the percentage of growth inhibition was determined by cell counting with a haemocytometer and trypan blue exclusion method. Final measurements were corrected for the negative control.

3.6.2. MTS TEST

To assess the possible cytotoxicity of the processed gellan gum hydrogels, MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium) test was used according to ISO/EN 10993 part 5 guidelines, which determines whether cells are metabolically active.[26] This cytotoxicity test is based on the bioreduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) (Cell Titer 96® Aq_{ueous} Solution Cell Proliferation Assay, Promega, USA), into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. Latex rubber was used as positive control for cell death, due to its high cytotoxicity to cells, and culture medium was used as a negative control. A rat lung fibroblasts cell line – L929, acquired from the European Collection of Cell Cultures (ECACC), was used for the studies. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany; Heat Inactivated) and 1% of antibiotic-antimycotic mixture. The samples were incubated in culture medium for 24h at 37ºC with constant shaking, as well as latex. Cultured L929 cells were trypsinised using trypsin-EDTA (Gibco, Invitrogen Corporation) and plated at a density of $6.6x10^4$ cells/well into 96-well micrometer plates (200 µl/well). The plates were incubated for 24 hours at 37ºC in a humidified atmosphere of 5% CO_2 in air. Afterwards, the medium was replaced by the extracts previously obtained, using culture medium as a negative control. After 72 hours, the cell culture was incubated with MTS (using culturing medium without phenol red) for further 3 hours at 37ºC in a humidified atmosphere of 5% CO_2 in air. Culture medium with MTS was then transferred to new wells. The optical density (OD) which is directly proportional to the cellular activity, being a measure of mitochondrial acitivity, was read on a multiwell microplate reader (Synergy HT, Bio-TeK Instruments, US) at 490 nm. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3.

4. CELL ISOLATION AND EXPANSION

4.1. CELLS FOR CYTOTOXICTY ASSAYS

A rat lung fibroblasts cell line – L929-, acquired from the European Collection of Cell Cultures (ECACC), was used for the cytotoxicity tests in chapters III and VI. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO)

supplemented with 10% foetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% of an antibiotic-antimycotic mixture (Sigma, St. Louis, MO). Trypsin (Sigma, St. Louis, MO) was used to detach the cells from the culture flasks before the experiments were conducted.

4.2. EXPANSION OF BMC9 CELLS (MOUSE MESENCHYMAL PROGENITOR CELL LINE)

The BMC9 cell line has been shown to exhibit four mesenchymal cell phenotypes: chondrocytic, adipocytic, stromal (supports osteoclasts formation), and osteoblastic.[27] The cells were grown as monolayer cultures in a culture medium consisting of α-MEM medium (Sigma, St. Louis, MO), 10% Foetal Bovine Serum (FBS), and 1% A/B (penicillin G sodium 10000 U/ml, amphotericine B (Fungizone) 25 μg/ml, streptomycin sulfate 10000 μg/ml, in 0.85% saline).

4.3. ISOLATION AND EXPANSION OF BOVINE ARTICULAR CHONDROCYTES

Full thickness hyaline cartilage was harvested from bovine metacarpophalangeal joint of adult animals (18-24 months) within 4 hours of slaughter. Chondrocytes were isolated by sequential enzymatic digestion and their numbers expanded in monolayer culture as described previously.[28] Chondrocytes were seeded at 50,000-100,000 cells/cm^2 and cultured in basic medium [Dulbecco's Modified Eagle's Medium, containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 μg/ml streptomycin, 20 mM L-alanyl glutamine, MEM non-essential amino acids, and 10% (v/v) foetal calf serum (Biosera S1800; NWPLS; Heat Inactivated)], supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). The expanded chondrocytes were used in the works described on chapters IV and V.

4.4. ISOLATION AND EXPANSION OF HUMAN NASAL CHONDROCYTES

Nasal cartilage was harvested from the nasal septum of adult patients (40-65 years) undergoing reconstructive surgery. This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and always sampled upon patient informed consent. Chondrocytes were isolated by enzymatic digestion with posterior collection. The human nasal septum cartilage free from all surrounding tissue was placed in a Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2-3 mm. The pieces were washed in sterile PBS solution, immersed in 20 ml of trypsin-EDTA solution, and incubated for 30 min at 37ºC on a rotator. Trypsin was removed and the pieces washed with basic DMEM. Then, 20 ml of filter sterilised collagenase type II solution (2mg/ml) in basic medium was added, and the mixture incubated for approximately 12 hours at 37ºC on a rotator. The digested tissue and cell suspension solution was centrifuged at

200xg for 7 min and the supernatant removed. The cell pellet was washed with PBS and the cells centrifuged as before. The procedure was repeated and the cells were ressuspended in PBS and counted using a hemocytometer. They were again centrifuged, the supernatant removed, and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium, containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 µg/ml streptomycin, 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human nasal chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.[28] The expanded chondrocytes were used in the work described on chapter VI.

4.5. ISOLATION AND EXPANSION OF HUMAN ARTICULAR CHONDROCYTES

Human articular cartilage was harvested from the femoral head and condyles of adult patients (40-65 years) undergoing knee arthroplasty surgery. This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and upon patient informed consent. Chondrocytes were isolated by enzymatic digestion; In detail, the human articular cartilage, free from all surrounding tissue, was placed in a Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2-3 mm. The pieces were washed in sterile PBS solution, immersed in 20 ml of trypsin-EDTA solution, and incubated for 30 min at 37ºC under agitation. Trypsin was removed and the tissue pieces washed with basic Dulbeco's Modified Eagle Medium (DMEM). Then, 20 ml of sterile collagenase type II solution (2mg/ml) in basic medium was added, and the mixture incubated for approximately 12 hours at 37ºC under agitation. The digested tissue and cell suspension solution was centrifuged at 200g for 7 min and the supernatant discarded. The cell pellet was washed with PBS and centrifuged again under the same conditions. Cells were again centrifuged, the supernatant removed, and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium, containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 µg/ml streptomycin, 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human nasal chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.[28] The expanded chondrocytes were used in the work described on chapter VII and VIII.

4.6. ISOLATION AND EXPANSION OF RABBIT ADIPOSE TISSUE DERIVED PROGENITOR CELLS

Rabbit adipose tissue was obtained from the intrascapular region of 10-11 weeks old /2.4-2.6 Kg female New Zealand White rabbits. Briefly, the rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corp., Finland). After the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the intrascapular region and disinfected with chlorhexidine (Lifo-Scrub®, B. Braun Melsungen AG, Germany). An incision was performed (reaching a maximum of 2 cm) in the intrascapular region and adipose tissue from this region was collected to a falcon tube containing sterile phosphate-buffered saline solution (PBS) with 10% antibiotic (antibiotic-antimycotic Gibco 15240). The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. The collected tissue was washed in sterile PBS to remove contaminating debris and red blood cells. The adipose samples were then incubated in a 1 mg/ml collagenase type II solution prepared in PBS for 60-90 minutes at 37°C with constant agitation. The processed adipose tissue was afterwards filtered and the released cells collected in a falcon tube and centrifuged at 1200 rpm for 10 minutes. The formed cell pellet was washed in culture medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% (v/v) foetal bovine serum (FBS, Biochrom, Berlin, Germany; Heat Inactivated), 1% antibiotic (antibiotic-antimycotic Gibco 15240), and centrifuged as before. The obtained cell pellet was again ressuspended in culture medium, and seeded in tissue culture polystyrene flasks. Rabbit adipose tissue derived progenitor cells (ASC) were incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion. Once the adequate cell number was reached, the cells were divided in two groups: one group subjected to a chondrogenic predifferentiation period consisting of DMEM, sodium pyruvate $1.0x10^{-3}$ M, ascorbate-2-phosphate 0.17 mM, proline 0.35 mM, ITS 1X, and supplemented with 10 ng/ml TGF-β1for 2 days followed by 100 ng/ml BMP-2 (R&D BioSystems, USA) for 3 days prior to *in vivo* implantation (ASC + GF); another group cultured with the same medium but without TGF-β1 and BMP-2 (ASC). The expanded cells were used in the work described on chapter IX.

4.7. ISOLATION AND EXPANSION OF RABBIT ARTICULAR CHONDROCYTES

Rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corp., Finland). After the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the joint area of

the left posterior leg and disinfected with chlorhexidine (Lifo-Scrub®, B. Braun Melsungen AG, Germany). Incisions were performed through the skin and muscle to access the articular capsule. Articular cartilage was harvested from the femoral condyles of the rabbits and washed in sterile phosphate buffered saline (PBS) with 1% antibiotic (antibiotic-antimycotic Gibco 15240). The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. The cartilage pieces were immersed in trypsin-EDTA solution for 15-20 min at 37ºC under constant agitation. Trypsin was removed, the pieces were washed with DMEM and sterile collagenase type II solution (2mg/ml) prepared in basic medium was added. The mixture was kept for 8-10 hours at 37ºC under constant agitation. The digested mixture was filtered using a 100 µm filter and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cell pellet washed with PBS and the cells counted using a hemocytometer. Cells were then collected by centrifugation and ressuspended in expansion medium consisting of DMEM, containing 10 mM HEPES buffer pH 7.4, 1% antibiotic (antibiotic-antimycotic Gibco 15240), 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human articular chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.[28] The expanded chondrocytes were used in the work described on chapter IX.

5. CELL AND MATERIALS CULTURING

5.1. BMC9 CELLS SEEDING AND CULTURING ON CHITOSAN/POLYBUTYLENE SUCCINATE SCAFFOLDS (COMPRESSION MOULDING AND SALT LEACHING)

In Chapter III, direct contact assays were performed with a mouse mesenchymal progenitor cell line (BMC9). When the adequate cell number was obtained, cells at passage 9 (P9) were trypsinized, centrifuged, and ressuspended in cell culture medium. Cells were seeded at a density of $5x10^5$cells/scaffold ($5x5x5mm^3$) under static conditions, using for this purpose aliquots of 15 µl loaded on top of the scaffolds that had been previously placed in 24 well culture plates. Two hours after seeding, 1 ml of culture medium was added to each well and the cell seeded scaffolds were allowed to develop for periods up to 3 weeks, in a humidified atmosphere at 37ºC, containing 5% CO_2, under chondrogenic differentiation inducing medium. This culturing medium

consisted of DMEM (Sigma, St. Louis, MO), dexamethasone (Sigma, St. Louis, MO) $1.0x10^{-4}$ M, sodium pyruvate (Sigma, St. Louis, MO) $1.0x10^{-3}$ M, ascorbate-2-phosphate (Sigma, St. Louis, MO) 0.17 mM, proline (Sigma, St. Louis, MO) 0.35 mM, ITS 1X (Sigma, St. Louis, MO), and hBMP-2 (R&D BioSystems, CA) 100ng/ml. The culture medium was changed every 3 to 4 days until the end of the experiment.

5.2. BOVINE CHONDROCYTES SEEDING AND CULTURING ON FIBRE SCAFFOLDS (C-PBS AND SPCL)

Scaffolds used in the works described in chapters IV and V were seeded with chondrocytes at passage P1 as described previously.[28] In brief, confluent cultures of chondrocyte cultures were harvested by trypsinisation. Scaffolds were threaded onto stainless steel wires and suspended in spinner flasks containing a stirred suspension of chondrocytes ($0.5x10^6$ cells/ml; 4 scaffolds per spinner flask) for 72 h to allow the chondrocytes to penetrate the scaffolds. The resultant cell/scaffold constructs were transferred to sterile, non-tissue culture treated Petri dishes and incubated for 4 days in basic medium supplemented with 10ng/ml bFGF to allow expansion of cell numbers on the scaffolds. The constructs were then cultured with basic medium supplemented with 1µg/ml insulin and 50µg/ml L-ascorbic acid to stimulate chondrogenesis. Throughout the culture period the constructs were gently shaken using an orbital shaker. The constructs were incubated until 42 days of culture (6 weeks), replacing the medium every 2-3 days. Polyglycolic acid (PGA) non-woven scaffolds (Albany international, Bury, Lancashire, UK) were used as a comparator.

5.3. HUMAN NASAL CHONDROCYTES ENCAPSULATION AND CULTURING IN GELLAN GUM AND AGAROSE HYDROGELS

Human nasal chondrocytes were expanded until an adequate cell number was obtained for cells encapsulation. In the work described in chapter VII, the cells were encapsulated at passage 1 in gellan gum hydrogels and in agarose type VII (A6560; Sigma, St. Louis, MO) hydrogels, the latter being used as controls. Regarding gellan gum the procedure was the following. Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. A sterile $CaCl_2$ solution was added to obtain a final concentration of 0.03% (w/v). The temperature was progressively decreased to 40ºC and stabilised at this stage always under constant stirring. Human nasal chondrocytes were detached by trypsinisation, mixed with expansion medium, and centrifuged at 200xg for 7 min. The

supernatant was removed and the cells were ressuspended in warm sterile PBS solution, counted using and hemocytometer, and finally centrifuged at 200xg for 7 min. The supernatant was discarded and the cells pellet kept at the bottom of the falcon tube. The gellan gum 0.7% (w/v) with $CaCl_2$ 0.03% (w/v) solution was added to the cells pellet and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of 1 $x10^6$ cells/ml. Gellan discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 1-2 minutes to form a solid gel, and then discs of Ø 6±0.01 mm x 5.5±0.46 mm height were cut using a borer. Regarding the agarose hydrogels, the procedure is detailed elsewhere.[24] Briefly, a sterile agarose type VII low Tm 4% (w/v) solution prepared in sterile PBS was heated to 70ºC for 30 seconds, until complete dissolution. The solution was added to a human nasal chondrocytes pellet prepared as described for the gellan gum encapsulation and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of $1x10^6$ cells/ml. Agarose discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 20 minutes to form a solid gel, and then discs were cut using a borer. The experiments were repeated 3 times independently.

5.4. HUMAN ARTICULAR CHONDROCYTES ENCAPSULATION AND CULTURING IN GELLAN GUM HYDROGELS

In the work described in chapter VII, human articular chondrocytes were expanded and encapsulated at passage 1 in gellan gum hydrogels using the following procedure. Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. A sterile $CaCl_2$ solution was added to obtain a final concentration of 0.03% (w/v). The temperature was progressively decreased to 42ºC and stabilised at this value for posterior use always under constant stirring. Human articular chondrocytes were detached by trypsinisation, mixed with expansion medium, and centrifuged at 200g for 7 min. The supernatant was removed and the cells were ressuspended in warm sterile PBS solution, counted using an hemocytometer, and finally centrifuged at 200g for 7 min. The supernatant was discarded and the cells pellet kept at the bottom of the falcon tube. The gellan gum 1% (w/v) with $CaCl_2$ 0.03% (w/v) solution was added to the pellet of cells and the mixture ressuspended for complete homogenization of the cells within the matrix. Gellan gum discs containing $8x10^6$ cells/ml human articular chondrocytes were produced by casting this mixture

into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 1-2 minutes to form a solid gel. Discs of Ø 6±0.01 mm x 5.5±0.46 mm height were cut using a borer. The discs were cultured in expansion medium for 7 days which was afterwards replaced by differentiation medium for 49 days. The differentiation medium was prepared by replacing the bFGF in the expansion medium with insulin (1µg/ml) and ascorbic acid (50 µg/ml). The culture medium was changed every 3-4 days.

5.5. HUMAN ARTICULAR CHONDROCYTES ENCAPSULATION IN GELLAN GUM HYDROGELS (*IN VIVO T*ESTS)

Human articular chondrocytes were expanded and encapsulated at passage 2 in gellan gum hydrogels (Chapter VIII) using the following procedure. Briefly, gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1.25% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. The temperature was progressively decreased to 42ºC and stabilised always under constant stirring. Human articular chondrocytes were detached by trypsinisation, mixed with culture medium, and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cells were ressuspended in sterile PBS solution, counted using and hemocytometer, and finally centrifuged as before. The cell number was calculated so that the final concentration after encapsulation was of $5x10^6$ cells/ml. The supernatant was discarded and the cells pellet kept at the bottom of the tube were ressuspended in PBS. The gellan gum 1.25% (w/v) solution was extensively mixed with the chondrocytes suspension for complete homogeneous dispersion within the gel. Gellan gum with the encapsulated cells was allowed to gel in a cylindrical mould for 2-3 minutes. Discs of Ø 3 mm x 3 mm height were cut using a sterile blade and kept in sterile PBS before the implantation procedure. Gellan gum discs with no cells encapsulated were also prepared using the same procedure and used as controls.

6. *IN VIVO* IMPLANTATION

6.1. SUBCUTANEOUS IMPLANTATION OF GELLAN GUM HYDROGELS IN MICE

As part of the work described in chapter VII, gellan gum discs were prepared under sterile conditions following a methodology described.[29] Gellan gum 1% (w/v) (Ø 6±0.01 mm x 5.5±0.46 mm height) discs were subcutaneously implanted in the dorsal part of Balb/c mice (2-3 months

with an average weight of 20 g) during periods of up to 21 days. Six female mice were used (2 for each period of implantation). Each animal was anaesthetized with a mixture of 5:1 Imalgene® 1000 (Merial Toulouse, France) and Domitor® (1.25 mg/mouse, and 25 μg/mouse, respectively) prepared in physiological serum. Under surgical sterile conditions, 2 medial and ventral incisions (approximately 0.7 cm) containing the subcutis and the *Panniculus Carnosus* (skin smooth muscle) were performed in the dorsum of the mice. Craniolateral oriented pockets (2 per incision) were subcutaneously created by blunt dissection. Into these pockets, the Gellan gum discs were inserted (4 discs per animal) and the *Panniculus carnosus* and the skin were carefully sutured. The animals were kept with food and water *ad libitum* during all time of implantation.

6.2. SUBCUTANEOUS IMPLANTATION OF GELLAN GUM HYDROGELS WITH ENCAPSULATED HUMAN ARTICULAR CHONDROCYTES IN NUDE MICE

Gellan gum hydrogels discs with encapsulated human articular chondrocytes were subcutaneously implanted in the back of nude mice, wihtin the work described in chapter VIII. Six 4-week-old female Balb/C nude mice female with an average weight of 20 g (Charles River Laboratories Inc. USA) were anaesthetized with a mixture of ketamine (1.2 mg/mouse s.c., Imalgene® 1000, Merial, Lyon, France) and medetomidine (20 μg/mouse s.c., Domitor®, Orion Corp., Finland) prepared in physiological serum. After the confirmation of analgesia/anaesthesia two incisions were performed (reaching a maximum of 1.5 cm each) being one in the intrascapular region and another in the lumbar region. With the help of a forceps two side pockets were created through each of the incisions and gellan gum discs with encapsulated chondrocytes and with no encapsulated cells (control) were subcutaneously implanted. Four discs were implanted per animal, being two on the anterior region and other two on the posterior region. The incision sites were sutured and the mice transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. After 1 week and 4 weeks post implantation, mice were euthanized (n=3 for each time point) by exposure to a saturated carbon dioxide environment and the gellan gum discs were surgically recovered and processed for histological analysis, biochemical, and molecular analyses.

6.3. *IN VIVO* INJECTION IN RABBIT ARTICULAR CARTILAGE DEFECTS OF GELLAN GUM-CELLS SYSTEMS

Chondrogenic pre-differentiated rabbit adipose tissue derived progenitor cells (ASC + GF), non chondrogenic pre-differentiated rabbit adipose tissue derived progenitor cells (ASC) and rabbit articular chondrocytes (AC) were expanded until an adequate cell number was obtained for cells encapsulation and used at passage 2 under the following procedure (chapter IX). Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1.25% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. The temperature was progressively decreased to 42ºC and stabilised always under constant stirring. Articular cartilage full-thickness defects with a diameter of 4 mm were created in the medial septum of rabbit femur condyles. A 1 mm diameter hole was drilled to the subchondral bone in order to establish a link between the implant site and the subchondral bone marrow (Figure 1). Briefly, the rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corp., Finland). After the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the joint area of the right posterior leg and disinfected with chlorhexidine (Lifo-Scrub®, B. Braun Melsungen AG, Germany). Incisions were performed through the skin and muscle to access the articular capsule. The defects were created as detailed in Figure 1. The isolated cells were detached by trypsinisation, mixed with culture medium, and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cells were ressuspended in sterile PBS solution, counted using and hemocytometer, and finally centrifuged as before. The cell number was calculated so that the final concentration after encapsulation was $10x10^6$ cells/ml. The supernatant was discarded and the cells pellet kept at the bottom of the tube were ressuspended in a small amount of PBS. The gellan gum 1.25% (w/v) solution was mixed with the cell suspension for complete homogeneous dispersion within the gel and the mixture injected into the defect. A waiting time of 2-3 minutes was given for the gels to form *in situ*. Defects were also filled with gellan gum with no encapsulated cells and other defects were left empty. The experiments were conducted for periods of up to 8 weeks with data collection points at 1 week, 4 weeks, and 8 weeks. All test groups were performed in triplicates, except for rabbit articular chondrocytes which were performed in duplicates for each time point. The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink ad libitum without movement restrictions. At the established time points, the animals were euthanized by endovenous injection of an overdose of pentobarbital sodium (Eutasil® Ceva Sante Animale, France) and the defect sites were surgically

exposed. These were subjected to macroscopic observation and afterwards processed for histological and molecular analyses.

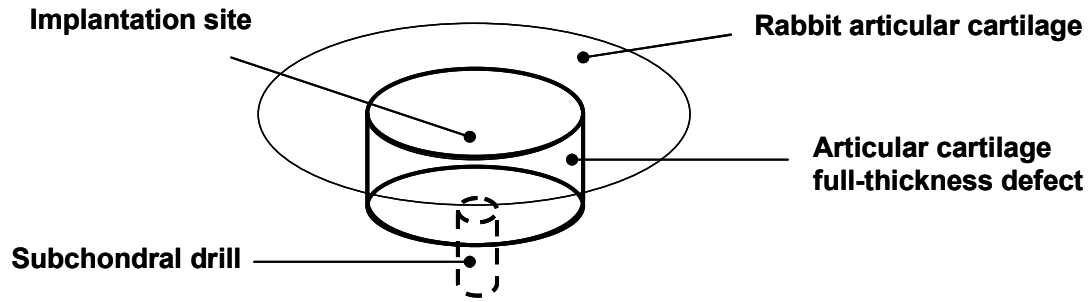

Figure II.1. Schematic representation of the articular cartilage defect created in the rabbits femoral condyles.

7. BIOLOGICAL EVALUATION

7.1. CELLULAR VIABILITY BY MTS TEST

The MTS test was performed in Chapter III to evaluate the viability of the cells seeded on the scaffolds at the different time periods, specifically 1, 2 and 3 weeks. Briefly, the procedure is as follows: the cell seeded C-PBS scaffolds (n=3) were rinsed in 0.15M phosphate-buffered saline (Sigma, St. Louis, MO) and immersed in a mixture consisting of serum-free cell culture medium and MTS reagent at 5:1 ratio. Incubation for 3h at 37ºC in a humidified atmosphere containing 5% CO_2 followed. After this, 100 µl were transferred to 96 well plates and the optical density (OD) determined at 490 nm. Controls consisting of scaffolds without any cells seeded were also used. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3; α=0.05.

7.2. CELL VIABILITY BY FLUORESCENT DYES

Cells viability during culturing was assessed using calcein AM staining in chapters VI and VII. Calcein AM (C3099, Invitrogen Corp.) is a fluorescence-based method for assaying cell viability and cytotoxicity in which the reagent is retained in cells that have intact membrane. Briefly, a calcein AM solution of 1/1000 was prepared in culture medium. One disc of each type of hydrogel with encapsulated chondrocytes was collected from the culturing plates and incubated in the

calcein AM solution for 15-30 min at 37ºC and afterwards washed in sterile PBS. For the samples of the work described in chapter VII, DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to DNA was used for counterstaining. The samples were observed under fluorescent microscopy (Zeiss HAL 100/HBO 100; Axiocam MRc5 (Zeiss).

7.3. CELL ADHESION AND MORPHOLOGY BY SCANNING ELECTRON MICROSCOPY (SEM)

Cell adhesion, morphology and average distribution were observed by SEM analysis in chapter III. The cell-scaffold constructs were washed in 0.15M phosphate-buffered saline and fixed in 2.5% glutaraldehyde (in phosphate-buffered saline). The constructs were then rinsed three times in phosphate-buffered saline, and subjected to a series of ethanol increasing conditions (30, 50, 70, 90, 100% ethanol), 10-15 minutes each, to allow dehydration of the samples. The samples were let to air dry afterwards, and then sputter coated with gold (JEOL JFC-1100) and analyzed with a Leica Cambridge S360 scanning electron microscope.

Scanning electron microscopy analysis was also applied in the works described in chapters IV and V in the following way. The tissue engineered constructs were washed in sterile PBS and immersed in 3% glutaraldehyde with 0.1 M cacodylate buffer pH 7.4) (AGAR) at room temperature for one hour. They were further washed three times in 0.1 M cacodylate buffer pH 7.4, and post fixed in 1% aqueous solution of osmium tetroxide for one hour. Finally, they were dehydrated in alcohols and let to dry. The samples were sputter coated with gold and observed using a Phillips XL-20 scanning electron microscope.

7.4. HISTOLOGICAL ANALYSIS

Several histological techniques were applied within the course of the works described in this thesis. As part of the work performed within chapters IV and V, the constructs were included in Optimal Cutting Temperature gel (OCT) (OCT compound BDH, Gurr®), frozen using liquid nitrogen and isopentane, stored at -20ºC for posterior cryosectioning. Tissue sections of 8 μm were taken and fixed using fresh 4% paraformaldehyde (Sigma, Co.). The slides were then washed in distilled water, let to dry and stored at 4ºC until the staining was performed. Hematoxylin-eosin stain was conducted in an automatic machine (Fume Cupboard; X219/E11/LEV1). In this procedure, the slides are stained in hematoxylin for a suitable time, optimised according to in-house procedures. The sections are washed in running tap water for 5 minutes or less, and afterwards differentiated in 1% acid alcohol, for 5-10 seconds. The slides are washed again in tap water for 5 minutes or less, and stained in 1% eosin for 10 minutes. They are again washed in tap water for 5 minutes, and dehydrated through alcohols and mounted in DPX

(Fluka/Sigma Co.). Toluidine blue staining was performed using standard histological methods in the following way. One drop of 1% toluidine blue was placed on each section for 2-3 seconds. Alcian blue staining was performed by rinsing the sections in 3% acetic acid and staining withy 1% alcian blue for 18 hours. Counterstaining was conducted with neutral red for 1 min. The sections were rinsed with distilled water. Dehydration through alcohols followed and the sections were then left to dry overnight, and mounted in DPX.

Within the works described in chapters VI and VII, samples were included in a methacrylate based compound for histological analysis. Hematoxylin-eosin (H&E), alcian blue and safranin-O staining on 4 μm thickness sections of the cell-scaffold constructs collected at different periods of culture. The samples were fixed in glutaraldehyde 2.5% (v/v), for 30-40 minutes at 4ºC and washed in PBS. Histological processing was performed using Tecnhovit 7100® (Heraeus Kulzer GmbH, DE) following the commercial procedure. Sections were cut using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH). H&E staining was performed using automatic processor (Leica Auto Stainer XL) according to in-house methodology (Leica TP1020-1, Leica MicroSystems GmbH). Histological staining with alcian blue and safranin-O was performed using standard histological methods. The slides were afterwards washed 3 times in distilled water, quickly dehydrated through 95% and 100% ethanol and then cleared in Histoclear® (National Diagnostics) and mounted using Microscopy Entellan® (Merck) for observation.

In chapters VII, VIII and IX, paraffin embedding of the samples was applied for histological analysis. Common histological analysis was performed on 4 μm thickness sections of the explants collected at different periods of culture. Hematoxylin-eosin (H&E) was conducted to observe general cell morphology and overall distribution, and alcian blue was performed to evaluate extracellular matrix components deposition, namely proteoglycans (glycosaminoglycans). Briefly, the constructs were carefully dissected from the subcutaneous tissue of nude mice and collected in eppendorf tubes. They were immediately fixated in formalin for 30-40 minutes and washed in PBS. Histological processing was conducted by dehydrating the samples in increasing ethanol concentrations, embedding them in paraffin and cutting sections for posterior analysis using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH). H&E staining was performed using an automatic processor according to in-house methodology (Leica TP1020-1, Leica MicroSystems GmbH) and alcian blue staining was performed using standard histological methods. The slides were washed afterwards in distilled water, dehydrated through increasing ethanol concentrations, and finally cleared in xylene substitute and mounted using Microscopy Entellan® (Merck & Co., Inc., USA) for observation.

7.5. HISTOLOGICAL SCORING (PINEDA SCORING SYSTEM)

The Pineda scoring system was used for histological evaluation of the explants collected after determined implantation periods in the work described in chapter IX.[30] The degree and the quality of healing in all defects was assessed and scored blindly by 3 independent researchers. The graded parameters included filling of defect, reconstruction of osteochondral junction, matrix staining, and cell morphology. The maximum possible score in the Pineda scoring system is 0 points with a minimum of 14 points. Detailed information can be found in Table 1.

Table II.1. Cartilage repair score by Pineda and co-workers.[30]

Characteristics	score
Filling of defect	
125%	1
100%	0
75%	1
50%	2
25%	3
0%	4
Reconstruction of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced staining	1
Significantly reduced staining	2
Faint staining	3
No stain	4
Cell morphology	
Normal	0
Most hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage, but mostly nonchondrocytic cells	3
Nonchondrocytic cells only	4

7.6. QUANTIFICATION OF PROTEOGLYCAN CONTENT

Proteoglycans are key components of the cartilage extracellular matrix being crucial for the mechanical functionality of the tissue. The 1,9-dimethylmethylene blue (DMB) metachromatic

assay is a standard procedure to quantitatively assess the production of these molecules and was performed based on previous descriptions on the works presented in chapters IV, V, and VIII.[31] Proteoglycans were determined by measuring the level of sulfated glycosaminoglycans (GAGs). GAG levels can be quantified in solution using DMB since the mechanical entanglement of this reagent with GAGs generates a peak shift at $A_{525\text{-}530}$ that can be measured spectrophotometrically. Briefly, the constructs were carefully collected in eppendorf tubes. The samples were grinded with a mortar and pestle and immersed in a digestion solution with papain and N-acetyl cysteine at 60ºC for approximately 3 hours. The tubes were centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected for biochemical analysis. A chondroitin sulfate standard solution was prepared in water and kept at 4ºC. The samples and the chondroitin sulfate standards were placed in a 96 well round-bottomed plate, DMB solution was added to each well, and the optical density was measured using a microplate reader at 530 nm. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3. Small variations were performed on the incubation times depending on the type of sample analysed.

7.7. REALTIME PCR ANALYSES

The experimentakl work within chapters VII, VIII, IX, involved the use of molecular approaches to study the transduction of cartilage specific markers during the time of the studies. Realtime PCR is semiquantitative/quantitative method that measures in real time the quantity of a given codifying molecule in a sample, enabling to determine their regulatory profile, that can be up or downregulated, or alternatively exhibit no significant variation. Within the works shown in chapters VII to IX, samples were collected at defined time periods, quickly frozen in liquid nitrogen, and stored at −80°C until the analysis was performed. RNA was extracted using TRIzol® (Invitrogen) according to the provided technical datasheet. Briefly, 3 samples of each condition were grinded and mechanically homogenized with a mortar and pestle in TRIzol reagent, being each condition performed in duplicate. Afterwards, chloroform was added and the samples centrifuged to establish a three-phase composition in the tube. The aqueous phase was collected and put in a new tube where isopropanol was added. The samples were centrifuged, the supernatant discarded and the pellet washed with 75% ethanol. After a final centrifugation the samples were allowed to air-dry, and suspended in ultrapure water for posterior analysis. The amount of isolated RNA and A260/280 ratio was determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies). After these determinations, 1µg of RNA of each sample was reverse transcribed into cDNA using the IScript™ cDNA synthesis kit (Biorad) in a MJ Mini™ Personal Thermal Cycler (Biorad). Cartilage related markers were chosen to evaluate the chondrogenic

phenotype of the cultured systems. These included Sox9, collagen type I, collagen type II, collagen type X, and aggrecan, using GAPDH as the housekeeping gene for normalization. The expression of each gene was normalized to the GAPDH value in that sample. The relative gene expression quantification was performed using the $2^{-\Delta\Delta Ct}$ (Livak) method,[32] considering that:

$$2^{-\Delta\Delta Ct} = \text{Normalized expression ratio}$$

All the primer sequences were generated using Primer3™ software[33] and acquired from MWG Biotech™. More details can be found in Table 1. Real-time PCR was performed using an MJ Mini™ Personal Thermal Cycler (Biorad) machine and SYBR Green IQ™ Supermix (Biorad) to detect amplification variations. The analysis of the results was performed with MJ Opticon Monitor 3.1 software (Biorad). Some changes were performed in independent works in relation to the analytical apparatus.

7.8. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND TYPE II

Collagen types I and II were detected immunohistochemically (chapters IV and V) using monoclonal antibodies against collagen types I and II (Southern Biotechnology, UK), as previously described[31]. Briefly, fixed sections were washed with PBS and pre-treated with hyaluronidase (10 mg/ml), followed by pronase (2 mg/ml). The slides were washed thoroughly in PBS and treated with 3% hydrogen peroxide in 50% methanol, followed by washing in Tris-buffered saline (TBS) and blocking with 3% bovine serum albumin (BSA). Incubation with the primary antibody (collagen type I and collagen type II) (UNLB) followed. The remaining protocol is as described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). The slides were washed in water for 5 minutes, counterstained with Mayer's haematoxylin, and mounted with DPX mounting medium. Controls were performed using normal goat serum instead of the primary antibodies, which was included in the kit. Polyglycolic acid (PGA) non-woven scaffolds (Albany international, Bury, Lancashire, UK) were used as controls.

The work presented in chapter VIII exhibited slight variations when compared with this procedure as described above. Collagen types I and II were detected immunohistochemically with monoclonal antibodies against collagen types I and II (Southern Biotechnology, UK) using the Vectastain® Universal Elite ABC Kit PK-7200 (Vector Laboratories Ltd, UK) and DAB Substrate Kit for Peroxidase SK-4100 (Vector Laboratories Ltd, UK) according to the suppliers indications. Briefly, paraffin sections on the collected explants were deparaffinised and hydrated through

decreasing ethanol concentrations. The sections were treated with 3% hydrogen peroxide in 50% methanol for 5 minutes, washed in PBS buffer, and incubated in pre-diluted blocking serum. The incubation with the primary antibodies and negative control followed (collagen type I 1:100; collagen type II 1:20, normal horse serum 1:100). The remaining protocol is as described in the Vectastain® Elite ABC Kit. The sections were further incubated with the DAB substrate at room temperature until suitable staining develops according the suppliers indications. The sections were counterstained with neutral red, dehydrated through increasing ethanol concentrations, and finally cleared in Xylene substitute ® (National Diagnostics) and mounted using Microscopy Entellan® (Merck) for observation.

7.9. WESTERN BLOT FOR COLLAGEN TYPE II

Production of collagen type II by the cell-scaffold systems described in the work presented in chapter III was analysed by western blotting. For the protein extraction, the cell-scaffold constructs (n=3) were washed in 0.15 M phosphate-buffered saline, lysed in 750 µl of lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, and Triton X-100), and sonicated three times at 40 kV (15 s). After sonication the C-PBS scaffolds were removed and the resulting suspension was centrifuged for 10 min at 13000 rpm, 4ºC at the end of which the formed pellet was discarded. The supernatants containing the protein fraction were stored for quantification. Western blot was performed using the protein extracts collected at 3 weeks. A 5% stacking polyacrylamide gel (30% acrylamide mix; 1.0 MTris (pH 6.8); 10% SDS; 10% ammonium persulfate; TEMED) and a 8% resolving gel (30% acrylamide mix; 1.5 M Tris (pH 8.8); 10% SDS; 10% ammonium persulfate; TEMED) were prepared. The collected supernatant was heated at 100ºC for 5 minutes in the water bath and then an aliquot (15.64 µl) was loaded in the gels and subjected to electrophoresis (30 mA, 3 hours), and electrotransferred to a Hybond P membrane (Amersham Biosciences, Piscataway, NJ). Afterwards, the membrane was washed in phosphate-buffered saline-T, submerged in Coomassie blue (isopropyl alcohol 0.25% (v/v); acetic acid 0.1% (v/v); Coomassie Brilliant Blue R250 (CBB) 2 g/L, and left overnight. The membrane was washed in Ponceau solution and the transfer from the gel to the membrane was visually confirmed. Membranes were then incubated with a blocking solution of 5% (wt/v) powdered milk in TBS (Tris base 2.42 g/L; NaCl 8 g/L; HCl 3.8 g/L) with Triton X-100 0.002% (v/v), for 1 hour under constant stirring, at room temperature. Incubation with an equal solution followed, but altering the powdered milk concentration to 2.5% (wt/v). This solution also included the primary antibody against collagen type II (University of Iowa, Iowa City, IA) at a 1:500 dilution. The membrane was left overnight with constant stirring, at 4ºC. The membrane was then washed three times in

phosphate-buffered saline-T under stirring, and incubated with the secondary antibody (1/1000) for 1 hour at room temperature, under stirring. The secondary antibody was diluted in the same solution as the one described above for the primary antibody. The membranes were washed three times in phosphate-buffered saline-T and passed to a phosphate-buffered saline solution. The immune complex was detected by incubation of the membrane as described in SuperSignal® Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA).

8. STATISTICAL ANALYSIS

Statistical analyses were conducted using Student's two tailed t-test assuming unequal variances and ρ-values below 0.05 were considered statistically significant. Regarding specific dynamic mechanical analyses and rheological measurements, statistical analysis were performed using confidence intervals based on the experimental results, with a confidence level of 99%.

REFERENCES

[1.] Freier, T., Koh, H. S., Kazazian, K., and Shoichet, M. S., Controlling cell adhesion and degradation of chitosan films by N-acetylation, *Biomaterials* 26 (29), 5872-8, 2005.

[2.] Madihally, S. V. and Matthew, H. W. T., Porous chitosan scaffolds for tissue engineering, *Biomaterials* 20 (12), 1133-1142, 1999.

[3.] Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering A* In Press, Corrected Proof, 2005.

[4.] Mahmoudifar, N. and Doran, P. M., Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors, *Biomaterials* In Press, Corrected Proof.

[5.] Devlieghere, F., Vermeulen, A., and Debevere, J., Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables, *Food Microbiology* 21 (6), 703-714, 2004.

[6.] Silva, R. M., Malafaya, P. B., Mano, J. F., and Reis, R. L., Bioactive composite chitosan membranes to be used in bone regeneration applications, *Bioceramics 15* 240-2, 423-426, 2003.

[7.] Lyoo, W. S., Kim, J. H., Yoon, W. S., Ji, B. C., Choi, J. H., Cho, J., Lee, J., Yang, S. B., and Yoo, Y., Effects of polymer concentration and zone drawing on the structure and properties of biodegradable poly(butylene succinate) film, *Polymer* 41 (26), 9055-9062, 2000.

[8.] Henry, E. W., Chiu, T.-H., Nyilas, E., Brushart, T. M., Dikkes, P., and Sidman, R. L., Nerve regeneration through biodegradable polyester tubes, *Experimental Neurology* 90 (3), 652-676, 1985.

[9.] Han, J.-A. and Lim, S.-T., Structural changes of corn starches by heating and stirring in DMSO measured by SEC-MALLS-RI system, *Carbohydrate Polymers* 55 (3), 265-272, 2004.

[10.] Funami, T., Kataoka, Y., Omoto, T., Goto, Y., Asai, I., and Nishinari, K., Food hydrocolloids control the gelatinization and retrogradation behavior of starch. 2b. Functions of guar gums with different molecular weights on the retrogradation behavior of corn starch, *Food Hydrocolloids* 19 (1), 25-36, 2005.

[11.] Marques, A. P., Reis, R. L., and Hunt, J. A., An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats, *Macromolecular Bioscience* 5 (8), 775-785, 2005.

[12.] Gomes, M. E., Godinho, J. S., Tchalamov, D., Cunha, A. M., and Reis, R. L., Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties, *Materials Science and Engineering: C* 20 (1-2), 19, 2002.

[13.] Salgado, A. J., Coutinho, O. P., and Reis, R. L., Novel Starch-Based Scaffolds for Bone Tissue Engineering: Cytotoxicity, Cell Culture, and Protein Expression, *Tissue Engineering* 10 (3-4), 465-474, 2004.

[14.] Marques, A. P. and Reis, R. L., Hydroxyapatite reinforcement of different starch-based polymers affects osteoblast-like cells adhesion/spreading and proliferation, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 25 (2), 215-229, 2005.

[15.] Li, W. J., Danielson, K. G., Alexander, P. G., and Tuan, R. S., Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(?-caprolactone) scaffolds, *Journal of Biomedical Materials Research - Part A* 67 (4), 1105-1114, 2003.

[16.] Li, W.-J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K. G., Hall, D. J., and Tuan, R. S., A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells, *Biomaterials* 26 (6), 599, 2005.

[17.] Freed, L. E., Vunjak-Novakovic, G., Biron, R. J., Eagles, D. B., Lesnoy, D. C., Barlow, S. K., and Langer, R., Biodegradable polymer scaffolds for tissue engineering, *Biotechnology (N Y)* 12 (7), 689-93, 1994.

[18.] Jansson, P.-E., Lindberg, B., and Sandford, P. A., Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea, *Carbohydrate Research* 124 (1), 135-139, 1983.

[19.] Moorhouse R., Colegrove G.T., Sandford P.A., Baird J.K., and Kang K.S., *PS-60: a new gel-forming polysaccharide* D.A. Brandt, Washington DC, 1981.

[20.] Kang, K. S., Colegrove T. & Veeder, G.T., USA, 1982.

[21.] Grasdalen, H. and Smidsrod, O., Gelation of gellan gum, *Carbohydrate Polymers* 7 (5), 371-393, 1987.

[22.] Gomes, M. E., Sikavitsas, V. I., Behravesh, E., Reis, R. L., and Mikos, A. G., Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds, *J Biomed Mater Res A* 67 (1), 87-95, 2003.

[23.] Salgado, A. J., Coutinho, O. P., and Reis, R. L., Novel starch-based scaffolds for bone tissue engineering: cytotoxicity, cell culture, and protein expression, *Tissue Eng* 10 (3-4), 465-74, 2004.

[24.] Mauck, R. L., Yuan, X., and Tuan, R. S., Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture, *Osteoarthritis and Cartilage* 14 (2), 179-189, 2006.

[25.] Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering: A* 403 (1-2), 57-68, 2005.

[26.] ISO10993, d., Biological compatibility of medical devices. 5. Test for citotoxicity: *In vitro* methods, December 1992.

[27.] Dennis, J. E., Merriam, A., Awadallah, A., Yoo, J. U., Johnstone, B., and Caplan, A. I., A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse, *J Bone Miner Res* 14 (5), 700-9, 1999.

[28.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[29.] JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, and RL Reis, Gellan gum: A new biomaterial for cartilage tissue engineering applications *submitted*, 2008.

[30.] Pineda, S., Pollack, A., Stevenson, S., Goldberg, V., and Caplan, A., A semiquantitative scale for histologic grading of articular cartilage repair, *Acta Anat (Basel)* 143 (4), 335-40, 1992.

[31.] Kafienah, W., Sims, T.J., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[32.] Livak, K. J. and Schmittgen, T. D., Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method, *Methods* 25 (4), 402-408, 2001.

[33.] http://frodo.wi.mit.edu/.

SECTION 3.

CHAPTER III.

Assessment of the Suitability of Chitosan/PolyButylene Succinate Scaffolds Seeded with Mouse Mesenchymal Progenitor Cells for a Cartilage Tissue Engineering Approach

CHAPTER III.

Assessment of the Suitability of Chitosan/PolyButylene Succinate Scaffolds Seeded with Mouse Mesenchymal Progenitor Cells for a Cartilage Tissue Engineering Approach*

ABSTRACT

In this work, scaffolds derived from a new biomaterial originated by the combination of a natural and a synthetic material, were tested for assessing their suitability for cartilage tissue engineering applications. In order to obtain a better outcome result in terms of scaffolds overall properties, different blends of natural and synthetic materials were created and details regarding this may be found elsewhere.[1] Chitosan and polybutylene succinate 50/50 (wt%) were melt blended using a twin screw extruder and processed into 5x5x5mm scaffolds (C-PBS) by compression moulding with salt leaching. Micro-computed tomography (μ-CT) analysis estimated an average of 66.29% porosity and 92.78 % interconnectivity degree for the presented scaffolds. The salt particles used ranged in size between 63-125 μm, retrieving an average pore size of 251.28 μm. Regarding the mechanical properties, the compressive modulus was of 1.73±0.4 MPa (Esec 1%). Cytotoxicity evaluation revealed that the leachables released by the developed porous structures were not harmful to the cells and hence were non cytotoxic. Direct contact assays were carried using a mouse bone marrow derived mesenchymal progenitor cell line (BMC9). Cells were seeded at a density of $5x10^5$cells/scaffold and allowed to grow for periods up to 3 weeks, under chondrogenic differentiating conditions. Scanning electron microscopy (SEM) analysis revealed the cells were able to proliferate and colonize the scaffolds structure, and MTS test demonstrated cell viability during the time of the experiment. Finally, western blot performed for collagen type II, a natural cartilage extracellular matrix component, showed that this protein was being expressed by the end of 3 weeks, which seems to indicate that the BMC9 cells were being differentiated towards the chondrogenic pathway. These results indicate the adequacy of these newly developed C-PBS scaffolds for supporting cells growth and differentiation towards the chondrogenic pathway, suggesting they should be considered for further studies in the cartilage tissue engineering field.

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1. INTRODUCTION

Trauma and disease of bone and joints, frequently involving structural damage to both the articular cartilage surface and the subchondral bone, result in severe pain and disability for millions of people worldwide. Such problems were initially addressed by performing different surgical procedures, which included debridement, drilling, abrasion arthroplasty, and microfracture.[2,3] Some of these clinical experiments turned out to be successful in some cases, and are still being performed in hospitals and clinics throughout the world, but most of their outcomes are variable and dependent on a wide range of factors that can limit its widen application.[4,5]

Tissue engineering was brought up as a new way to address these problems and grew as a new field of knowledge not only in cartilage regeneration but also in several other types of tissue.[6-8] The fundamental goal of tissue engineering is to develop biological substitutes that restore, maintain or improve tissue function and to apply these to clinical scenarios where tissue is lost through trauma or disease.[9] The cells support structure – a scaffold, should serve as a three-dimensional template for initial cell attachment and subsequent tissue formation, both *in vitro* and *in vivo*. It must not trigger strong immunological responses nor cause severe cytotoxicity effects, and should present mechanical properties similar to the tissue of interest.[6]

Scaffolds are made of materials that can be broadly divided into synthetic and natural.[10,11] Synthetic polymers include the polylactides, such as polylactic acid (PLA)[10] and polyglycolic acid (PGA),[12] although a wide range of others such as polyethylene oxide (PEO),[13] poly(lactic-co-glycolic) PLGA,[14] and poly-2-hydroxyethyl methacrylate (PHEMA)[15] exist. Even though they possess some controllable and advantageous features, such as reproducible manufacturing at a large scale and controlled degradation time, they also have several disadvantages, like the lack of cell-recognition signals. Furthermore, specific features of some polymers, like the acidic by-products release by PLA for example, pose additional difficulties to their use.[16]

Natural origin materials seem to overcome some of those limitations.[17,18] Most of them are normal components of the tissue to be regenerated, possess specific sites for cell recognition, and share some similarities with the native tissue components.[19-21] Collagen,[20] hyaluronic acid,[19] chitosan,[11,21] and starch,[22] are examples of those materials. The conjugation of natural and synthetic materials in the fabrication of a scaffold taking advantage of the individual features of each one is a strategy that has been tried by different research groups. Chen et al. produced a hybrid mesh of PLGA and collagen that enabled the aggregation of mesenchymal stem cells and provided them with a microenvironment that allowed chondrogenic differentiation to occur.[23] In

another study, Wang et al fabricated a hybrid matrix based on polyglycolide and chitosan that allowed fibroblast proliferation and revealed promising for further tissue engineering applications.[24]

In this work, scaffolds of a blend of chitosan, a natural polymer derived from the deacetylation of chitin, and polybutylene succinate, a synthetic polymer, were produced and put through preliminary *in vitro* tests. The rationale is to combine the biological properties of chitosan with the mechanical support conferred by polybutylene succinate expecting this will render a better performance to the tissue engineered construct once implanted. Chitosan is a partially deacetylated derivative of chitin, which is the primary structural polymer in arthropod exoskeletons, shells of crustaceans, and the cuticles of insects.[25] Chitosan is a semi-crystalline polysaccharide that is normally insoluble in aqueous solutions above pH 7. However, in dilute acids (pH 6), the free amine groups are protonated and the molecule becomes soluble. This pH-dependent solubility provides a convenient mechanism for processing under mild conditions.[26] Chitosan is reported to be non-toxic, biodegradable and biocompatible[27], and has structural similarities to glycosaminoglycans, which are structural components of the cartilage extracellular matrix.[28] It serves different applications and its use ranges from the food industry to the biomedical and pharmaceutical fields.[29,30] Polybutylene succinate (PBS) is one of the most accessible biodegradable polymers, and has been extensively studied for its potential use as a future conventional plastic, serving also as a support for different approaches in the medical field.[31,32] It is an aliphatic polyester presenting good processability and flexibility, and having degradation products that are non-toxic and can enter the metabolic cycles of bioorganisms. Its conjugation with chitosan aims at providing mechanical support to the scaffold, which should be advantageous considering the clinical scenario of constant load-bearing efforts in articular cartilage. Little research has been conducted in the melt blending of synthetic polyesters and chitosan,[27] and the preliminary results described herein for their use as potential scaffolds for cartilage regeneration are important. The herein developed C-PBS scaffolds were seeded with cells originated from a mouse mesenchymal stem cell line (BMC9)[33] and cultured under chondrogenic inductive conditions, in order to assess their suitability for cartilage tissue engineering approaches.

2. MATERIALS AND METHODS

2.1. SCAFFOLDS PRODUCTION AND PROCESSING

The chitosan/polybutylene succinate 50/50 (%wt) blend was compounded in a twin screw extruder. The details of the processing conditions are summarized elsewhere.[27] The methodology used for the scaffolds production was based on compression moulding followed by salt leaching. Before using it in the scaffolds processing, the salt was grinded and sieved to obtain particles with size between 63 μm<d<125 μm. The compounded polymeric blend was ground, mixed with salt and compression molded into discs. The salt content was 80% by weight. The discs were cut into 5x5x5 mm cubes. These cubes were then immersed in distilled water to leach out the salt, dried, and used for cell culture and proliferation studies after sterilization by ethylene oxide.

2.2. SCAFFOLDS CHARACTERIZATION

The C-PBS scaffolds structure was analysed by scanning electron microscopy (SEM) using a Leica Cambridge S360 (Leica Cambridge, Cambridge, UK). Micro-computed tomography equipment (SkyScan, Belgium) was used for more detailed analysis of the morphology of the developed scaffolds and CT Analyser and CT Vol Realistic 3D Visualization were used as image processing softwares, both from SkyScan (Belgium), were used as image processing tools. Uniaxial compression tests were performed to assess the mechanical properties of the scaffolds (dry state) using a Universal tensile testing machine (Instron 4505 Universal Machine). The details regarding these methods are presented elsewhere.[1]

2.3. *IN VITRO* CYTOTOXICITY TESTS

To assess the short-term cytotoxicity of the developed C-PBS scaffolds, minimum essential medium (MEM) extraction and 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) tests, both within a 24 h extraction period, according to ISO/EN 10993 part 5 guidelines[34] were used, in order to establish the possible toxic effects of leachables released from the scaffolds during extraction. Latex rubber was used as positive control for cell death due to its high cytotoxicity to cells, and culture medium was used as a negative control representing the ideal situation for cell proliferation. The results are presented after normalisation wuth the negative control. The objectives of the MEM extraction test are to evaluate changes in

cell morphology and growth inhibition, whereas the MTS test determines whether cells are metabolically active.

2.3.1. CELL CULTURE

A rat lung fibroblasts cell line – L929-, acquired from the European Collection of Cell Cultures (ECACC), was used. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% foetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% of an antibiotic-antimycotic mixture (Sigma, St. Louis, MO). Trypsin (Sigma, St. Louis, MO) was used to detach the cells from the culture flasks before the experiments were conducted.

2.3.2. MEM EXTRACTION TEST

The ratio of material weight to extract fluid was constant and equal to 0.2g/ml for porous samples. For the positive control the ratio of material outer surface to extraction fluid was 2.5 cm^2/ml. Test material (n=6) and positive control were extracted for 24h at 37ºC, using complete culture medium as extraction fluid. Before the tests, culture medium was removed from the wells and an identical volume (2 ml) of extraction fluid was added. For the MEM extraction test, the cells were seeded in 24 well plates (n=3) at a density of $1.25x10^5$ cells/well. They were incubated for 24h at 37ºC, in a humidified atmosphere with 5% CO_2 after this. Cell response was evaluated after 24, 48, and 72h of incubation. Confluence of the monolayer, degree of floating cells, and changes in morphology were analyzed by visual observation. After 72h, the percentage of growth inhibition was determined by cell counting with a haemocytometer and trypan blue exclusion method. Final measurements were corrected for the negative control.

2.3.3. MTS TEST

For the MTS test, cells were seeded in 96 well plates (n=6) at a density of $1.8x10^4$cells/well. They were incubated for 24h at 37ºC, in a humidified atmosphere with 5% CO_2 after this. As referred, the MTS was performed to evaluate the cytotoxic effects of the developed scaffolds. A kit CellTiter 96 One solution Cell Proliferation Assay kit (Promega, Madison, WI) was used. It is based on the bioreduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS), into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. Briefly, the procedure was conducted as follows: the extraction procedure was the same described previously for the MEM extraction test, using 200 µl of extraction fluid per well.

After 72 h, the extraction fluid was removed and 200 µl of a mixture containing serum-free culture medium without phenol red, and MTS was added to each well. Cells were then incubated for 3h at 37ºC in a humidified atmosphere containing 5% CO_2. After this time, optical density (OD) was measured with a plate reader (Molecular Devices, SunnyVale, CA) at 490 nm. The mean OD value obtained was standardized taking into account the values for the negative control. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3; α=0.05.

2.4. DIRECT CONTACT ASSAYS

2.4.1. CELL SEEDING AND CULTURING

For the direct contact assays, a mouse mesenchymal progenitor cell line (BMC9) was used. The BMC9 cell line has been shown to exhibit four mesenchymal cell phenotypes: chondrocytic, adipocytic, stromal (supports osteoclasts formation), and osteoblastic.[33] The cells were grown as monolayer cultures in a culture medium consisting of α-MEM medium (Sigma, St. Louis, MO), 10% Foetal Bovine Serum (FBS), and 1% A/B (penicillin G sodium 10000 U/ml, amphotericine B (Fungizone) 25 µg/ml, streptomycin sulfate 10000 µg/ml, in 0.85% saline). When the adequate cell number was obtained, cells at passage 9 (P9) were trypsinized, centrifuged, and ressuspended in cell culture medium. Cells were seeded at a density of $5x10^5$cells/scaffold ($5x5x5mm^3$) under static conditions, using for this purpose aliquots of 15 µl loaded on top of the scaffolds that had been previously placed in 24 well culture plates. Two hours after seeding, 1 ml of culture medium was added to each well and the cell seeded scaffolds were allowed to develop for periods up to 3 weeks, in a humidified atmosphere at 37ºC, containing 5% CO_2, under chondrogenic differentiation inducing medium. This culturing medium consisted of DMEM (Sigma, St. Louis, MO), dexamethasone (Sigma, St. Louis, MO) $1.0x10^{-4}$ M, sodium pyruvate (Sigma, St. Louis, MO) $1.0x10^{-3}$ M, ascorbate-2-phosphate (Sigma, St. Louis, MO) 0.17 mM, proline (Sigma, St. Louis, MO) 0.35 mM, ITS 1X (Sigma, St. Louis, MO), and hBMP-2 (R&D BioSystems, CA) 100ng/ml. The culture medium was changed every 3 to 4 days until the end of the experiment.

2.4.2. CELLULAR VIABILITY BY MTS TEST

The principle of the MTS test has been already previously described in the Materials and Methods part, for the *in vitro* cytotoxicity tests. The MTS test was performed for the cell seeded scaffolds for different time periods, specifically 1, 2 and 3 weeks. Briefly, the procedure is as

follows: the cell seeded C-PBS scaffolds (n=3) were rinsed in 0.15M phosphate-buffered saline (Sigma, St. Louis, MO) and immersed in a mixture consisting of serum-free cell culture medium and MTS reagent at 5:1 ratio. Incubation for 3h at 37ºC in a humidified atmosphere containing 5% CO_2 followed. After this, 100 µl were transferred to 96 well plates and the optical density (OD) determined at 490 nm. Controls consisting of scaffolds without any cells seeded were also used. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3; α=0.05.

2.4.3. CELL ADHESION AND MORPHOLOGY BY SCANNING ELECTRON MICROSCOPY (SEM)

Cell adhesion, morphology and average distribution were observed by SEM analysis. The cell-scaffold constructs were washed in 0.15M phosphate-buffered saline and fixed in 2.5% glutaraldehyde (in phosphate-buffered saline). The constructs were then rinsed three times in phosphate-buffered saline, and subjected to a series of ethanol increasing conditions (30, 50, 70, 90, 100% ethanol), 10-15 minutes each, to allow dehydration of the samples. The samples were let to air dry afterwards, and then sputter coated with gold (JEOL JFC-1100) and analyzed with a Leica Cambridge S360 scanning electron microscope.

2.4.4. WESTERN BLOT: COLLAGEN TYPE II

For the protein extraction, the cell-scaffold constructs (n=3) were washed in 0.15 M phosphate-buffered saline, lysed in 750 µl of lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, and Triton X-100), and sonicated three times at 40 kV (15 s). After sonication the C-PBS scaffolds were removed and the resulting suspension was centrifuged for 10 min at 13000 rpm, 4ºC at the end of which the formed pellet was discarded. The supernatants containing the protein fraction were stored for quantification. Western blot was performed using the protein extracts collected at 3 weeks. A 5% stacking polyacrylamide gel (30% acrylamide mix; 1.0 MTris (pH 6.8); 10% SDS; 10% ammonium persulfate; TEMED) and a 8% resolving gel (30% acrylamide mix; 1.5 M Tris (pH 8.8); 10% SDS; 10% ammonium persulfate; TEMED) were prepared. The collected supernatant was heated at 100ºC for 5 minutes in the water bath and then an aliquot (15.64 µl) was loaded in the gels and subjected to electrophoresis (30 mA, 3 hours), and electrotransferred to a Hybond P membrane (Amersham Biosciences, Piscataway, NJ). Afterwards, the membrane was washed in phosphate-buffered saline-T, submerged in Coomassie blue (isopropyl alcohol 0.25% (v/v); acetic

acid 0.1% (v/v); Coomassie Brilliant Blue R250 (CBB) 2 g/L, and left overnight. The membrane was washed in Ponceau solution and the transfer from the gel to the membrane was visually confirmed. Membranes were then incubated with a blocking solution of 5% (wt/v) powdered milk in TBS (Tris base 2.42 g/L; NaCl 8 g/L; HCl 3.8 g/L) with Triton X-100 0.002% (v/v), for 1 hour under constant stirring, at room temperature. Incubation with an equal solution followed, but altering the powdered milk concentration to 2.5% (wt/v). This solution also included the primary antibody against collagen type II (University of Iowa, Iowa City, IA) at a 1:500 dilution. The membrane was left overnight with constant stirring, at 4ºC. The membrane was then washed three times in phosphate-buffered saline-T under stirring, and incubated with the secondary antibody (1/1000) for 1 hour at room temperature, under stirring. The secondary antibody was diluted in the same solution as the one described above for the primary antibody. The membranes were washed three times in phosphate-buffered saline-T and passed to a phosphate-buffered saline solution. The immune complex was detected by incubation of the membrane as described in SuperSignal® Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA).

3. RESULTS

3.1. SCAFFOLDS CHARACTERIZATION

As seen in the SEM and μ-CT images (Figs. 1 and 2, respectively), the structure of the processed C-PBS scaffolds appear to be quite interconnected (Fig. 2) and with suitable pore size to provide support for cell growth and development. Micro-computed tomography analysis estimated an average of 66.29% (±2.55%) porosity and 92.78% (±1.69%) interconnectivity degree for these C-PBS scaffolds. The salt particles used ranged in size between 63-125 μm, retrieving an average pore size of 251.28 μm (±61. 9 μm). In terms of mechanical properties, the scaffolds exhibited a compressive modulus of 1.73±0.4 MPa (Esec 1%).

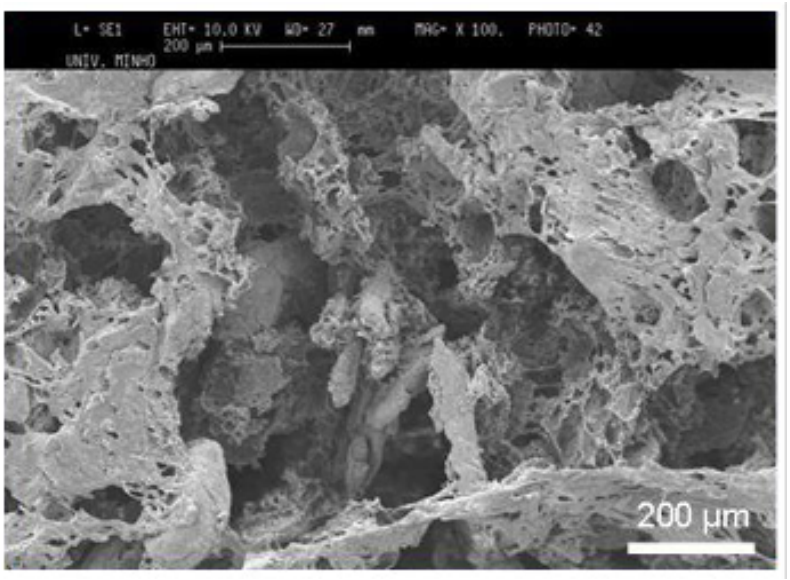

Figure III.1. SEM micrograph showing the surface of a chitosan-polybutylene succinate scaffold 50:50 (wt%), with 80% porosity.

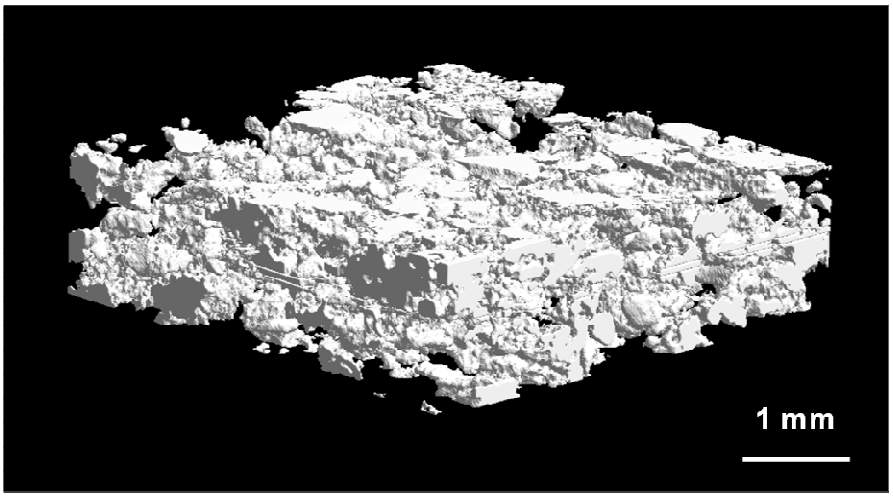

Figure III.2. µ-CT image evidencing the structure and interconnectivity of the processed chitosan-polybutylene succinate scaffold 50:50 (wt%), with 80% porosity scaffolds.

3.2. *IN VITRO* CYTOTOXICITY TESTS (MEM EXTRACTION AND MTS TESTS)

Regarding the MEM extraction test, the results showed that the materials did not cause any morphological changes or induce any deleterious alteration to the metabolic activity of L929 cells and thus, can be considered non cytotoxic. There was no growth inhibition detected after the 72h time period (0.0±0.0%), when using the trypan blue exclusion method. The negative control did not affect cell proliferation and morphology and a monolayer of spread cells was observed. The

toxic effect of the positive control (latex) was evident, given the severe changes on morphology and the inability of cells to proliferate. The extracts from the C-PBS scaffolds did not exert any deleterious effect on L929 cells morphology, presenting morphological and proliferative features similar to those encountered for the negative controls (data not shown). Concerning the MTS test, L929 cells were able to metabolize the MTS into a brown formazan product after a 72 h incubation period with the collected extracts and the values obtained were similar to the negative control (Fig. 3).

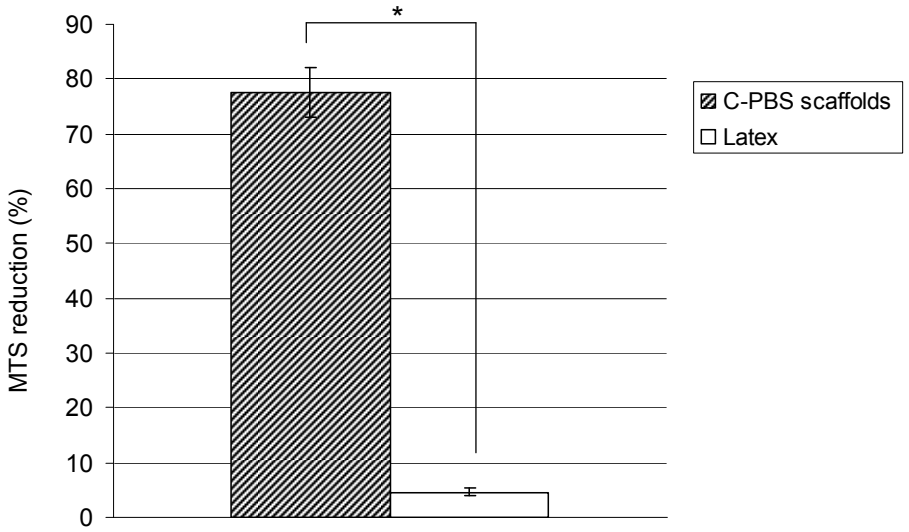

Figure III.3. Graphical representation of the results obtained after cytotoxicity evaluation of the processed scaffolds (MTS test) using L929 cells, derived from a rat lung fibroblast cell line. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3; α=0.05.

3.3. DIRECT CONTACT ASSAYS

Direct contact assays were performed to evaluate BMC9 cells response to the C-PBS scaffolds. MTS was performed to assess cells viability at defined time periods, specifically 1, 2 and 3 weeks. Figure 4 represents the obtained results where it can be observed that the cells were able to remain viable within the C-PBS scaffolds during the whole time of the experiments. The values were higher than the control for all time points and had a significant increase ($p < 0.05$) from 1 week to 2 weeks, which is a good indicator of cell viability. At 3 weeks, a significant decrease ($p < 0.05$) is observed in the OD values.

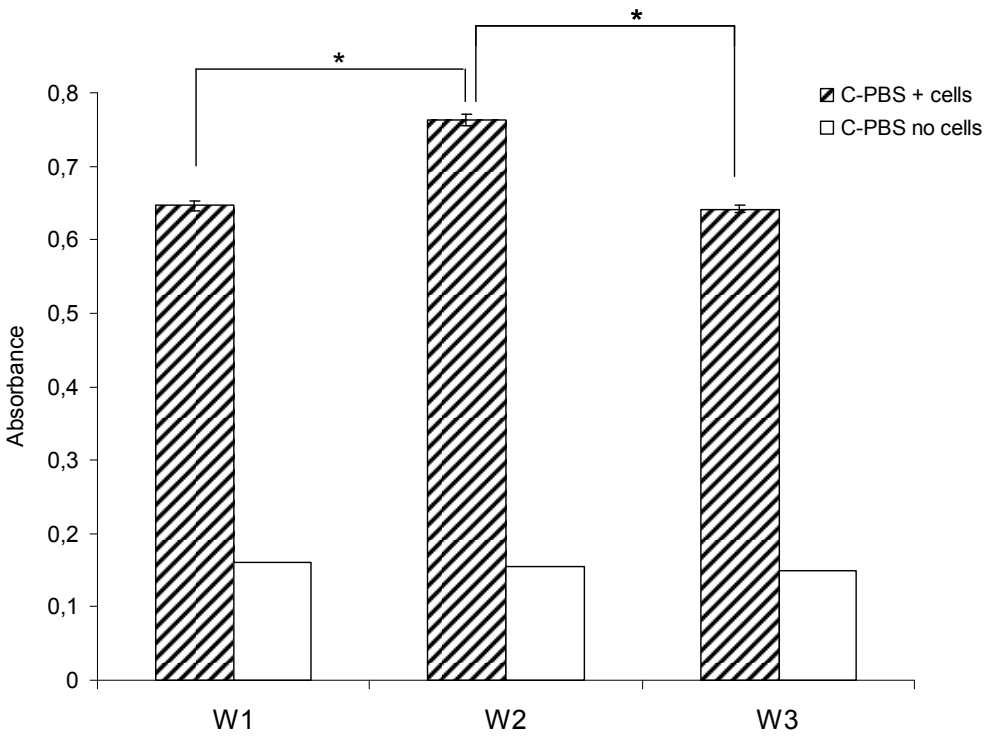

Figure III.4. Graphical representation of the results obtained after performing MTS test with samples taken at 1, 2, and 3 weeks of culturing. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3; α=0.05.

SEM analysis evidenced that BMC9 cells were well adhered onto the C-PBS scaffolds surface, and appeared to be morphologically normal throughout the whole time of the experiments. Furthermore, a morphological transition in these cells was observed from an initial fibroblastic-like shape, to a round-shaped phenotype, which is a feature present in articular cartilage chondrocytes that underwent culturing under 2D conditions.[35] From the SEM picture at 1 week, it is clear that cells adhered to the scaffolds and spread along its structure (Fig. 5A) forming multilayers (Fig. 5B). Cell morphology is clearly fibroblastic-like, with extensive cell-to-cell interactions.

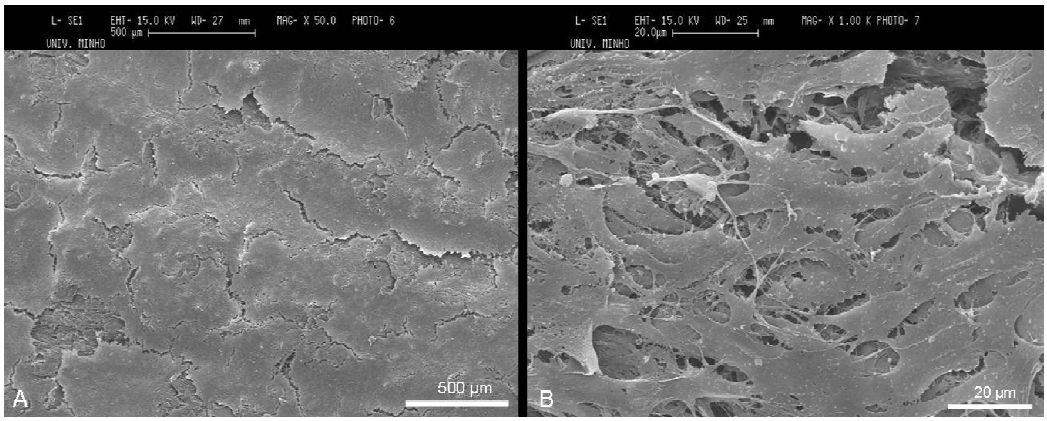

Figure III.5. SEM micrographs for 1 week of culturing, showing that BMC9 cells were able to adhere and appear to remain viable within the scaffolds structure.

After 2 weeks of culture, cell morphology passed from the initial fibroblastic-like shape with some evident cytoplasmic membrane extensions, to a round-shaped phenotype, as evidenced on Fig. 6A, 6B. SEM analysis also revealed that the chondrocyte-like cells were widely present in the pores of the scaffolds, as shown by a representative example in Fig. 6E.

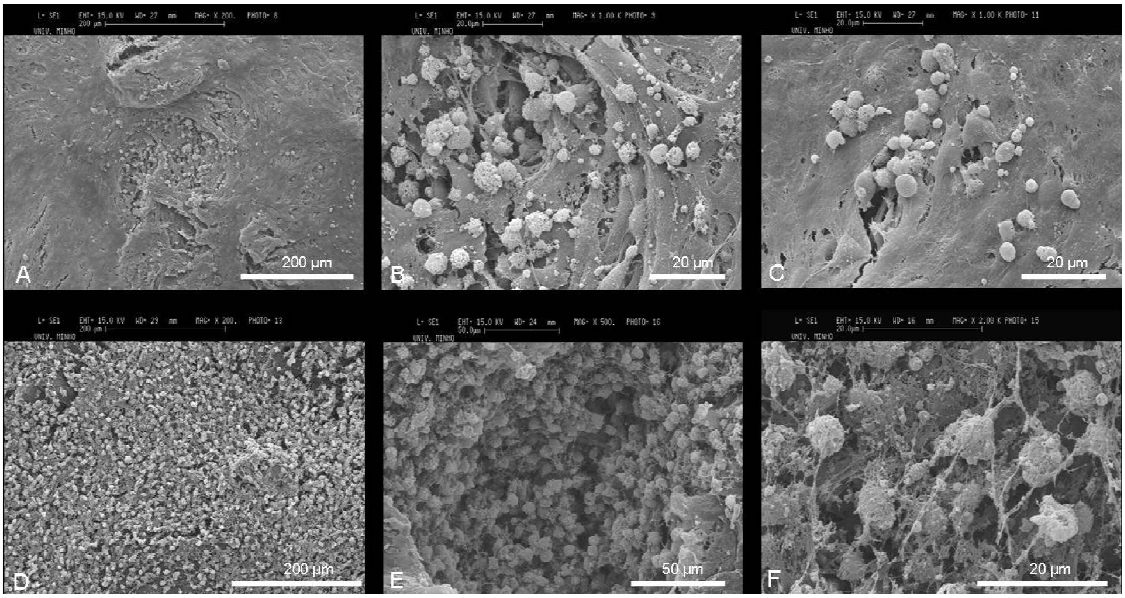

Figure III.6. SEM micrographs for 2 weeks of culturing. We can observe the cell morphological transition, passing from the initial fibroblastic-like shape with some evident cytoplasmic membrane extensions, to a round-shaped phenotype, as evidenced in (Fig. 6A, 6B).

After 3 weeks, almost all the cells exhibited a round-shaped phenotype and were widely distributed throughout the scaffold (Fig. 7). Furthermore, on the cells surface, some structures previously described as collagen fibrils[36] were observed (Fig. 7D, arrows). Even though speculative, one may correlate this observation with the results shown afterwards for collagen type II expression, performed by western blot immunological analysis.

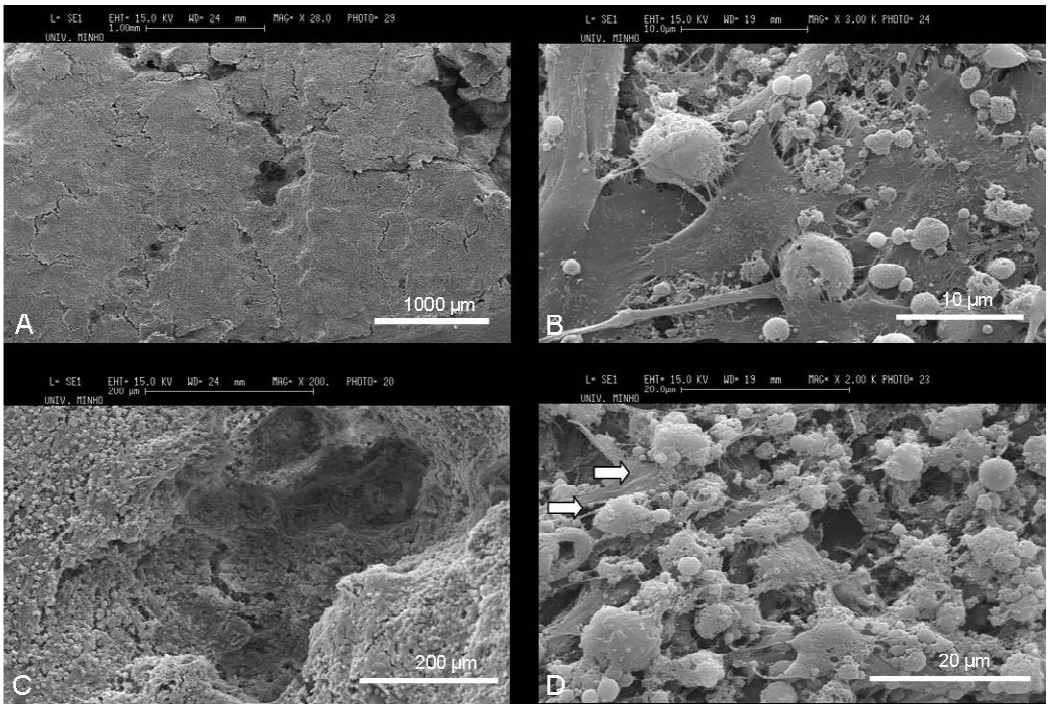

Figure III.7. SEM micrographs showing BMC9 cells onto the developed scaffolds at 3 weeks. Most cells exhibited a round-shaped phenotype and were widely distributed throughout the scaffold (Fig. 7C). Structures previously described as collagen fibrils are indicated by white arrows.

Western blot analysis at 3 weeks (Fig. 8) demonstrated that collagen II was being expressed, which is a good indicator for the successful differentiation of the BMC9 cells towards the chondrogenic pathway, and suggests at the same time, that cartilage-like extracellular matrix (ECM) was being produced as suggested from the SEM micrographs observations (Fig. 7). The band obtained correlates with data presented in the literature for collagen type II protein[37,38].

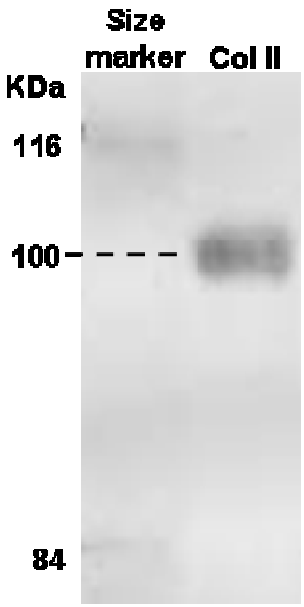

Figure III.8. Western blot analysis performed for collagen type II. The band obtained correlates with data presented in the literature for collagen type II protein (approximately 100 kDa). Collagen type II expression is a good indicator for the successful differentiation of the BMC9 cells towards the chondrogenic lineage.

4. DISCUSSION

In this work, scaffolds produced from a blend of chitosan and polybutylene succinate were tested for assessing their suitability for cartilage tissue engineering applications. The scaffolds were characterized to evaluate their potential for future uses in the cartilage regeneration field by employing techniques such as μ-CT and performing their mechanical behaviour evaluation. Its cytotoxicity was determined and in a final stage the direct contact with BMC9 cells under chondrogenic differentiating conditions was conducted as well as analysis of their differentiation status. Considering the salt particles sizes used (63-125 μm), one would expect that the created pores after the salt leaching step will be within this range. Nevertheless, some disperse particle agglomerations take place during processing, creating therefore bigger pore sizes, which may arise as an advantage regarding interconnectivity. In fact, results show the scaffolds present a high range of pore sizes exhibiting a 251.28 μm average pore size with a $\pm$61.9 μm standard deviation. These measurements show that pores of less than 100 μm and more than 300 μm are also present in the scaffolds granting them greater versatility in terms of pore size profiles for cells to distribute. The average porosity of 66.29% ($\pm$2.55%) is also adequate and quite interesting

considering the processing technology used, while the 92.78% (±1.69%) interconnectivity degree renders these scaffolds with a quite interconnected structure, thereby enabling seeded cells to proliferate and establish communication paths throughout the 3D support, as well as allowing nutrients and metabolic waste flow to be conducted. Such data prompts these C-PBS scaffolds to go through further screenings in order to evaluate their potential in the generation of a functional cartilage tissue engineered construct. The mechanical properties of the scaffolds disclosed values that are in the range of those shown in the literature for human articular cartilage[39] which is an indication of the suitability of the C-PBS scaffolds in terms of mechanical performance for cartilage regeneration strategies. In an *in vivo* scenario, the authors expect a balance between chitosan absorption by the organism, and cells proliferation and tissue ingrowth, in such a way that the mechanical support and biological role of the structures is constantly maintained. Cytotoxicity evaluation performed using MEM and MTS tests showed that the C-PBS were non cytotoxic. The leachables released from the scaffolds did not cause any severe alteration to the L929 cells metabolism thereby reinforcing their adequacy for moving through further studies in the regeneration of cartilaginous tissues. The following step involved the direct contact of cells with the fabricated C-PBS scaffolds. The cells used were derived from a mouse mesenchymal stem cell line (BMC9) and these were cultured under chondrogenic inductive conditions onto the C-PBS scaffolds, assessing in this way the suitability of these systems in future cartilage regeneration studies. Bone morphogentic protein type 2 (BMP-2) was used as the promoting agent for chondrogenic differentiation. BMP-2 is a protein belonging to the TGF-β superfamily that can stimulate the chondrogenic lineage development of cells from mesenchymal origin and its inclusion was a key factor in cells differentiation. The cell seeding was performed using the cell drop seeding method, which is currently used for seeding cells into scaffolds. Although it is disadvantageous in terms of cells seeding homogeneity throughout the support structures, it is a good way to perform a first evaluation of how the cells react once in contact with the proposed scaffolds. In the case of the C-PBS, although this method gives rise to an inhomogeneous cells distribution, it was observed that the scaffolds surface were highly colonised throughout the time of the experiments. When analysing the MTS results, it is possible to observe that the values were higher than the control for all time points and had a significant increase ($p < 0.05$) from 1 week to 2 weeks, which is a good indicator of cell viability. This may be the consequence of two factors: either the cells are proliferating and thereby more MTS is metabolized, justifying therefore the higher OD values; or the proliferation rates are not that high, but on the other hand the mitochondrial machinery is highly active, converting therefore higher amounts of MTS. Any combination of these two situations is possible, but either mechanism is indicative of cell viability

within the scaffolds structure. This fact is highly relevant for the following steps because although the leachables released from the scaffolds did not present any *in vitro* cytotoxicity, the direct cell contact with the structures could be affected by factors such as surface chemistry, topography, wetabillity, which was shown not to occur. At 3 weeks, a significant decrease ($p < 0.05$) is observed in the OD values, which may be directly related to normal changes in cells metabolism, given that the active protein synthesis normally associated with this stage usually implies a decrease in cellular proliferation. As the mitochondria have a prominent role in cell growth, the reduction in their activity leads to a diminished metabolization of the MTS. The C-PBS scaffolds appear to be able to support BMC9 cells proliferation and differentiation towards the chondrogenic lineage, once subjected to the specific medium used which contained BMP-2. This occurrence was observed by SEM after 2 weeks of culture, since the cells passed from an initial fibroblast to a more round chondrocytes-like morphology. In fact, this situation is similar to the one obtained when growing primary culture chondrocytes under a 2D environment for extended periods of time. Initially, the chondrocytes dedifferentiate and adopt a more fibroblastic-like phenotype, with alteration in protein expression, decreasing for example collagen type II expression, and increasing collagen type I.[35,40] Nevertheless, once confluence is reached under a 2D environment, cells start to pack themselves into multilayers, reflecting a consequence of high cell density culturing, and start to regain their globular morphology. In fact, this multilayered arrangement somehow mimics a 3D environment such as the one created under pellet or 3D support cultures. Once this change takes place, protein expression patterns also modify, and for example, collagen type II levels increase again. In this work, this change in morphology is an indication for a possible BMC9 cells chondrogenic lineage differentiation. This was an expected result due to the culturing of the cells in a 3D environment supplied with BMP-2. Furthermore, on the cells surface, some structures previously described as collagen fibrils[36] were observed (Fig. 7D, arrows). Even though speculative, one may correlate this observation with the results shown afterwards for collagen type II expression, performed by western blot immunological analysis. Finally, western blot analysis after 3 weeks (Fig. 8) demonstrated that collagen II was being expressed, which is a good indicator for the successful differentiation of the BMC9 cells towards the chondrogenic pathway, and suggests at the same time, that cartilage-like extracellular matrix (ECM) was being produced as suggested from the SEM micrographs observations (Fig. 7). The band obtained correlates with data presented in the literature for collagen type II protein[37,38]. Given the mesenchymal progenitor origin of BMC9 cells, which have not been shown to normally express collagen type II, this result leads to believe that the cells were actually being directed towards the chondrogenic lineage. As a preliminary screening, such suggestions are indeed

important for the future application of these systems in cartilage regeneration approaches. The results obtained so far show these C-PBS scaffolds have fulfilled the basic requirements to be put through sequential testing. This work revealed that they can support the growth and differentiation of undifferentiated cells and create an environment suitable for their chondrogenic differentiation. These results can be considered as a base for following experiments that can combine undifferentiated cells from other sources, direct them towards the chondrogenic lineage, and culture them *in vitro* for adequate periods so that a functional tissue engineered construct is formed. This possesses the mechanical stability provided by polybutylene succinate and the biological similarity properties conferred by chitosan.

5. CONCLUSIONS

In the present work, it was observed that scaffolds made of a blend of chitosan-polybutylene succinate 50:50 (%wt) are adequate to be used in cartilage tissue engineering approaches. These scaffolds were shown to present mechanical properties and morphological features suitable for cell development and to be non-cytotoxic and cytocompatible. Direct contact assays evidenced that cells from a mouse mesenchymal progenitor cell line (BMC9) were able to adhere to the scaffolds surface and penetrate its pores, as well as remaining viable for at least 3 weeks of culturing. SEM analysis indicated the cells were directed towards the chondrogenic lineage due to observed morphological transitions occurring around 2 weeks of culture. This was one of the expected outcomes, due to the 3D environment onto which the cells were cultured, as well as the specific medium used which contained BMP-2. BMC9 cells chondrogenic differentiation was further corroborated by the collagen type II expression obtained after 3 weeks of culturing. The obtained data so far presents good perspectives for the use of chitosan and polybutylene succinate scaffolds in cartilage regeneration approaches.

REFERENCES

1. Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering: A* 403 (1-2), 57-68, 2005.

2. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L., Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, *N Engl J Med* 331 (14), 889-95, 1994.

3. Gross, A. E., Cartilage resurfacing: filling defects, *J Arthroplasty* 18 (3 Suppl 1), 14-7, 2003.

4. Duda, G. N., Maldonado, Z. M., Klein, P., Heller, M. O., Burns, J., and Bail, H., On the influence of mechanical conditions in osteochondral defect healing, *J Biomech* 38 (4), 843-51, 2005.

5. Richardson, J. B., Caterson, B., Evans, E. H., Ashton, B. A., and Roberts, S., Repair of human articular cartilage after implantation of autologous chondrocytes, *J Bone Joint Surg Br* 81 (6), 1064-8, 1999.

6. Salgado, A. J., Gomes, M. E., Chou, A., Coutinho, O. P., Reis, R. L., and Hutmacher, D. W., Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 20 (1-2), 27-33, 2002.

7. Chen, G., Sato, T., Ohgushi, H., Ushida, T., Tateishi, T., and Tanaka, J., Culturing of skin fibroblasts in a thin PLGA-collagen hybrid mesh, *Biomaterials* 26 (15), 2559-66, 2005.

8. Holmes, T. C., de Lacalle, S., Su, X., Liu, G., Rich, A., and Zhang, S., Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds, *Proc Natl Acad Sci U S A* 97 (12), 6728-33, 2000.

9. Langer, R. and Vacanti, J. P., Tissue engineering, *Science* 260 (5110), 920-6, 1993.

10. Jung, Y., Kim, S. S., Kim, Y. H., Kim, S. H., Kim, B. S., Kim, S., and Choi, C. Y., A poly(lactic acid)/calcium metaphosphate composite for bone tissue engineering, *Biomaterials* 26 (32), 6314-22, 2005.

11. Malafaya, P. B. and Reis, R. L., Porous bioactive composites from marine origin based in chitosan and hydroxylapatite particles, *Bioceramics 15* 240-2, 39-42, 2003.

12. Zammaretti, P. and Jaconi, M., Cardiac tissue engineering: regeneration of the wounded heart, *Current Opinion in Biotechnology* 15 (5), 430-434, 2004.

13. Koegler, W. S. and Griffith, L. G., Osteoblast response to PLGA tissue engineering scaffolds with PEO modified surface chemistries and demonstration of patterned cell response, *Biomaterials* 25 (14), 2819-30, 2004.

14. Yang, Y., Basu, S., Tomasko, D. L., Lee, L. J., and Yang, S. T., Fabrication of well-defined PLGA scaffolds using novel microembossing and carbon dioxide bonding, *Biomaterials* 26 (15), 2585-94, 2005.

15. Hoffman, A. S., Hydrogels for biomedical applications, *Adv Drug Deliv Rev* 54 (1), 3-12, 2002.

16. Kim, B. S. and Mooney, D. J., Development of biocompatible synthetic extracellular matrices for tissue engineering, *Trends Biotechnol* 16 (5), 224-30, 1998.

17. Gomes, M. E., Ribeiro, A. S., Malafaya, P. B., Reis, R. L., and Cunha, A. M., A new approach based on injection moulding to produce biodegradable starch-based polymeric scaffolds: morphology, mechanical and degradation behaviour, *Biomaterials* 22 (9), 883-889, 2001.

18. Solchaga, L. A., Dennis, J. E., Goldberg, V. M., and Caplan, A. I., Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage, *J Orthop Res* 17 (2), 205-13, 1999.

19. Solchaga, L. A., Temenoff, J. S., Gao, J., Mikos, A. G., Caplan, A. I., and Goldberg, V. M., Repair of osteochondral defects with hyaluronan- and polyester-based scaffolds, *Osteoarthritis Cartilage* 13 (4), 297-309, 2005.

20. Boccafoschi, F., Habermehl, J., Vesentini, S., and Mantovani, D., Biological performances of collagen-based scaffolds for vascular tissue engineering, *Biomaterials*, 2005.

21. Suh, J. K. and Matthew, H. W., Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review, *Biomaterials* 21 (24), 2589-98, 2000.

22. Reis, R. L., Cunha, A. M., Allan, P. S., and Bevis, M. J., Mechanical behavior of injection-molded starch-based polymers, *Polymers for Advanced Technologies* 7 (10), 784-790, 1996.

23. Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T., Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold, *Biochemical and Biophysical Research Communications* 322 (1), 50, 2004.

24. Wang, Y.-C., Lin, M.-C., Wang, D.-M., and Hsieh, H.-J., Fabrication of a novel porous PGA-chitosan hybrid matrix for tissue engineering, *Biomaterials* 24 (6), 1047-1057, 2003.

25. Freier, T., Koh, H. S., Kazazian, K., and Shoichet, M. S., Controlling cell adhesion and degradation of chitosan films by N-acetylation, *Biomaterials* 26 (29), 5872-8, 2005.

26. Madihally, S. V. and Matthew, H. W. T., Porous chitosan scaffolds for tissue engineering, *Biomaterials* 20 (12), 1133-1142, 1999.

27. Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering A* In Press, Corrected Proof, 2005.

[28.] Mahmoudifar, N. and Doran, P. M., Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors, *Biomaterials* In Press, Corrected Proof.

[29.] Devlieghere, F., Vermeulen, A., and Debevere, J., Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables, *Food Microbiology* 21 (6), 703-714, 2004.

[30.] Silva, R. M., Malafaya, P. B., Mano, J. F., and Reis, R. L., Bioactive composite chitosan membranes to be used in bone regeneration applications, *Bioceramics 15* 240-2, 423-426, 2003.

[31.] Lyoo, W. S., Kim, J. H., Yoon, W. S., Ji, B. C., Choi, J. H., Cho, J., Lee, J., Yang, S. B., and Yoo, Y., Effects of polymer concentration and zone drawing on the structure and properties of biodegradable poly(butylene succinate) film, *Polymer* 41 (26), 9055-9062, 2000.

[32.] Henry, E. W., Chiu, T.-H., Nyilas, E., Brushart, T. M., Dikkes, P., and Sidman, R. L., Nerve regeneration through biodegradable polyester tubes, *Experimental Neurology* 90 (3), 652-676, 1985.

[33.] Dennis, J. E., Merriam, A., Awadallah, A., Yoo, J. U., Johnstone, B., and Caplan, A. I., A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse, *J Bone Miner Res* 14 (5), 700-9, 1999.

[34.] ISO10993, d., Biological compatibility of medical devices. 5. Test for citotoxicity: *In vitro* methods, December 1992.

[35.] Cancedda, R., Dozin, B., Giannoni, P., and Quarto, R., Tissue engineering and cell therapy of cartilage and bone, *Matrix Biol* 22 (1), 81-91, 2003.

[36.] Fuss, M., Ehlers, E. M., Russlies, M., Rohwedel, J., and Behrens, P., Characteristics of human chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge under different culture conditions. A light, scanning and transmission electron microscopy study, *Ann Anat* 182 (4), 303-10, 2000.

[37.] Gartland, A., Mechler, J., Mason-Savas, A., MacKay, C. A., Mailhot, G., Marks, S. C., and Odgren, P. R., In vitro chondrocyte differentiation using costochondral chondrocytes as a source of primary rat chondrocyte cultures: An improved isolation and cryopreservation method, *Bone* 37 (4), 530-544, 2005.

[38.] Estrada, L. E., Dodge, G. R., Richardson, D. W., Farole, A., and Jimenez, S. A., Characterization of a biomaterial with cartilage-like properties expressing type X collagen generated in vitro using neonatal porcine articular and growth plate chondrocytes, *Osteoarthritis and Cartilage* 9 (2), 169-177, 2001.

[39.] von der Mark, K., Kirsch, T., Nerlich, A., Kuss, A., Weseloh, G., Gluckert, K., and Stoss, H., Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy, *Arthritis Rheum* 35 (7), 806-11, 1992.

[40.] Woodfield, T. B. F., Malda, J., de Wijn, J., Peters, F., Riesle, J., and van Blitterswijk, C. A., Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique, *Biomaterials* 25 (18), 4149-4161, 2004.

[41.] Cancedda, R., Descalzi Cancedda, F., and Castagnola, P., Chondrocyte differentiation, *Int Rev Cytol* 159, 265-358, 1995.

[42.] Kirsch, T., Harrison, G., Golub, E. E., and Nah, H.-D., The Roles of Annexins and Types II and X Collagen in Matrix Vesicle-mediated Mineralization of Growth Plate Cartilage, 2000, pp. 35577-35583.

SECTION 3.

CHAPTER IV.

Novel Melt-Processable Chitosan-Polybutylene Succinate Fibre Scaffolds for Cartilage Tissue Engineering

CHAPTER IV.

Novel Melt-Processable Chitosan-Polybutylene Succinate Fibre Scaffolds for Cartilage Tissue Engineering*

ABSTRACT

Novel chitosan/polybutylene succinate fibre-based scaffolds (C-PBS) were seeded with bovine articular chondrocytes in order to assess the suitability of these materials for cartilage tissue engineering. These are new melt-processable materials that are able to combine both chitosan and polybutylene succinate in a 3D scaffold which is expected to perform better as a tissue engineered construct. Chondrocytes were seeded onto C-PBS scaffolds using spinner flasks under dynamic conditions, and cultured under orbital rotation for a total of 6 weeks. Non-woven polyglycolic acid (PGA) felts were used as reference materials. Tissue engineered constructs were characterized by scanning electron microscopy (SEM), hematoxylin-eosin (H&E), toluidine blue and alcian blue staining, immunolocalisation of collagen types I and II, and dimethylmethylene blue (DMB) assay for glycosaminoglycans (GAG) quantification at different time points. SEM showed that the chondrocytes had typical morphology, with colonisation at the surface and within the pores of the C-PBS scaffolds. These observations were supported by routine histology. Toluidine blue and alcian blue stains as well as immunohistochemistry for collagen types I & II, provided qualitative information on the composition of the engineered extracellular matrix. More pronounced staining was observed for collagen type II than collagen type I. Similar results were observed with constructs engineered on PGA scaffolds. These also exhibited higher amounts of matrix glycosaminoglycans, as determined from the GAG assay. The central region of PGA constructs contained fewer cells and little matrix, most likely as a result of necrosis due to limited mass-transfer and/or acidic products of degradation of PGA. This feature was not detected with C-PBS constructs, suggesting improved biocompatibility or improved mass-transfer due to pore size or more limited growth of tissues.

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1. INTRODUCTION

Articular cartilage is a tissue with unique features in terms of biological structure and functionality. It has a structural function in the skeletal system as a weight bearing tissue present at the proximal and distal epiphyses of bones which creates smooth gliding areas that can both absorb shocks and loads in an efficient way. Articular cartilage is avascular and possesses a low ratio of cells to extracellular matrix (ECM), being the cells embedded in an extensive network constituted mostly of collagen and proteoglycans. Collagen type II and proteoglycans (aggrecan) are the main contributors to cartilage mechanical function.[1-3] Collagen II is responsible for the tensile properties and the proteoglycans for resistance to compression.[4,5] Articular cartilage has a low metabolic turnover and poor regenerative potential and so trauma or degeneration of the tissue may cause restriction of normal locomotion, pain and reduction of quality of life.[2,6-9]

Different approaches have been developed to treat these conditions, but the most commonly used therapies frequently result in limited success.[10-13] The use of tissue engineering technologies to generate a functional tissue graft which can be used to repair the damaged cartilage is a very active field of research.[14-17] Ideally, scaffold materials should have adequate mechanical properties and degradation kinetics, be biocompatible and have a controlled geometry and 3D structure.[8,18,19]

Fibre based scaffolds are very attractive due to their highly interconnected structure, allowing cells to infiltrate throughout the network and potentially enhancing formation of extracellular matrix throughout the scaffold.[19,20] Many different materials have been proposed for producing fibre based scaffolds.[21,22] Woodfield et al studied PEGT/PBT scaffolds produced by a fibre deposition technique and seeded with bovine articular chondrocytes.[21] In another study, a benzyl ester derivative of hyaluronic acid (HYAFF®11) was combined with human nasoseptal chondrocytes in tissue engineering procedures of cartilage reconstruction.[20] Gomes et al. have also proposed the use of different starch-based blends for bone tissue engineering applications.[23]

Scaffolds for tissue engineering can be synthetic, or natural materials, or a blend of both types.[20,21,24,25] These last might arguably combine the best properties of both types of materials in a single structure, therefore increasing the probability of a better regenerative performance.

In the present study, some of the above concepts were combined by culturing bovine articular chondrocytes with novel chitosan/polybutylene succinate (C-PBS) fibre-based scaffolds. These materials have been previously used by our group by combining mouse mesenchymal progenitor cells with C-PBS compression moulded scaffolds for cartilage regeneration.[26]

Chitosan is a natural material produced by a partial alkaline deacetylation of chitin, the main structural polymer in arthropods exoskeletons and shells of crustaceans[27]. It is a semi-crystalline polysaccharide that is normally insoluble in aqueous solutions above pH 7. In dilute acids, the free amine groups are protonated and the molecule becomes soluble, enabling it to be processed under mild acetic acid conditions.[28] Chitosan is reported to be non-toxic, biodegradable and biocompatible[29], and has structural similarities to glycosaminoglycans, which are structural components of the cartilage extracellular matrix.[30] It is currently used in diverse applications in the food industry and biomedical field.[31-33] Polybutylene succinate (PBS) is a synthetic biodegradable polymer that has been studied for a range of applications extending from its use as a future conventional biodegradable plastic, to components in medical devices.[34,35] PBS presents good processing and mechanical properties flexibility, having degradation products that are non-toxic and can be metabolised by living organisms.[29] The evaluation of PBS *in vitro* with rat osteoblasts showed that it had good cytocompatibility and sustained both osteoblast proliferation and differentiation.[36]

This study is the first report on the suitability of new chitosan/polybutylene succinate fibre scaffolds for cartilage tissue engineering by culturing bovine articular chondrocytes on the scaffolds for up to 6 weeks. In brief, the scaffold fibre structure allowed the cells to efficiently colonise both the outer periphery and central regions of the scaffolds. Analysis of the cell morphology and extracellular matrix suggested good potential for C-PBS scaffolds application in cartilage tissue engineering.

2. MATERIALS AND METHODS

2.1. SCAFFOLDS PRODUCTION AND CHARACTERIZATION

The scaffolds were prepared from fibres of a chitosan/polybutylene succinate (C-PBS) blend. The processing details are described elsewhere.[37] Briefly, the raw materials (chitosan and PBS) were compounded on a twin screw extruder in a ratio of 50/50 wt%. The polymeric fibres were obtained by further re-extruding the C-PBS blend using a microextruder. Afterwards, the processed fibres were packed in an appropriate mould, compressed, and heated above the melting temperature (Tm) of the thermoplastic for a determined residence period, thereby allowing the fibres to bond and consequently to obtain a mechanically stable macroporous fibre mesh structure. The

scaffolds were further cut in a cylindrical shape (Ø 7 mm x thickness 1.5 mm). The scaffold morphology was assessed by scanning electron microscopy (Leica-Cambridge S-360, Germany) and micro-computed tomography (SkyScan, Belgium). The mechanical properties were determined using a Universal mechanical testing machine (Instron 4505, UK). Mechanical testing was performed under compression using a crosshead speed of 5mm/min and the results averaged from tests conducted in at least five specimens.

2.2. ISOLATION AND EXPANSION OF BOVINE ARTICULAR CHONDROCYTES

Full thickness hyaline cartilage was harvested from bovine metacarpophalangeal joint of adult animals (18-24 months) within 4 hours of slaughter. Chondrocytes were isolated by sequential enzymatic digestion and their numbers expanded in monolayer culture as described previously.[38] Chondrocytes were seeded at 50,000-100,000 cells/cm^2 and cultured in basic medium [Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Co, USA) containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 µg/ml streptomycin, 20 mM L-alanyl glutamine, MEM non-essential amino acids, and 10% (v/v) foetal calf serum (Biosera S1800; NWPLS; Heat Inactivated)], supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK).

2.3. CHONDROCYTE CULTURE ON 3D C-PBS FIBRE SCAFFOLDS

Chondrocytes, at passage P1, were used for all experiments. Scaffolds were seeded with cells as described previously.[38] In brief, confluent cultures of chondrocyte cultures were harvested by trypsinisation. Scaffolds were threaded onto stainless steel wires and suspended in spinner flasks containing a stirred suspension of chondrocytes (0.5x10^6 cells/ml; 4 scaffolds per spinner flask) for 72 h to allow the chondrocytes to penetrate the scaffolds. The resultant cell/scaffold constructs were transferred to sterile, non-tissue culture treated Petri dishes and incubated for 4 days in basic medium supplemented with 10ng/ml bFGF to allow expansion of cell numbers on the scaffolds. The constructs were then cultured with basic medium supplemented with 1 g/ml insulin and 50 g/ml L-ascorbic acid (Sigma-Aldrich Co, USA) to stimulate chondrogenesis. Throughout the culture period the constructs were gently shaken using an orbital shaker. The constructs were incubated until 42 days of culture (6 weeks), replacing the medium every 2-3 days. Polyglycolic acid (PGA) non-woven scaffolds (Albany International Ltd, UK) were used as a comparator. These presented the same dimensions and followed the same procedures as those of C-PBS.

2.4. SCANNING ELECTRON MICROSCOPY

The C-PBS constructs were washed in sterile PBS and immersed in 3% glutaraldehyde with 0.1 M cacodylate buffer pH 7.4) at room temperature for one hour. They were further washed three times in 0.1 M cacodylate buffer pH 7.4, and post fixed in 1% aqueous solution of osmium tetroxide for one hour. Finally, they were dehydrated in alcohols and let to dry. The samples were sputter coated with gold and observed using a Phillips XL-20 scanning electron microscope. Complementarily, some stereolight microscopy images were collected from samples in culture to obtain a macroscopic view of the cultured constructs after 42 days of culture.

2.5 HISTOLOGY

Constructs were harvested at pre-defined culturing periods, until up to 6 weeks of total culturing time. The constructs were bisected and one half mounted in the cryoprotectant Optimal Cutting Temperature (OCT, BDH, Gurr®), and frozen using liquid nitrogen and isopentane. The remaining construct half was stored at -20^{o}C for quantitative determination of glycosaminoglycan content. Tissue sections of 8 μm were taken and fixed using fresh 4% paraformaldehyde. Hematoxylin-eosin stain was conducted in an automatic machine (Fume Cupboard; X219/E11/LEV1). Histological staining with toluidine blue and alcian blue staining was performed using standard histological methods. Polyglycolic acid (PGA) non-woven scaffolds were used as controls.

2.6. QUANTIFICATION OF PROTEOGLYCAN CONTENT

Proteoglycans were determined by measuring the level of sulfated glycosaminoglycans (GAGs) using 1,9-dimethylmethylene blue (Sigma-Aldrich Co, USA) metachromatic assay as previously described[39]. GAG levels in solution can be quantified using the basic dye, 1,9- dimethylmethylene blue (DMB) which binds to glycosaminoglycans generating a metachromatic shift that peaks at $A_{525\text{-}530}$ and can be measured spectrophotometrically. Briefly, the constructs were immersed in a digestion solution with papain and N-acetyl cysteine, and incubated at 60ºC overnight. After the digestion was completed, the tubes were centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and stored at 4ºC until the GAG assay was performed. A chondroitin

sulfate standard solution was prepared in water and kept refrigerated. The samples and chondroitin sulfate standards were placed in a 96 well round-bottomed plate, DMB solution was added to each well, and the optical density measured using a microplate reader, at 530 nm. Polyglycolic acid (PGA) non-woven scaffolds were used as controls.

2.7. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND TYPE II

Collagen types I and II were detected immunohistochemically using monoclonal antibodies against collagen types I and II (Southern Biotechnology, UK), as previously described[39]. Briefly, fixed sections were washed with PBS and pre-treated with hyaluronidase (10 mg/ml), followed by pronase (2 mg/ml) (Sigma-Aldrich Co, USA). The slides were washed thoroughly in PBS and treated with 3% hydrogen peroxide in 50% methanol, followed by washing in Tris-buffered saline (TBS) and blocking with 3% bovine serum albumin (BSA). Incubation with the primary antibody (collagen type I and collagen type II) (UNLB) followed. The remaining protocol is as described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). The slides were washed in water for 5 minutes, counterstained with Mayer's haematoxylin, and mounted with DPX mounting medium. Controls were performed using normal goat serum instead of the primary antibodies, which was included in the kit. Polyglycolic acid (PGA) non-woven scaffolds were used as controls.

3. RESULTS

3.1. SCAFFOLDS CHARACTERIZATION

The morphology and internal structure of the novel C-PBS fibre mesh scaffolds used in this study was investigated using SEM (Figure. 1a.). Micro-computed tomography (μCT) was also performed and representative 2D and 3D μCT images of a C-PBS fibre mesh scaffold are shown in Figures 1b-c., respectively.

Morphological analysis of the C-PBS structures shows that the fibre mesh scaffolds exhibit good interconnectivity and possess an adequate 3D structure for cells to be seeded and cultured towards the generation of a functional tissue engineered construct. The porosity (46,1 ± 1,8 % estimated by μCT) should enable cells to penetrate into the bulk of the scaffold, while also

enhancing nutrient diffusion and removal of metabolic waste products. Morphological studies also revealed a characteristic surface roughness (see Fig. 1.a) and microporosity (Fig. 1.c) of the C-PBS fibres. Mechanical testing has shown that the scaffolds possess a compression modulus of 32.6±12.8 MPa, which is higher than the values described for human articular cartilage.[40]
Further details may be found elsewhere.[41]

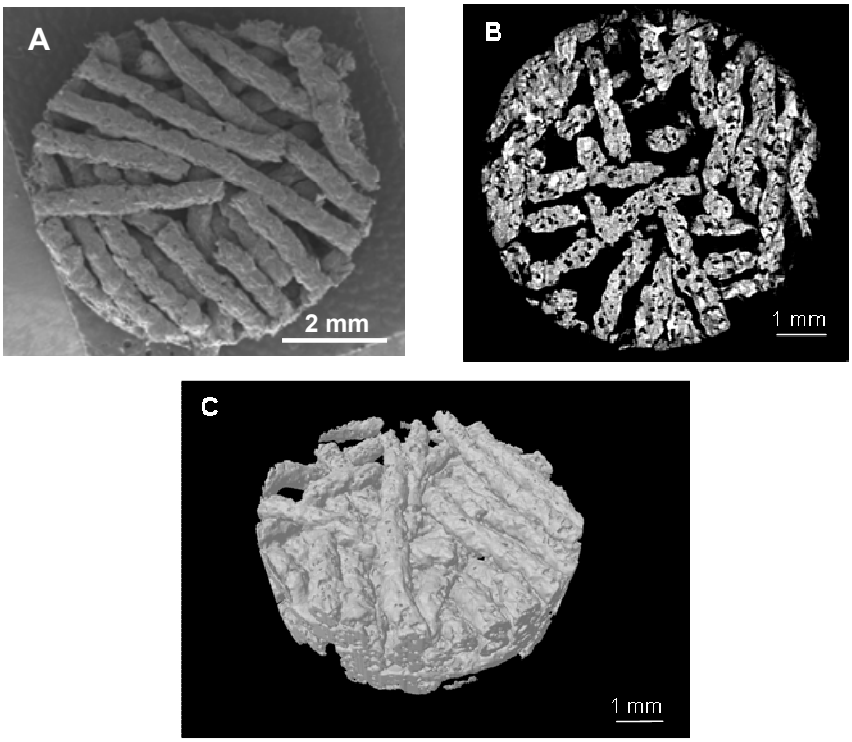

Figure IV.1. a) SEM micrograph of the upper surface of a representative C-PBS (50/50 wt%) fibre mesh scaffold; b) and c) 2D and 3D μCT images, respectively.

3.2. STEREOLIGHT MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

In Figure 2a) a macroscopic view of one construct after 6 weeks of culture is shown. It can be observed that a considerable amount of new cartilage-like tissue has been formed during culture. The opaque matrix that can be visualised in the figure is macroscopically very similar to the one encountered in native bovine articular cartilage at the time of cell isolation. A good adhesion and integration of the newly formed tissue with the scaffold structure was noticed upon handling and macroscopical observation.

SEM observations of the C-PBS tissue engineered constructs after 6 weeks of culture were performed. The micrographs on Figure 2b) show that the bovine articular chondrocytes had completely penetrated the scaffolds being present in both the surface and inner regions. Also the cellular morphology is the typical of healthy cultured chondrocytes with the cells being widely and homogeneously dispersed throughout the structures.

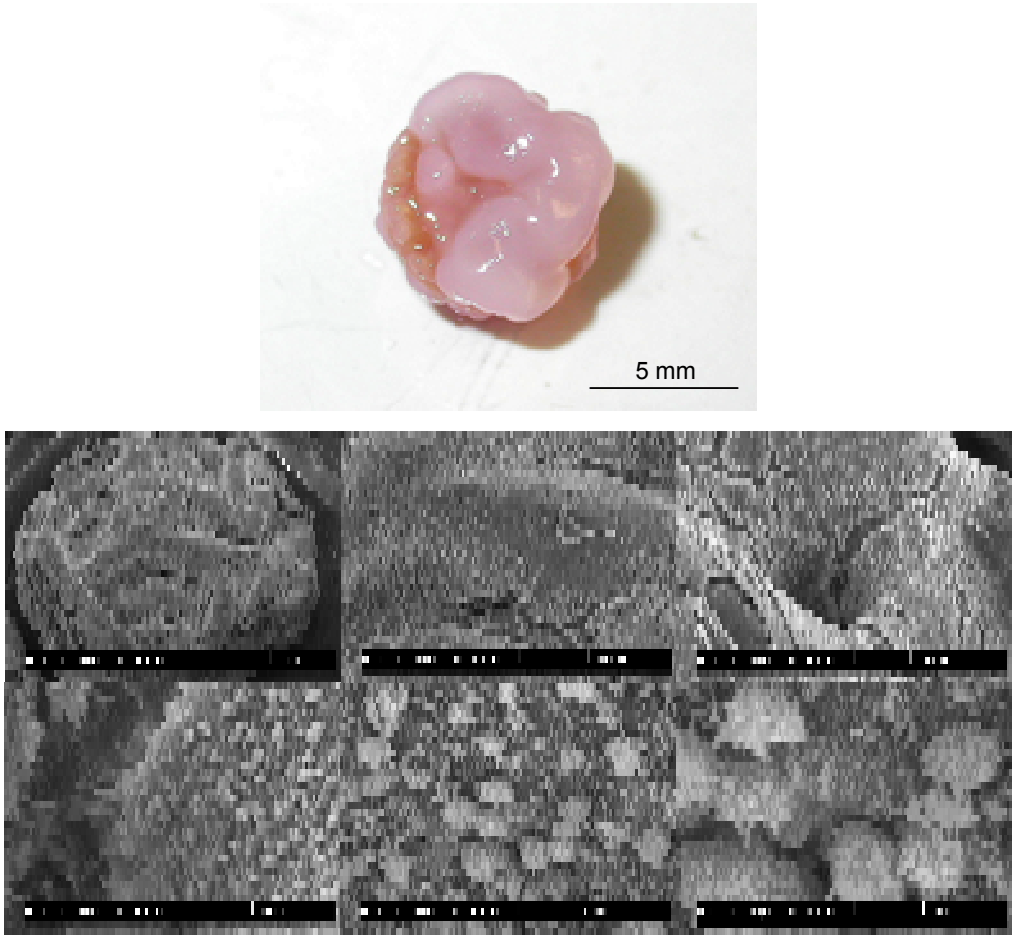

Figure IV.2. a) Stereolight microscopy of a tissue engineered C-PBS construct after 6 weeks of culture. b) Scanning electron microscopy images showing C-PBS constructs at 6 weeks of culture. The cells were homogeneously distributed with typical morphology of healthy cultured chondrocytes.

3.3. HISTOLOGY

Figure 3 shows different histological sections taken after 6 weeks of culture for both C-PBS (A-C) and PGA (D-F) constructs stained with H&E. It is possible to observe once more the chondrocytes homogeneous distribution throughout the scaffolds being widely present in both inner and outer regions. Cells and ECM were observed between the C-PBS fibres (3.A-C) and an apparent integration of cell mass, ECM and the scaffold structure was also seen (Figure 3.A). These results corroborate those presented previously for SEM analyses.

A similar chondrocyte and extracellular matrix distribution were observed in the PGA constructs. However, a central area of extensive necrosis was detected for the different analysed tissue engineered constructs (Figure 3.D) which is in agreement with previous data present in the literature that correlates the accumulation of degradation products with toxic effects to cells.[42]

C-PBS and PGA tissue engineered constructs were stained with toluidine blue and alcian blue (Figures 4 and 5, respectively). These stains were used to show the distribution of proteoglycans in the constructs. The proteoglycan content of the constructs was also measured quantitatively by determining the glycosaminoglycan content with 1,9-dimethylmethylene blue. Figures 4 and 5 show the staining profiles for the histological sections of the C-PBS (A-C) and PGA (D-F) tissue engineered constructs after 6 weeks of culture using toluidine blue and alcian blue, respectively.

From the histological analysis, it is possible to observe a positive staining in the pericellular areas indicating that the chondrocytes produced an extracellular matrix containing proteoglycans. Both C-PBS and PGA tissue engineered constructs showed sulfated glycosaminoglycans in the newly synthesized ECM. In addition, the histology of C-PBS shown by H&E, toluidine blue, and alcian blue staining (Figures 3a-c, 4a-c, and 5 a-c, respectively) more closely resembled the morphological structure observed for native articular cartilage than that found in the for PGA constructs (Figures 3d-f, 4 d-f, and 5 d-f, respectively).

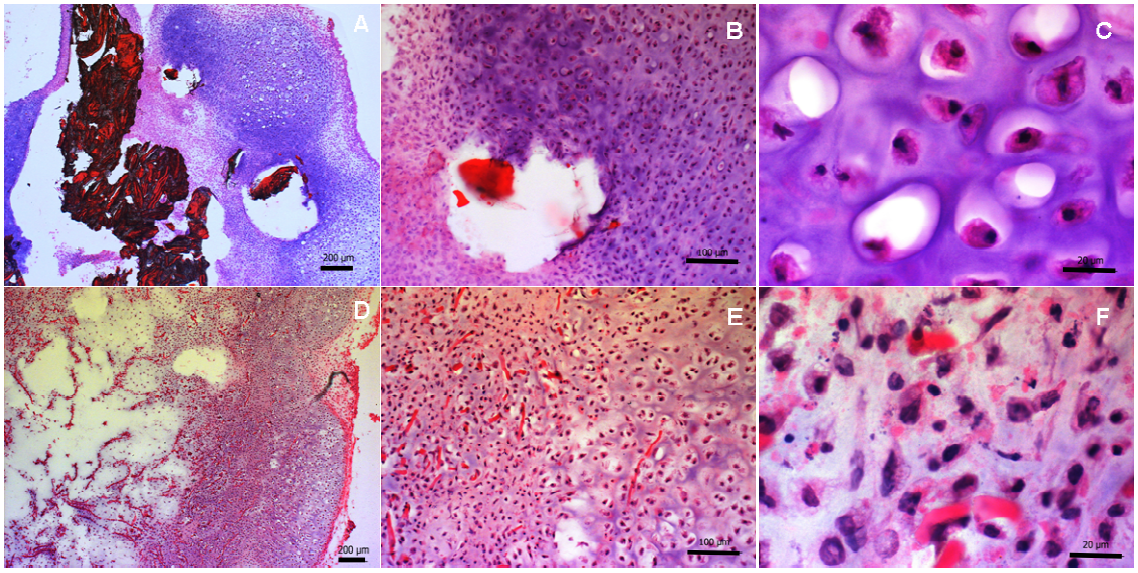

Figure IV.3. Light microscopy images of histology sections obtained from C-PBS constructs collected after 6 weeks of culture stained with hematoxylin-eosin (A-C). Figures D-F show images obtained from PGA constructs after similar incubation conditions. Scale bar: 200 µm (A,D); 100 µm (B,E); 20 µm (C,F).

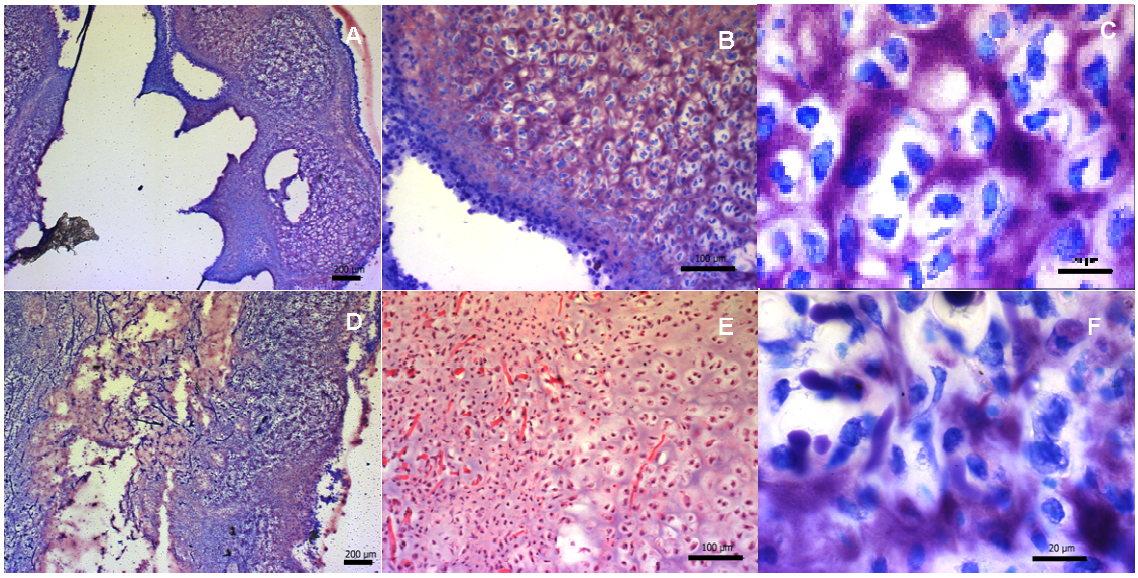

Figure IV.4. Light microscopy images of histology sections obtained from C-PBS constructs collected after 6 weeks of culture stained with toluidine blue (A-C). Figures D-F show sections obtained from PGA constructs after similar incubation conditions. Scale bar: 200 µm (A,D); 100 µm (B,E); 20 µm (C,F).

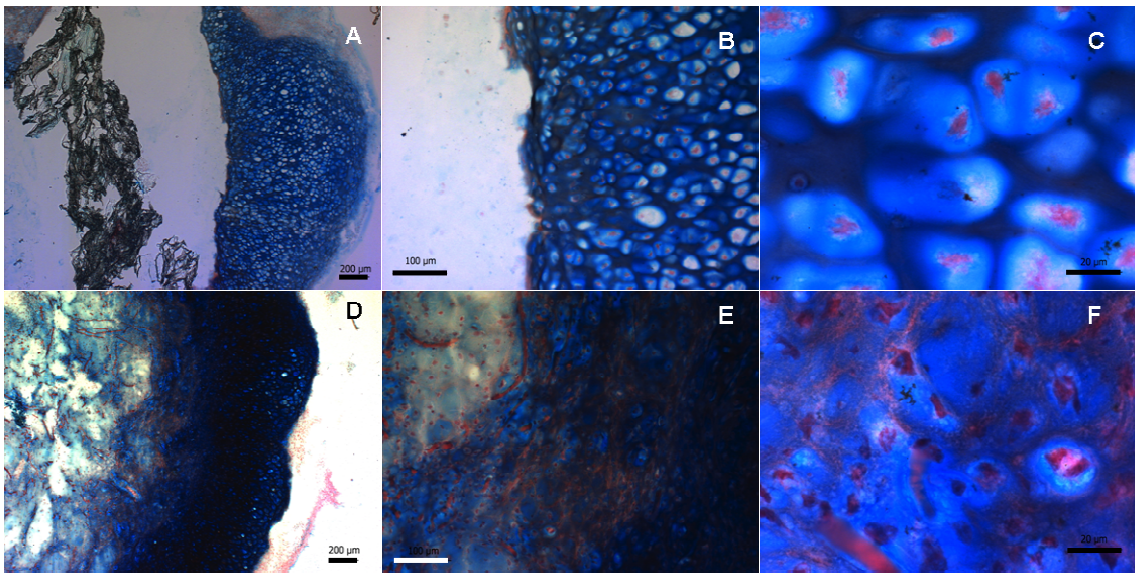

Figure IV.5. Light microscopy images of histology sections obtained from C-PBS constructs collected after 6 weeks of culture stained with alcian blue (A-C). Figures D-F show sections obtained from PGA constructs after similar incubation conditions. Scale bar: 200 µm (A,D); 100 µm (B,E); 20 µm (C,F).

3.4. QUANTIFICATION OF PROTEOGLYCAN CONTENT

The proteoglycan content of the constructs was measured quantitatively by determining the glycosaminoglycan content with 1,9-dimethylmethylene blue. Glycosaminoglycan content was found to increase steadily during the 6 weeks of culture for both C-PBS and PGA-based constructs (Figure 6). When analysing the patterns of GAGs variation in the C-PBS systems, a continuous increase was detected which is a positive result in terms of the formation of a cartilage-like engineered tissue. The same pattern of GAG increase was observed for the PGA controls but these exhibited higher levels of glycosaminoglycan deposition when compared to C-PBS.

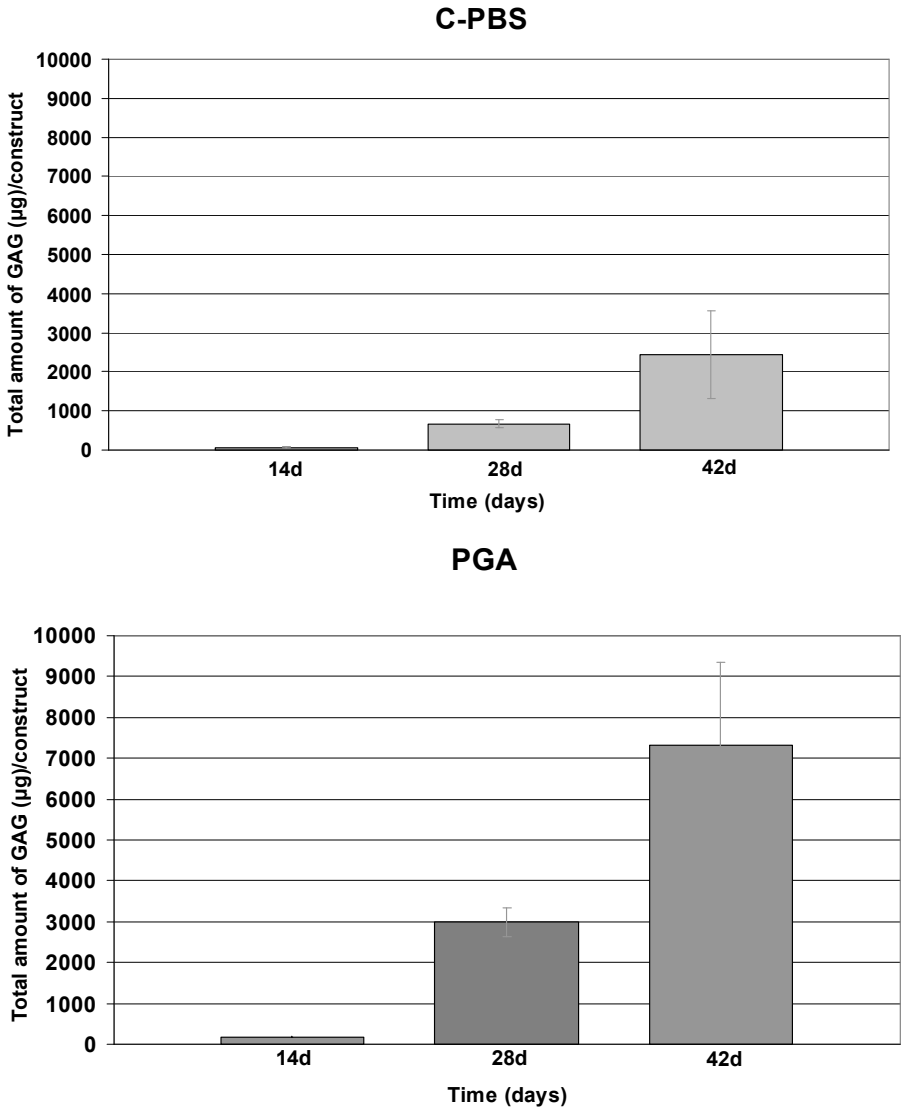

Figure IV.6. Graphical representation of the results obtained from DMB assay for proteoglycans content quantification of C-PBS (top) and PGA (bottom) tissue engineered constructs after 2, 4, and 6 weeks of culture.

3.5. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND TYPE II

Immunolocalisation of collagen type II was conducted in order to assess its expression patterns in both C-PBS and PGA constructs, being an indication of their suitability and future performance for cartilage regeneration. Also to confirm that in fact a cartilage-like tissue engineered construct of hyaline nature was being formed, collagen type I expression was verified.

Figure 7.a-c shows the immunolocalisation of collagens type I and II in C-PBS constructs. Dense staining for collagen type II was observed with some less dense staining for collagen type I also present which is a good indication for a hyaline cartilage ECM formation. The PGA constructs also gave a similar pattern of collagen type I and II distribution. Again, comparison of the morphologies of the C-PBS and PGA constructs with the native tissue showed that C-PBS constructs more closely resembled the cellular morphology observed in native cartilage. The presence of structures similar to lacunae and isogenous groups that can be found in native cartilage are seen (Figure 7.b). Such structures were not observed in the PGA constructs (Figure 7.E).

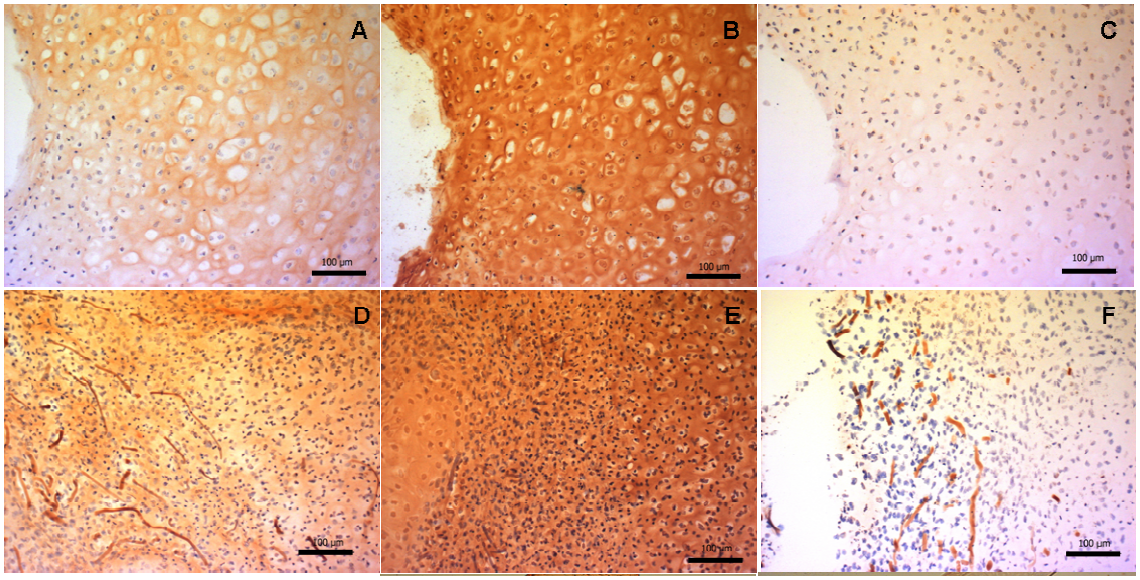

Figure IV.7. Light microscopy images showing immunolocalisation of collagens type I and type II in histology sections of C-PBS (A-C) and PGA constructs (D-F) after 6 weeks of culture. Images show collagen type I, collagen type II, and normal goat serum negative control (left, middle, and right column, respectively).

4. DISCUSSION

In this study, novel melt-processable fibre scaffolds of chitosan and polybutylene succinate (C-PBS) were combined with primary cultures of bovine articular chondrocytes and originally tested for cartilage tissue engineering applications. This is the first time that these types of structures were investigated in a detailed systematic study focusing on cartilage tissue engineering. The

combination of chitosan and polybutylene succinate is expected to render some advantages by the making use of the individual advantages of each type of material. Chitosan is non-toxic, biodegradable and biocompatible[29], and has structural similarities to components of the cartilage extracellular matrix. Polybutylene succinate presents good processing characteristics and mechanical properties flexibility and thereby is expected to develop an important role in mechanical sustainability of the tissue engineered structure once put in an in vivo scenario. Fibre based scaffolds are generally considered adequate for cells seeding and culturing *in vitro* since they usually enable a good cellular dispersion and colonisation while allowing renewal of nutrients and release of toxic metabolites. C-PBS scaffolds appear to contain these features. The scaffolds were quite interconnected and mechanically stable in solution which allows long term culturing and thereby creates the possibility for a tissue engineered cartilage construct to be formed. This is supported by the compression modulus determined that may offer an advantage in terms of enhanced mechanical stability after the onset of scaffold biodegradation. The morphological studies conducted using SEM and μCT also revealed a characteristic surface roughness (see Fig. 1.a) and microporosity (Fig. 1.c) of the C-PBS fibres which might have a positive effect in cell adhesion.[43] The SEM observations of the C-PBS tissue engineered constructs after 6 weeks of culture showed that the chondrocytes efficiently attached and penetrated the scaffolds being present in both the surface and inner regions. Such results suggest that the C-PBS scaffolds, in combination with the dynamic seeding conditions, enabled a homogeneous cell distribution. Also, macroscopic observation revealed that a considerable amount of new cartilage-like tissue had formed, clearly demonstrating the adequacy of C-PBS scaffolds in the support of bovine articular chondrocytes development and ECM synthesis. These were further corroborated by histological observations which granted a wider insight on the tissue engineered constructs phenotype. Cells and ECM were observed between the fibres of the C-PBS scaffolds (Fig. 3.A-C) being a good indication of cytocompatibility and efficiency of the structures. An apparent integration of cell mass, ECM and the scaffold structure was seen. This observation suggests that these tissue engineered constructs may preserve integrity *in vivo*, thereby allowing them to withstand the mechanical stresses which occur in native articular cartilage *in vivo*. The mechanical properties previously determined resulted in a compression modulus of 32.6±12.8 MPa (see Results 3.1) which should theoretically be sufficient for supporting the mechanical stresses acting on the native articular tissue *in vivo*. A similar chondrocyte and extracellular matrix distribution were observed in the PGA constructs. However, a central area of extensive necrosis was detected for the different analysed tissue engineered constructs (Figure 3.D). The necrotic tissue may have

been a result of high cell density and poor nutrient exchange, or necrosis related to acidic by-products produced in the degradation of PGA. These observations correlate well with data previously reported in the literature.[42,44] PGA releases acidic by-products in culture after defined immersion periods in aqueous solutions, as a result of hydrolytic chemical scission of the ester backbone. Its biodegradation occurs by non-specific hydrolytic chain scission at the ester bonds, resulting into glycolic acid residues that may substantially decrease locally the solution pH and affect cell development in active degradation sites. Moreover, it is well known that such materials instead of surface erosion show a bulk erosion degradation process which can produce an autocatalytic effect.[44,45] The nature of the newly formed cartilage is another key point when pursuing articular cartilage tissue engineering strategies. Hyaline cartilage is present in the articular cartilaginous parts developing important roles in both physiological and functional terms. The hyaline-like nature of the formed ECM was evaluated by assessing proteoglycan deposition using toluidine blue and alcian blue stain, as well as quantification of proteoglycan contents using DMB assay, and by comparing collagen type I and II expression. Proteoglycans are important ECM molecules since they enable cartilage to bind water and account for the necessary compressive stiffness and elasticity that are critical for the correct functioning of articular joints.[39] Figures 4 and 5 show the staining profiles for the histological sections of the C-PBS (a-c) and PGA (d-f) tissue engineered constructs after 6 weeks of culture using toluidine blue and alcian blue, respectively. It is possible to observe a positive staining in the pericellular areas indicating that the chondrocytes produced an extracellular matrix containing proteoglycans. The presence of these molecules is crucial since they can bind to various extracellular matrix molecules, cell-cell adhesion molecules, and growth factors.[46] Moreover, they have an important role in terms of water retention which is paramount for cartilage mechanical performance.[47] Both C-PBS and PGA tissue engineered constructs showed sulfated glycosaminoglycans presence in the newly synthesized ECM and this identification is a good indicator towards the formation of a cartilage-like tissue. In addition, the histology of C-PBS shown by H&E, toluidine blue, and alcian blue staining (Figures 3a-c, 4a-c, and 5a-c, respectively) more closely resembled the morphological structure observed for native articular cartilage than that found for PGA constructs (Figures 3d-f, 4d-f, and 5d-f, respectively). When analysing the patterns of GAGs variation in the C-PBS systems using the DMB assay, a continuous increase was detected (Figure 6a). This is another positive result towards the formation of a cartilage-like engineered tissue and is in agreement with the previous data obtained for toluidine blue and alcian blue histological analysis (see Results 3.3). The same pattern of GAG increase was observed for the PGA controls (Figure 6b).

However, higher levels of glycosaminoglycan deposition were observed for these when compared to C-PBS. Nonetheless, the histological analysis mainly focused on glycosaminoglycans (toluidine blue and alcian blue staining) did not reveal any clear qualitative difference between both structures. In fact, both cells morphological features and staining pattern of C-PBS tissue engineered constructs more closely resemble the one found in the native tissue when compared to the PGA. Although the chondrocytes within the PGA matrices may be synthesizing GAG in larger amounts, the variation encountered between the two systems does not correlate well with the histological findings. This difference may relate to a less efficient extraction process for glycosaminoglycans or to lower rates of glycosaminoglycans synthesis in the C-PBS scaffolds, although these hypotheses should be confirmed in future studies. Immunological methods were finally employed to detect the presence of collagen type II and compare it with collagen type I. Collagen type II is the most important major protein produced by chondrocytes and a good marker of tissue engineered hyaline-like cartilage. It is important for the weight bearing function of cartilage,[48,49] as it provides the tensile strength of the tissue,[39] forming a dense network of fibres responsible for retaining the aggrecan during compressive loading. Collagen type II expression patterns in both C-PBS and PGA constructs were analysed, being an indication of their suitability and future performance for cartilage regeneration. Also to confirm that in fact a cartilage-like tissue engineered construct of hyaline nature was being formed, collagen type I expression was verified. Figure 7.a-c shows the immunolocalisation of collagens type I and II in C-PBS constructs. Dense staining for collagen type II was observed with some less dense staining for collagen type I also present. These results suggest that the re-differentiation process was occurring although a complete mature chondrocyte phenotype has not yet settled. The PGA constructs also gave a similar pattern of collagen type I and II distribution. Again, qualitative comparison of the morphologies of the C-PBS and PGA constructs with the native tissue showed that C-PBS constructs more closely resembled the cellular morphology observed in native cartilage. The presence of structures similar to lacunae-like structures that can be found in native cartilage are seen (Figure 7.b). Such structures were not observed in the PGA constructs (Figure 7e). The overall analyses performed suggest that chondrocytes efficiently adhered and proliferated in the C-PBS scaffolds presenting a homogeneous distribution and extracellular matrix formation. The C-PBS scaffolds enabled the formation of a hyaline-like cartilaginous tissue (collagen type II and proteoglycans) indicating good potential for its use as a scaffold material for cartilage regenerative strategies. Nonetheless, the potential application of these scaffolds is not limited to the used processing and manufacturing methodologies. Complimentary approaches to

their use may involve the production of fibres with smaller diameter and the incorporation of bioactive agents of interest seeking in this way to develop structures with higher potential for future cartilage tissue engineering applications.

5. CONCLUSIONS

This study showed that new chitosan/polybutylene succinate (C-PBS) fibre scaffolds can support bovine articular chondrocyte adhesion, proliferation and differentiation, for up to 6 weeks *in vitro.* The cells adhered and proliferated within the 3D supports while expressing common cartilage differentiation markers, such as proteoglycans (glycosaminoglycans) and collagen type II. These C-PBS scaffolds were compared with non-woven polyglycolic acid (PGA) scaffolds cultured using the same parameters. Although the overall cell-materials interactions were similar between the two scaffold types, the PGA constructs presented a central area depleted of cells, which may either be a result of acidic by-products release from their hydrolytic degradation, or necrosis induced by high cellular densities which could compromise their clinical application. Instead, C-PBS tissue engineered constructs did not show any regions poorly colonised by cells, and in contrast, showed homogeneous cell colonization throughout the scaffold structure. Moreover, when compared to PGA, the histomorphology of the C-PBS constructs more closely resembled that of the native cartilage. Both C-PBS and PGA enabled the formation of an extracellular matrix by the chondrocytes as shown from the qualitative and quantitative determination of proteoglycans and immunolocalisation of collagens. Concerning the quantification of proteoglycans content, although a trend of increasing GAGs deposition along the periods of culture was observed in both C-PBS and PGA scaffolds, the latter exhibited higher amounts of synthesized glycosaminoglycans for the various analysed time-points. In summary, this work clearly demonstrates that new C-PBS fibre based scaffolds have good potential to be useful as matrices for cartilage tissue engineering approaches.

REFERENCES

1. Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T., Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold, *Biochem Biophys Res Commun* 322 (1), 50-5, 2004.

2. Temenoff, J. S. and Mikos, A. G., Review: tissue engineering for regeneration of articular cartilage, *Biomaterials* 21 (5), 431-40, 2000.

3. Cancedda, R., Dozin, B., Giannoni, P., and Quarto, R., Tissue engineering and cell therapy of cartilage and bone, *Matrix Biol* 22 (1), 81-91, 2003.

4. Grodzinsky, A. J., Electromechanical and physicochemical properties of connective tissue, *Crit Rev Biomed Eng* 9 (2), 133-99, 1983.

5. Zhu, W., Mow, V. C., Koob, T. J., and Eyre, D. R., Viscoelastic shear properties of articular cartilage and the effects of glycosidase treatments, *J Orthop Res* 11 (6), 771-81, 1993.

6. Lee, C. R., Breinan, H. A., Nehrer, S., and Spector, M., Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior in vitro, *Tissue Eng* 6 (5), 555-65, 2000.

7. Gugala, Z. and Gogolewski, S., In vitro growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold, *Journal of Biomedical Materials Research* 49 (2), 183-191, 2000.

8. D. A. Grande, C. H., G. Naughton, R. Schwartz, Ryhana Manji, Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts, *Journal of Biomedical Materials Research* 34 (2), 211-220, 1997.

9. Coleman, R. M., Case, N. D., and Guldberg, R. E., Hydrogel effects on bone marrow stromal cell response to chondrogenic growth factors, *Biomaterials* In Press, Corrected Proof.

10. Gugala, Z. and Gogolewski, S., In vitro growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold, *J Biomed Mater Res* 49 (2), 183-91, 2000.

11. Yaylaoglu, M. B., Yildiz, C., Korkusuz, F., and Hasirci, V., A novel osteochondral implant, *Biomaterials* 20 (16), 1513-1520, 1999.

12. Giannoni, P., Pagano, A., Maggi, E., Arbico, R., Randazzo, N., Grandizio, M., Cancedda, R., and Dozin, B., Autologous chondrocyte implantation (ACI) for aged patients: development of the proper cell expansion conditions for possible therapeutic applications, *Osteoarthritis Cartilage* 13 (7), 589-600, 2005.

[13.] Hunziker, E. B., Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects, *Osteoarthritis and Cartilage* 10 (6), 432-463, 2002.

[14.] Malafaya, P. B., Pedro, A. J., Peterbauer, A., Gabriel, C., Redl, H., and Reis, R. L., Chitosan particles agglomerated scaffolds for cartilage and osteochondral tissue engineering approaches with adipose tissue derived stem cells, *Journal of Materials Science: Materials in Medicine* V16 (12), 1077-1085, 2005.

[15.] Sams, A. E. and Nixon, A. J., Chondrocyte-laden collagen scaffolds for resurfacing extensive articular cartilage defects, *Osteoarthritis and Cartilage* 3 (1), 47-59, 1995.

[16.] Li, W.-J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K. G., Hall, D. J., and Tuan, R. S., A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells, *Biomaterials* 26 (6), 599-609, 2005.

[17.] Slivka MA, L. N., Kieswetter K, Niederauer GG., Porous, resorbable, fiber-reinforced scaffolds tailored for articular cartilage repair., *Tissue Engineering* 7 (6), 767-80, 2001

[18.] Moroni, L., de Wijn, J. R., and van Blitterswijk, C. A., 3D fiber-deposited scaffolds for tissue engineering: Influence of pores geometry and architecture on dynamic mechanical properties, *Biomaterials* 27 (7), 974-985, 2006.

[19.] Malda, J., Woodfield, T. B. F., van der Vloodt, F., Wilson, C., Martens, D. E., Tramper, J., van Blitterswijk, C. A., and Riesle, J., The effect of PEGT/PBT scaffold architecture on the composition of tissue engineered cartilage, *Biomaterials* 26 (1), 63-72, 2005.

[20.] J. Aigner, J. T. P. H. D. C. A. P. C. H. E. K. A. N., Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester, *Journal of Biomedical Materials Research* 42 (2), 172-181, 1998.

[21.] Woodfield, T. B. F., Malda, J., de Wijn, J., Peters, F., Riesle, J., and van Blitterswijk, C. A., Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique, *Biomaterials* 25 (18), 4149-4161, 2004.

[22.] J. Aigner, J. T., P. Hutzler, D. Campoccia, A. Pavesio, C. Hammer, E. Kastenbauer, A. Naumann Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester, *J Biomed Mater Res* 42 (2), 172-81, 1998.

[23.] Gomes, M. E., Azevedo, H. S., Moreira, A. R., Ella, V., Kellomaki, M., and Reis, R. L., Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: structure, mechanical properties and degradation behaviour, *J Tissue Eng Regen Med* 2 (5), 243-52, 2008.

[24.] Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering: A* 403 (1-2), 57-68, 2005.

[25.] Neves, N. M., Kouyumdzhiev, A., and Reis, R. L., The morphology, mechanical properties and ageing behavior of porous injection molded starch-based blends for tissue engineering scaffolding, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 25 (2), 195-200, 2005.

[26.] Oliveira, J. T., Correlo, V. M., Sol, P. C., Costa-Pinto, A. R., Malafaya, P. B., Salgado, A. J., Bhattacharya, M., Charbord, P., Neves, N. M., and Reis, R. L., Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach, *Tissue Eng Part A* 14 (10), 1651-61, 2008.

[27.] Freier, T., Koh, H. S., Kazazian, K., and Shoichet, M. S., Controlling cell adhesion and degradation of chitosan films by N-acetylation, *Biomaterials* 26 (29), 5872-8, 2005.

[28.] Madihally, S. V. and Matthew, H. W. T., Porous chitosan scaffolds for tissue engineering, *Biomaterials* 20 (12), 1133-1142, 1999.

[29.] Correlo, V. M., Boesel, L. F., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Properties of melt processed chitosan and aliphatic polyester blends, *Materials Science and Engineering A* In Press, Corrected Proof, 2005.

[30.] Mahmoudifar, N. and Doran, P. M., Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors, *Biomaterials* In Press, Corrected Proof.

[31.] Devlieghere, F., Vermeulen, A., and Debevere, J., Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables, *Food Microbiology* 21 (6), 703-714, 2004.

[32.] Silva, R. M., Malafaya, P. B., Mano, J. F., and Reis, R. L., Bioactive composite chitosan membranes to be used in bone regeneration applications, *Bioceramics 15* 240-2, 423-426, 2003.

[33.] Singh, D. R., AR., Biomedical applications of chitin, chitosan, and their derivatives., *J Macromol Sci C* 40, 69-83, 2000.

[34.] Lyoo, W. S., Kim, J. H., Yoon, W. S., Ji, B. C., Choi, J. H., Cho, J., Lee, J., Yang, S. B., and Yoo, Y., Effects of polymer concentration and zone drawing on the structure and properties of biodegradable poly(butylene succinate) film, *Polymer* 41 (26), 9055-9062, 2000.

[35.] Henry, E. W., Chiu, T.-H., Nyilas, E., Brushart, T. M., Dikkes, P., and Sidman, R. L., Nerve regeneration through biodegradable polyester tubes, *Experimental Neurology* 90 (3), 652-676, 1985.

[36.] Haiyan Li, J. C., Amin Cao, Junying Wang, in vitro Evaluation of Biodegradable Poly(butylene succinate) as a Novel Biomaterial, *Macromolecular Bioscience* 5 (5), 433-440, 2005.

[37.] Correlo, V. M., Boesel, L. F., Pinho, E., Costa-Pinto, A. R., Alves da Silva, M. L., Bhattacharya, M., Mano, J. F., Neves, N. M., and Reis, R. L., Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties, *J Biomed Mater Res A*, 2008.

[38.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[39.] Kafienah, W., Sims, T.J., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[40.] K. A. Athanasiou, M. P. R. J. A. B. T. I. M. V. C. M., Interspecies comparisons of in situ intrinsic mechanical properties of distal femoral cartilage, 1991, pp. 330-340.

[41.] VM Correlo, P. S., AR Costa-Pinto, ML Alves da Silva, JA Covas, M Bhattacharya, NM Neves, RL Reis, Novel Chitosan Based Fibreas and Fibre Mesh Scaffolds Produced by Melt Based Technologies, *submitted*, 2008.

[42.] M. S. Taylor, A. U. D., K. P. Andriano, J. Heller, Six bioabsorbable polymers: In vitro acute toxicity of accumulated degradation products, *Journal of Applied Biomaterials* 5 (2), 151-157, 1994.

[43.] Wan, Y., Wang, Y., Liu, Z., Qu, X., Han, B., Bei, J., and Wang, S., Adhesion and proliferation of OCT-1 osteoblast-like cells on micro- and nano-scale topography structured poly(l-lactide), *Biomaterials* 26 (21), 4453-4459, 2005.

[44.] Athanasiou, K. A., Niederauer, G. G., and Agrawal, C. M., Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/ polyglycolic acid copolymers, *Biomaterials* 17 (2), 93-102, 1996.

[45.] Athanasiou, K. A., Agrawal, C. M., Barber, F. A., and Burkhart, S. S., Orthopaedic applications for PLA-PGA biodegradable polymers, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 14 (7), 726-737, 1998.

[46.] Ruoslahti, E., Proteoglycans in cell regulation, *J Biol Chem* 264 (23), 13369-72, 1989.

47. Watanabe, H. and Yamada, Y., Chondrodysplasia of gene knockout mice for aggrecan and link protein, *Glycoconjugate Journal* 19 (4), 269-273, 2002.

48. Brodkin, K. R., Garcia, A. J., and Levenston, M. E., Chondrocyte phenotypes on different extracellular matrix monolayers, *Biomaterials* 25 (28), 5929-5938, 2004.

49. Cancedda, R., Descalzi Cancedda, F., and Castagnola, P., Chondrocyte differentiation, *Int Rev Cytol* 159, 265-358, 1995.

SECTION 3.

CHAPTER V.

A Cartilage Tissue Engineering Approach Combining Starch-Polycaprolactone Fibre Mesh Scaffolds With Bovine Articular Chondrocytes

CHAPTER V.

A Cartilage Tissue Engineering Approach Combining Starch-Polycaprolactone Fibre Mesh Scaffolds With Bovine Articular Chondrocytes*

ABSTRACT

In the present work we originally tested the suitability of corn starch-polycaprolactone (SPCL) scaffolds for pursuing a cartilage tissue engineering approach. Bovine articular chondrocytes were seeded on SPCL scaffolds under dynamic conditions using spinner flasks (total of 4 scaffolds per spinner flask using cell suspensions of $0.5x10^6$ cells/ml) and cultured under orbital agitation for a total of 6 weeks. Poly(glycolic acid) (PGA) non-woven scaffolds and bovine native articular cartilage were used as standard controls for the conducted experiments. PGA is a kind of standard in tissue engineering approaches and it was used as a control in that sense. The tissue engineered constructs were characterized at different time periods by scanning electron microscopy (SEM), hematoxylin-eosin (H&E) and toluidine blue stainings, immunolocalisation of collagen types I and II, and dimethylmethylene blue (DMB) assay for glycosaminoglycans (GAG) quantification assay. SEM results for SPCL constructs showed that the chondrocytes presented normal morphological features, with extensive cells presence at the surface of the support structures, and penetrating the scaffolds pores. These observations were further corroborated by H&E staining. Toluidine blue and immunohistochemistry exhibited extracellular matrix deposition throughout the 3D structure. Glycosaminoglycans, and collagen type I and II were detected. However, stronger staining for collagen type II was observed when compared to collagen type I. The PGA constructs presented similar features to SPCL at the end of the 6 weeks. PGA constructs exhibited higher amounts of matrix glycosaminoglycans when compared to the SPCL scaffolds. However, we also observed a lack of tissue in the central area of the PGA scaffolds. Reasons for these occurrences may include inefficient cells penetration, necrosis due to high cell densities, or necrosis related with acidic by-products degradation. Such situation was not detected in the SPCL scaffolds, indicating the much better biocompatibility of the starch based scaffolds.

*** This chapter is based on the following publication:**

A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes. JT Oliveira, A Crawford, JM Mundy, AR Moreira, ME Gomes, PV Hatton, RL Reis. Journal of Materials Science: Materials in Medicine (2007) 18:295–302.

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1. INTRODUCTION

Articular cartilage is an avascular supporting connective tissue, exhibiting a low metabolic rate and a low regenerative potential[1-3]. The ability of articular cartilage to function as a weight bearing tissue is dependent on the appropriate structural organisation and biochemical composition of the extracellular matrix, the two major components of which are collagen type II and proteoglycan.[3-5] The collagen is responsible for the tensile properties and the proteoglycans for compression resistance.[6,7] Articular cartilage is responsible for the correct functioning of the articulating skeleton, creating smooth gliding areas in the terminal parts of bones responsible for shock absorbance, load bearing and reduction of surface friction.[8] Trauma, aging related degeneration such as osteoarthritis, or developmental disorders, can result in pain and disability. Adult cartilage has limited self repair capacity and even when some regeneration exists, fibrocartilage-like tissue is frequently formed in the defect.[5,9,10] This type of cartilage possesses lower mechanical properties when compared to articular cartilage, compromising its functional role in weight bearing. Different strategies have been put forward to treat articular cartilage lesions. One common treatment in elderly patients is prosthetic joint replacement. Although successful, this invasive approach does not always provide long-term joint functionality due to loosening or limited life span of the prostheses[11]. Surgical procedures like osteotomy, perichondral grafting, interposition arthroplasty, and drilling, have been performed though the outcomes may be limited.[8,12] Several and different tissue engineering approaches are being conducted to regenerate cartilage, most of them based on seeding cells in a polymeric matrix. The materials used to serve as cells supports can be processed in various ways, including extrusion and moulding, among others. These procedures can generate porous structures (scaffolds) of different shapes and sizes, e.g. fibres with more regular or irregular surfaces with varying diameters, membranes, and others.[13] The materials used for tissue engineering may be broadly divided into synthetic and natural materials. Starch is a natural polymer made of a combination of two polymeric carbohydrates, amylose and amylopectin.[14,15] It has been put forward as a cell support material in combination with synthetic polymers such as polycaprolactone (PCL), polylactic acid (PLA), ethyl vinyl alcohol (EVOH), cellulose acetate (CA) giving rise to a blend that is expected to deliver better results. Several studies have been conducted with these materials, mainly in bone tissue engineering.[16-19] Polycaprolactone (PCL) is a synthetic semicrystalline biodegrabable polymer belonging to the family of poly-α-hydroxyl polyesters, that has also been used for such approaches.[20,21]

In this study, we have shown for the first time, the suitability of starch-polycaprolactone (SPCL) scaffolds for pursuing a cartilage tissue engineering approach. Bovine articular chondrocytes

were cultured on starch-polycaprolactone fibre scaffolds for periods of up to 6 weeks. Cells were initially seeded using spinner flask bioreactors and then cultured in an orbital shaker for the remaining time periods. The scaffold fibre structure allowed the cells to efficiently penetrate the bulk besides the colonization of the most outer parts. We have analysed cell distribution, morphology and extracellular matrix components deposition during the course of the experiments, and the results obtained are encouraging in indicating a utility of SPCL scaffolds for a tissue engineering cartilage regeneration strategy.

2. MATERIALS AND METHODS

2.1. SCAFFOLDS PRODUCTION

The methodology used to produce the scaffolds was melt spinning (to obtain the polymeric fibres) followed by fibre bonding.[22] This processing technique involves fibre packing in an appropriate mould, with posterior heating below the melting temperature (Tm) for a determined residence period that will allow the fibres to form a stable fibre mesh structure. The material used was a 30/70% (wt) blend of corn starch with polycaprolactone (SPCL). These scaffolds have already been shown previously to be suitable for conducting a bone tissue engineering approach[17,23]. The scaffolds produced were cut in a cylindrical shape, with dimensions of 7mm diameter x 3mm thickness. The porosity of the scaffolds was determined by microcomputerized tomography (μCT) (ScanCo Medical μCT 80, Bassersdorf, Switzerland) at a resolution of 10 mm, and using at least 3 samples. For comparison purposes, it should be stated that the PGA scaffolds dimensions were the same as those of SPCL. Both types of scaffolds were cut using a borer.

2.2. ISOLATION AND EXPANSION OF BOVINE ARTICULAR CHONDROCYTES

Full thickness hyaline cartilage was harvested from bovine metacarpophalangeal joint of adult animals (18-24 months) within 4 hours of slaughter. Chondrocytes were isolated by sequential enzymatic digestion as described previously.[24] The isolated cells were ressuspended in expansion medium (Dulbecco's Modified Eagle's Medium (Sigma Co.), containing 10 mM HEPES buffer pH 7.4 (Sigma Co.), 10000 units/ml penicillin/10000 μg/ml streptomycin (Sigma Co.), 20

mM L-alanyl glutamine (Sigma Co.), 1x MEM non-essential amino acids (Sigma Co.) and 10% (v/v) foetal calf serum (Biosera S1800; NWPLS; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK), and seeded on tissue culture treated Petri dishes at a density of 50,000-100,000 cells/cm^2. The dishes were incubated at 37ºC in a humidified atmosphere of 5% CO_2/95% air. The chondrocytes were allowed to expand until almost confluent, and then trypsinized and divide to other tissue culture treated Petri dishes using the same proportional relations.

2.3. CHONDROCYTE CULTURE ON 3D SPCL FIBRE SCAFFOLDS

Once the required cell number was achieved, confluent chondrocyte monolayers were harvested for seeding onto the polymeric scaffolds, as follows. The SPCL fibre scaffolds were allowed to equilibrate at room temperature in 10 ml of expansion medium. The chondrocytes were removed from the culture dishes by trypsinisation. The chondrocytes were pelleted by centrifuging at 200g for 7 min and the cell pellet ressuspended in expansion medium. The SPCL fibre scaffolds were placed on stainless steel wires in spinner flasks containing a suspension of chondrocytes with a concentration of $0.5x10^6$ cells/ml (4 scaffolds per spinner flask). The stirrer was set at a slow stirring of 75 rpm and the spinner flasks left for 72h to allow the cells to enter the scaffold material. After the seeding was completed, the chondrocytes/scaffold constructs were transferred to non-tissue culture treated sterile Petri dishes and 20 ml of fresh expansion medium added to each Petri dish. The Petri dishes were placed on the orbital shaker, and set at a gentle shaking speed of 50 rpm. The constructs were left for 72-96 h to allow further expansion of the cells on the scaffolds. The expansion culture medium was then removed and replaced with 20 ml of differentiation medium (Dulbecco's Modified Eagle's Medium (Sigma Co.), containing 10 mM HEPES buffer pH 7.4 (Sigma Co.), 10000 units/ml penicillin/10000 µg/ml streptomycin(Sigma Co.), 20 mM L-alanyl glutamine (Sigma Co.), MEM non-essential amino acids (Sigma Co.), and 10% (v/v) foetal calf serum (Biosera S1800; NWPLS; Heat Inactivated), supplemented with 1 µg/ml of insulin (Sigma Co.) and 50 µg/ml of L-ascorbic acid (Sigma Co.) to promote formation of a chondrogenic phenotype in the chondrocytes. The construct cultures were returned to the orbital shaker in the incubator and maintained at a speed of 50 rpm. The constructs were incubated until 42 days of culture, replacing the differentiation medium every 2-3 days.

2.4. SCANNING ELECTRON MICROSCOPY

The constructs were washed in sterile PBS and immersed in 3% glutaraldehyde (Sigma Co.) with 0.1 M cacodylate buffer pH 7.4) (AGAR) at room temperature for one hour. They were then washed three times in 0.1 M cacodylate buffer pH 7.4, and afterwards post fixed in 1% aqueous solution of osmium tetroxide (Fluka/Sigma Co.) for one hour. Finally, they were dehydrated in alcohols and let to dry. The samples were sputter coated with gold and observed using a Phillips XL-20 scanning electron microscope.

2.5 HISTOLOGICAL ANALYSIS (HEMATOXYLIN-EOSIN, TOLUIDINE BLUE)

Constructs were taken at specific culturing periods, until up to six weeks of total culturing time. The constructs were included in Optimal Cutting Temperature gel (OCT) (OCT compound BDH, Gurr®), frozen using liquid nitrogen and isopentane, stored at -20ºC for posterior cryosectioning. Tissue sections of 8 µm were taken and fixed using fresh 4% paraformaldehyde (Sigma, Co.). The slides were then washed in distilled water, let to dry and stored at 4ºC until the staining was performed. Hematoxylin-eosin stain was conducted in an automatic machine (Fume Cupboard; X219/E11/LEV1). In this procedure, the slides are stained in hematoxylin for a suitable time, optimised according to in-house procedures. The sections are washed in running tap water for 5 minutes or less, and afterwards differentiated in 1% acid alcohol, for 5-10 seconds. The slides are washed again in tap water for 5 minutes or less, and stained in 1% eosin for 10 minutes. They are again washed in tap water for 5 minutes, and dehydrated through alcohols and mounted in DPX (Fluka/Sigma Co.). Toluidine blue staining was performed using standard histological methods in the following way. One drop of 1% toluidine blue was placed on each section for 2-3 seconds. The sections were rinsed with distilled water. Dehydration through alcohols followed and the sections were then left to dry overnight, and mounted in DPX.

2.6. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND TYPE II

Collagen types I and II were detected immunohistochemically using monoclonal antibodies against collagen types I and II (Southern Biotechnology, UK), as previously described[25]. Briefly, fixed sections were washed with PBS and pre-treated with hyaluronidase (10 mg/ml) (Sigma Co.),

followed by pronase (2 mg/ml) (Fluka/Sigma Co.). The slides were then washed thoroughly in PBS and treated with 3% hydrogen peroxide (Sigma Co.) in 50% methanol (Aldrich), followed by washing in Tris-buffered saline (TBS) and blocking with 3% bovine serum albumin (BSA) (Sigma Co.) Incubation with the primary antibody (collagen type I and collagen type II) (UNLB) followed. The remaining protocol is as described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). The slides were afterwards washed in water for 5 minutes, counterstained with haematoxylin, and mounted with DPX mounting medium. Controls were performed using normal goat serum instead of the primary antibodies, which was included in the kit.

2.7. DIMETHYLMETHYLENE BLUE (DMB) ASSAY FOR GLYCOSAMINOGLYCANS QUANTIFICATION

Proteoglycans were determined by measuring the level of sulphated glcosaminoglycans (GAGs) using the dimethylmethylene blue metachromatic assay as described previously[25]. GAG levels in solution can be quantified by binding of the acidic polymer to the basic dye, 1,9-dimethylmethylene blue (DMB). The resulting metachromatic shift peaks at $A_{525\text{-}530}$ which can therefore be adapted for a spectrophotometric assay. Briefly, the constructs were immersed in a digestion solution with papain (Sigma Co.) and N-acetyl cysteine (Sigma Co.), and incubated at 60ºC overnight. After the digestion was completed, the tubes were centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and stored at 4ºC until the GAG assay was performed. A chondroitin sulphate standard solution (Sigma Co.) was prepared in water and kept refrigerated. The samples and chondroitin sulphate standards were placed in a 96 well round-bottomed plate, DMB solution was added to each well, and the optical density measured using a microplate reader, at 530 nm. Poly(glycolic acid) (PGA) non-woven scaffolds (Albany international, Bury, Lancashire, UK) were used as controls.

3. RESULTS AND DISCUSSION

3.1. SCAFFOLDS PRODUCTION

A microcomputerized tomography (μCT) image of a SPCL scaffold is shown in figure 1. It was observed that the fibre mesh network for support of cell growth and development presents good interconnectivity. The fibre network structure was advantageous for the dynamic seeding using spinner flasks. An extensive porous area (approximately 75% as estimated by μCT) is an advantage towards cells penetration into the bulk of the scaffold, while also enhancing nutrients diffusion and removal of metabolic wastes. The scaffolds thickness (3mm) is within the range of the values encountered for normal human articular cartilage.[26]

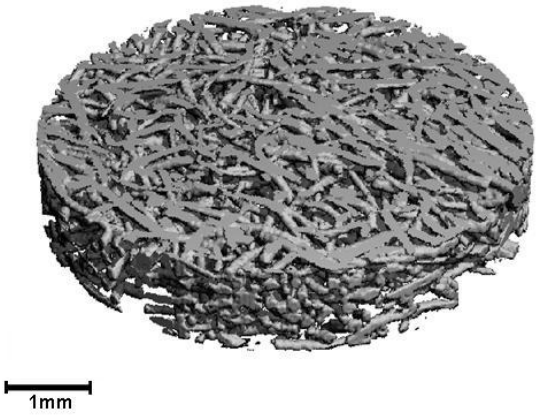


Figure V.1. Microcomputerized tomography image of a SPCL 30/70% (wt) fibre scaffold.

3.2. SCANNING ELECTRON MICROSCOPY

Scanning electron micrographs showed that the bovine articular chondrocytes extensively colonised the scaffold structure, being widely present at the surface and penetrating the various pores. We believe this is a consequence not only of the materials used, to which the cells adhere, but also to the scaffolds 3D arrangement, that in combination with the dynamic cells seeding using the spinner flasks, allowed the cells to spread and proliferate homogeneously throughout the entire construct. In fact, SPCL scaffolds have already been shown to be successful in bone tissue engineering approaches, in studies conducted with bone marrow stromal cells cultured in SPCL fibre based scaffolds, under dynamic conditions using bioreactors.[27] The results presented here for the first time using SPCL scaffolds in a cartilage tissue engineering approach constitute another evidence for the application of these supports in tissue engineering approaches.

Figures 2.1.A-C show at increasing magnifications, constructs collected after 2 weeks of culturing. Figure 2.1.A exhibits a global view over the cells-scaffold construct, showing that the cells were homogeneously distributed and had adhered uniformly, showing no difference between a fibre surface and the contact junction between two fibres. The morphology of the chondrocytes is the one of normal and healthy cells,[28,29] and these were forming multilayer as observed in figure 2.1.C. These observations indicate that with such an arrangement, the cells create a 3D environment which favours extracellular matrix formation. Figures 2.2.A-C shows the cultured scaffolds at week 4. Comparison of figures 2.1.A and 2.2.A, corresponding to weeks 2 and 4 of culturing, respectively, showed higher cell coverage in the latter, indicating that the cells have proliferated during these periods. Comparing the results at week 4 (Figures 2.2.A-C) with those from week 6 (2.3.A-C), no difference is observed. However, it can be observed that the cell coverage was extensive in both time periods. These observations might be explained by the exchange in culture medium supply that was performed. The supply of expansion medium containing bFGF during the initial periods, induces cell proliferation which enables obtaining a full cell coverage on the scaffolds[30]. The change to a differentiation medium containing insulin and L-ascorbic acid (after 7 days of culture) would induce a decrease in the proliferation rates and trigger the onset of redifferentiation, with consequent expression of extracellular matrix.[31]

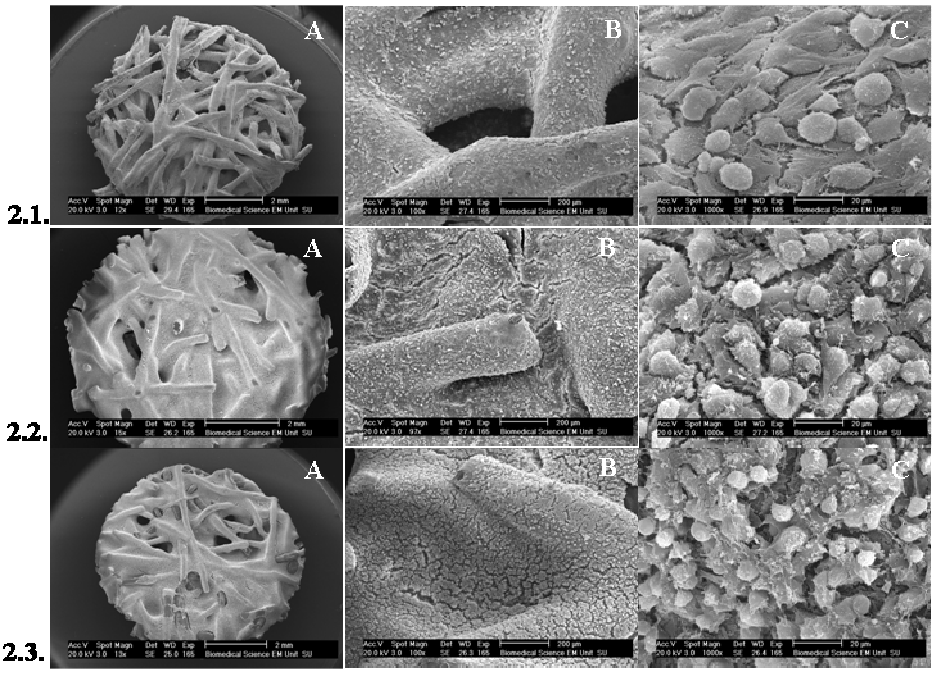


Figure V.2. Scanning electron microscopy images of SPCL scaffolds seeded with bovine articular chondrocytes and cultured for 14 days (2.1.), 28 days (2.2.), and 42 days (2.3.). B and C represent higher magnifications of A, showing in detail the cells morphology and arrangement.

3.3. HEMATOXYLIN-EOSIN AND TOLUIDINE BLUE

Figure 3 shows different histological sections of scaffolds taken at week 4 (3.1.A.) and week 6 (3.1.B-D). As shown previously in the SEM analysis, it can be observed an increase in cell mass from week 4 to week 6, when comparing figures 3.1.A and 3.1.B. Cells have also created a consistent adhesion interface with the SPCL fibres, as can be observed in figure 3.1.D (arrows). The histology processing usually leads the structures to contract, and the detachment of cell mass observed in some of the fibres is a result of that. The fact that continuity is observed at the cells-fibres interface allows us to predict the tissue engineered constructs may preserve its integrity *in vivo*, acting as one functional unit. It is also evident the cells presence in both bulk and more external areas of the scaffolds (figure 3B). Regarding the PGA scaffolds, the observations indicate that these exhibited higher cell proliferation when compared to SPCL in the initial periods. We also detected a central area within the scaffolds were cells were lacking. Reasons for these occurrences may be related with inefficient cells penetration, necrosis due to high cell densities, or necrosis related with acidic by-products degradation, as shown in figures 3E and 3F. This was observed by week 4 of culture and correlates with data reported in the literature. PGA, as well as other polyesters, release acidic by-products in culture after a determined time period, as a result of hydrolytic chemical scission of the ester backbone.[32] PGA biodegradation occurs by non-specific hydrolytic scission of their ester bonds, resulting into glycolic acid residues that may substantially decrease the solution pH and indirectly affect cell development.[33] Figures 4.1.A-D show toluidine blue stained histology sections of SPCL scaffolds seeded with bovine articular chondrocytes. Toluidine blue is a metachromatic stain that identifies glycosaminoglycans present in the extracellular matrix of hyaline cartilage. It is possible to observe a light purple staining at both time periods presented (Figures 4.1.A-D), which indicates that the chondrocytes had produced an extracellular matrix containing proteoglycans. Proteoglycans enable cartilage to bind water molecules and account for the property of compressive stiffness important for the correct functioning of articular joints.[25] Therefore, the identification of glycosaminoglycans in the cell extracellular matrix is a positive indicator towards the formation of a cartilage-like tissue. Concerning the PGA scaffolds, the results obtained from the staining with toluidine blue are very similar to those obtained for SPCL scaffolds. However, in PGA scaffolds no glycosaminoglycans presence was detected in the bulk area due to lack of cellular material (4.2.A-B), as previously

mentioned in H&E stained sections. Both tissue engineered constructs presented a staining profile very similar to the one encountered for native articular cartilage extracellular matrix (4.2.D).

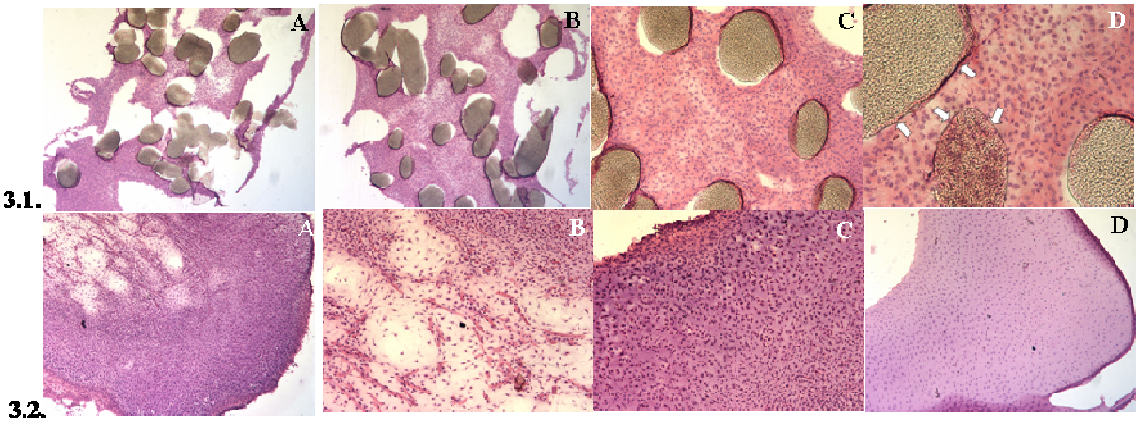


Figure V.3. Optical microscopy images of histology sections obtained from SPCL scaffolds seeded with bovine articular chondrocytes and stained with hematoxylin-eosin. The images shown correspond to samples collected after 28 days (3.1.A), and 42 days (3.1.B-D) of culture. Figures 3.2.A-C show optical microscopy images of histology sections obtained from PGA scaffolds seeded with bovine articular chondrocytes and stained in the same way (42 days). Figure 3.2.D represents native bovine articular cartilage control stained using the same technique.

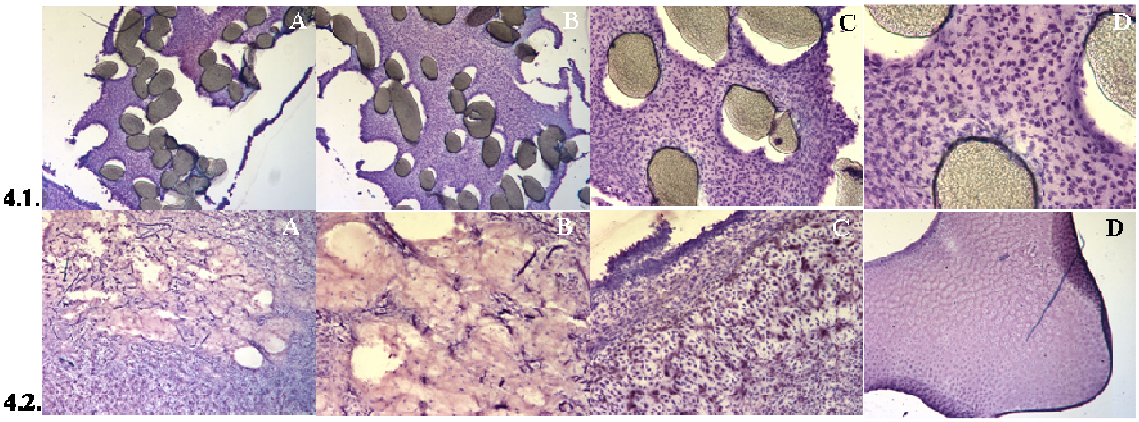


Figure V.4. Optical microscopy images of histology sections obtained from SPCL scaffolds seeded with bovine articular chondrocytes stained with toluidine blue. The images shown correspond to 28 days (4.1.A), and 42 days (4.1.B-D) of culturing. Optical microscopy images of histology sections of PGA scaffolds seeded with bovine articular chondrocytes and stained with toluidine blue are presented at 42 days (4.2.A-C). Figure 4.2.D shows native bovine articular cartilage stained with toluidine blue dye.

3.4. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND TYPE II

Four collagen types, namely, type II, IX, X, and XI, are traditionally considered specific for cartilage.[34] Collagen type II is the major protein produced by chondrocytes in articular cartilage, being involved in its weight bearing and adsorbing functions.[35] It was performed immunolocalisation of both collagen type I and collagen type II proteins in sections obtained from SPCL seeded scaffolds (Figure 5.1.). The results correspond to samples collected at week 6 of culture. A difference in the expression pattern can be noted, when comparing collagen type I and type II, with type II collagen displaying stronger antibody staining. When articular chondrocytes are isolated and expanded in 2D culture conditions, the cells expression profiles change. Collagen type II production is reduced and collagen type I is expressed. This process is described as dedifferentiation and it is a characteristic feature of chondrocytes grown in two dimensional cultures[34,36]. Once the cells reach confluence and begin to pack in multilayered 3D structures, they begin to re-differentiate. Re-differentiation is the process of regaining normal articular cartilage molecules synthesis, such as collagen type II, aggregan, and Sox-9, for example.[37,38] When *in vitro* differentiation is induced, type I collagen rapidly decreases during culture, and the levels of collagen type II and IX experience an increase.[34,35,39] Cell constructs studied herein presented stronger staining of collagen type II by week 4 (data not shown), which was maintained throughout the rest of the culture period. Collagen type II is the major structural macromolecule of hyaline cartilage, conferring tensile strength to the cartilage matrix and is thus a good marker of tissue engineered hyaline-like cartilage.[25] Considering this, the expression of these proteins, detected in the constructs cultured for 6 weeks, is another indication of the hyaline-like nature of these tissue engineered constructs. Regarding the PGA scaffolds, it was observed a higher intensity staining with collagen type II antibodies than with collagen type I. Predominance of collagen type II staining over collagen type I in both types of scaffolds inferred that bovine articular chondrocytes regained a chondrogenic phenotype on the SPCL and PGA scaffolds. These results correlated with the toluidine blue staining, which reveal that glycosaminoglycans were present in the newly elaborated extracellular matrix.

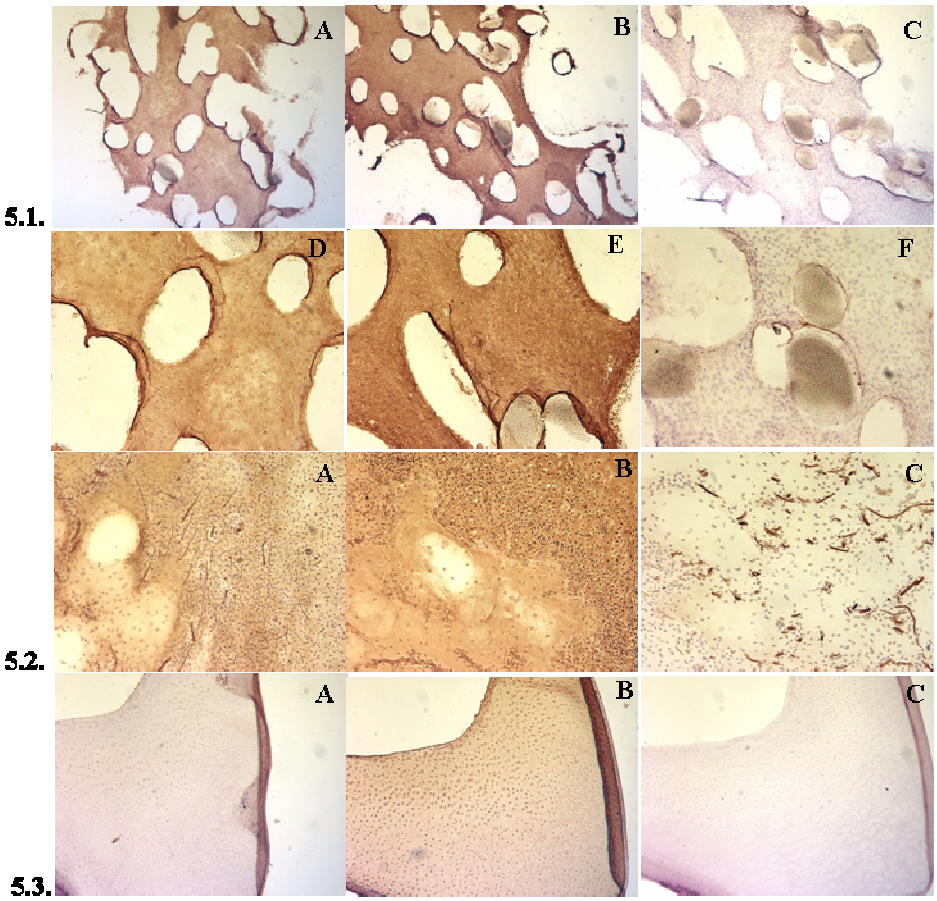
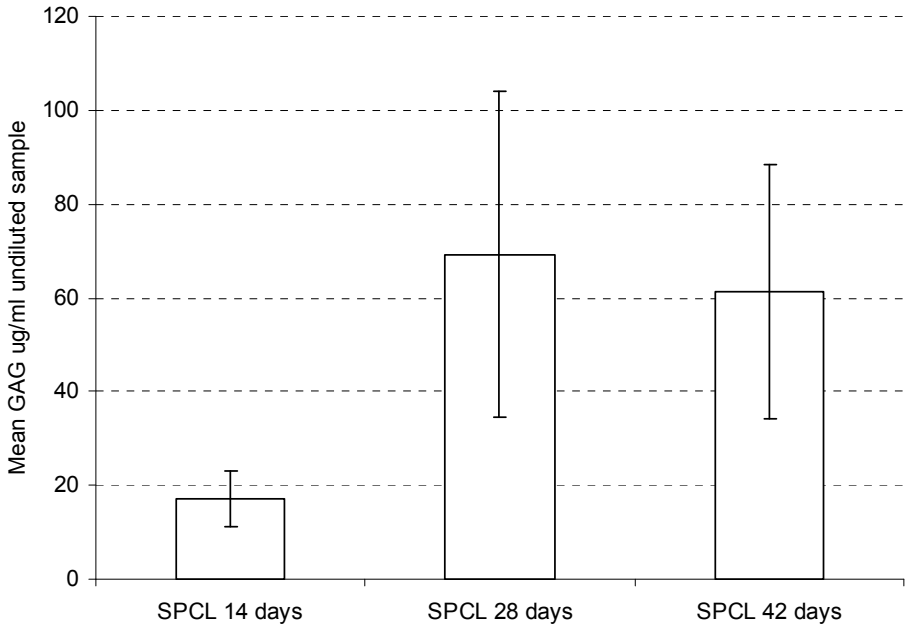


Figure V.5. Optical microscopy images presenting the results obtained from the immunolocalisation of collagen type I and type II in histology sections of SPCL scaffolds seeded with bovine articular chondrocytes. Images present results at 42 days for collagen type I, collagen type II, and normal goat serum-control (left to right, respectively) (5.1.A-C;D-F). The images are shown at different magnifications. Below, comparative results are shown for PGA scaffolds at 42 days for collagen type I, collagen type II and normal goat serum-control (left to right, respectively) (5.2.A-C). Figure 5.3.A-C represents native bovine articular cartilage stained using the same method (collagen type I, collagen type II, and normal goat serum-control, from left to right, respectively).

3.5. DMB ASSAY FOR GLYCOSAMINOGLYCANS QUANTIFICATION

The glycosaminoglycans (GAG) were quantified using the dimethylmethylene blue (DMB) assay. Comparing the results obtained with SPCL constructs with the ones generated for PGA, it can be observed a wide difference in glycosaminoglycans quantification. Given the similar results obtained with the toluidine blue staining for both types of scaffolds, the higher values for GAGs

concentration in PGA scaffolds may be a result of the apparently higher initial cell proliferation rates observed for the PGA scaffolds, when compared to SPCL. A higher cell number would result in an increase in the exrtracellular matrix components, as it is known that intercellular contacts exert extreme importance for chondrocytes to begin extracellular matrix deposition.[34,40] The fact that the cells were able to proliferate at higher rates in the PGA scaffolds may justify the higher glycosaminoglycans synthesis that was observed. However, this may be jeopardised by the tissue depletion observed in the central area of the PGA scaffolds, which can be a result of acidic by-products release, inefficient cells penetration or necrosis induced by high cellular densities that may lead to loss in mass transfer throughout the constructs. Furthermore, GAG deposition in SPCL may be achieved with some complementary strategies, such as the addition of certain growth factors. For example, it is described in the literature that bone morphogenetic protein 2 (BMP-2) and cartilage-derived morphogenetic protein 2 (CDMP-2) significantly enhance proteoglycans production on a chondrocytic cell line (MC615).[41] Also, it has been shown that insulin-like growth factor type I (IGF-I) increases the synthesis of proteoglycans in bovine articular chondrocytes.[31] Some of these molecules can be added to the culture medium or even included in the scaffolds.



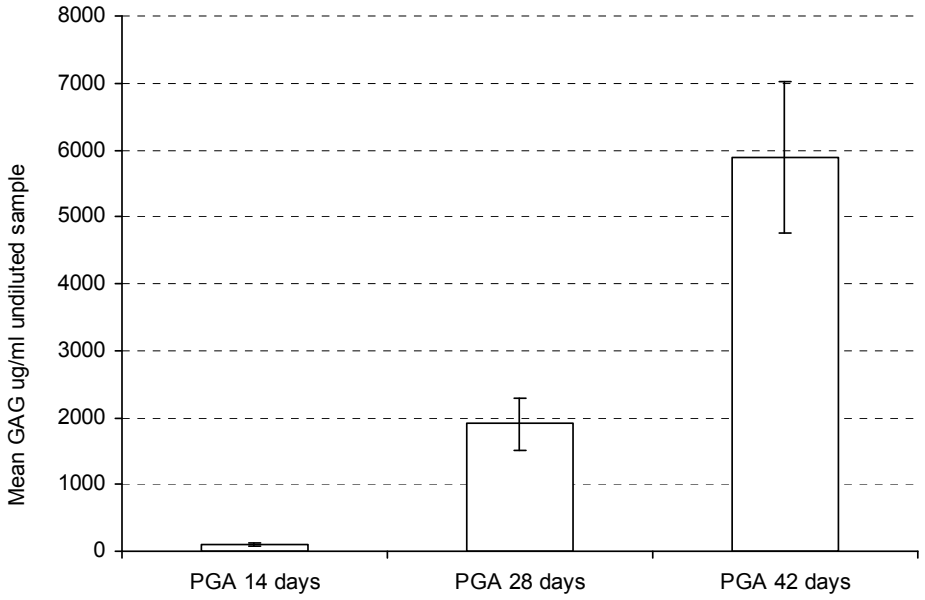


Figure V.6. Results obtained from DMB assay for GAGs quantification results for SPCL and PGA scaffolds at different time periods.

4. CONCLUSIONS

There is a great need for the development of clinically useful cartilage tissue engineering strategies. In this work, we have showed that SPCL scaffolds can support bovine articular chondrocytes adhesion, proliferation and differentiation, for up to 6 weeks of culturing. These scaffolds were compared with non-woven polyglycolic acid (PGA) scaffolds cultured using the same parameters. The PGA scaffolds presented a central area of cells depletion, which can be a result of acidic by-products release from their hydrolytic degradation, inefficient cells penetration or necrosis induced by high cellular densities. This can compromise the clinical application of these standard scaffolds. This situation was not observed in the SPCL scaffolds, which presented homogeneous cell colonization throughout the scaffold structure. The results obtained for toluidine blue staining and immunolocalisation of collagens type I and type II were very similar for both types of scaffold materials. Nevertheless, quantitatively, PGA scaffolds exhibited higher amounts of glycosaminoglycans, when compared to the SPCL scaffolds. In summary, the results obtained from this work, demonstrate that the SPCL fibre based scaffolds may constitute a valid

alternative and should be considered for further studies in the cartilage tissue engineering field, in addition to their already promising performance in the bone tissue engineering area.

REFERENCES

1. Salgado A.J., O. J. T., Pedro A.J., Reis R.L., Stem Cells in Bone and Cartilage Tissue Engineering, *Current Stem Cell Research & Therapy,* 1, 231-238, 2006.

2. Uematsu, K., Hattori, K., Ishimoto, Y., Yamauchi, J., Habata, T., Takakura, Y., Ohgushi, H., Fukuchi, T., and Sato, M., Cartilage regeneration using mesenchymal stem cells and a three-dimensional poly-lactic-glycolic acid (PLGA) scaffold, *Biomaterials* 26 (20), 4273, 2005.

3. Cancedda, R., Dozin, B., Giannoni, P., and Quarto, R., Tissue engineering and cell therapy of cartilage and bone, *Matrix Biol* 22 (1), 81-91, 2003.

4. Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T., Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold, *Biochem Biophys Res Commun* 322 (1), 50-5, 2004.

5. Temenoff, J. S. and Mikos, A. G., Review: tissue engineering for regeneration of articular cartilage, *Biomaterials* 21 (5), 431-40, 2000.

6. Grodzinsky, A. J., Electromechanical and physicochemical properties of connective tissue, *Crit Rev Biomed Eng* 9 (2), 133-99, 1983.

7. Zhu, W., Mow, V. C., Koob, T. J., and Eyre, D. R., Viscoelastic shear properties of articular cartilage and the effects of glycosidase treatments, *J Orthop Res* 11 (6), 771-81, 1993.

8. Yaylaoglu, M. B., Yildiz, C., Korkusuz, F., and Hasirci, V., A novel osteochondral implant, *Biomaterials* 20 (16), 1513-1520, 1999.

9. Lee, C. R., Breinan, H. A., Nehrer, S., and Spector, M., Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior *in vitro*, *Tissue Eng* 6 (5), 555-65, 2000.

10. Gugala, Z. and Gogolewski, S., *In vitro* growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold, *Journal of Biomedical Materials Research* 49 (2), 183-191, 2000.

11. Giannoni, P., Pagano, A., Maggi, E., Arbico, R., Randazzo, N., Grandizio, M., Cancedda, R., and Dozin, B., Autologous chondrocyte implantation (ACI) for aged patients: Development of the proper cell expansion conditions for possible therapeutic applications, *Osteoarthritis and Cartilage* 13 (7), 589-600, 2005.

12. Gugala, Z. and Gogolewski, S., *In vitro* growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold, *J Biomed Mater Res* 49 (2), 183-91, 2000.

13. Hutmacher, D. W., Scaffolds in tissue engineering bone and cartilage, *Biomaterials* 21 (24), 2529-2543, 2000.

[14.] Han, J.-A. and Lim, S.-T., Structural changes of corn starches by heating and stirring in DMSO measured by SEC-MALLS-RI system, *Carbohydrate Polymers* 55 (3), 265-272, 2004.

[15.] Funami, T., Kataoka, Y., Omoto, T., Goto, Y., Asai, I., and Nishinari, K., Food hydrocolloids control the gelatinization and retrogradation behavior of starch. 2b. Functions of guar gums with different molecular weights on the retrogradation behavior of corn starch, *Food Hydrocolloids* 19 (1), 25-36, 2005.

[16.] Marques, A. P., Reis, R. L., and Hunt, J. A., An *in vivo* study of the host response to starch-based polymers and composites subcutaneously implanted in rats, *Macromolecular Bioscience* 5 (8), 775-785, 2005.

[17.] Gomes, M. E., Godinho, J. S., Tchalamov, D., Cunha, A. M., and Reis, R. L., Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties, *Materials Science and Engineering: C* 20 (1-2), 19, 2002.

[18.] Salgado, A. J., Coutinho, O. P., and Reis, R. L., Novel Starch-Based Scaffolds for Bone Tissue Engineering: Cytotoxicity, Cell Culture, and Protein Expression, *Tissue Engineering* 10 (3-4), 465-474, 2004.

[19.] Marques, A. P. and Reis, R. L., Hydroxyapatite reinforcement of different starch-based polymers affects osteoblast-like cells adhesion/spreading and proliferation, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 25 (2), 215-229, 2005.

[20.] Li, W. J., Danielson, K. G., Alexander, P. G., and Tuan, R. S., Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(?-caprolactone) scaffolds, *Journal of Biomedical Materials Research - Part A* 67 (4), 1105-1114, 2003.

[21.] Li, W.-J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K. G., Hall, D. J., and Tuan, R. S., A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells, *Biomaterials* 26 (6), 599, 2005.

[22.] Gomes, M. E., Sikavitsas, V. I., Behravesh, E., Reis, R. L., and Mikos, A. G., Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds, *J Biomed Mater Res A* 67 (1), 87-95, 2003.

[23.] Salgado, A. J., Coutinho, O. P., and Reis, R. L., Novel starch-based scaffolds for bone tissue engineering: cytotoxicity, cell culture, and protein expression, *Tissue Eng* 10 (3-4), 465-74, 2004.

[24.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[25.] Kafienah, W., Sims, T.J., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[26.] Thambyah, A., Nather, A., and Goh, J., Mechanical properties of articular cartilage covered by the meniscus, *Osteoarthritis and Cartilage* In Press, Corrected Proof.

[27.] Gomes, M. E., Sikavitsas, V. I., Behravesh, E., Reis, R. L., and Mikos, A. G., Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds, *Journal of Biomedical Materials Research Part A* 67A (1), 87-95, 2003.

[28.] Barry, J. J. A., Gidda, H. S., Scotchford, C. A., and Howdle, S. M., Porous methacrylate scaffolds: supercritical fluid fabrication and *in vitro* chondrocyte responses, *Biomaterials* 25 (17), 3559-3568, 2004.

[29.] Ciolfi, V. J. D., Pilliar, R., McCulloch, C., Wang, S. X., Grynpas, M. D., and Kandel, R. A., Chondrocyte interactions with porous titanium alloy and calcium polyphosphate substrates, *Biomaterials* 24 (26), 4761-4770, 2003.

[30.] Cuevas, P., Burgos, J., and Baird, A., Basic fibroblast growth factor (FGF) promotes cartilage repair *in vivo*, *Biochemical and Biophysical Research Communications* 156 (2), 611-618, 1988.

[31.] Luyten, F. P., Hascall, V. C., Nissley, S. P., Morales, T. I., and Reddi, A. H., Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants, *Arch Biochem Biophys* 267 (2), 416-25, 1988.

[32.] Shum, A. W. T. and Mak, A. F. T., Morphological and biomechanical characterization of poly(glycolic acid) scaffolds after *in vitro* degradation, *Polymer Degradation and Stability* 81 (1), 141-149, 2003.

[33.] Athanasiou, K. A., Agrawal, C. M., Barber, F. A., and Burkhart, S. S., Orthopaedic applications for PLA-PGA biodegradable polymers, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 14 (7), 726-737, 1998.

[34.] Cancedda, R., Descalzi Cancedda, F., and Castagnola, P., Chondrocyte differentiation, *Int Rev Cytol* 159, 265-358, 1995.

[35.] Brodkin, K. R., Garcia, A. J., and Levenston, M. E., Chondrocyte phenotypes on different extracellular matrix monolayers, *Biomaterials* 25 (28), 5929-5938, 2004.

[36.] Darling, E. M. and Athanasiou, K. A., Biomechanical Strategies for Articular Cartilage Regeneration, *Annals of Biomedical Engineering* 31 (9), 1114-1124, 2003.

[37.] Dozin, B., Malpeli, M., Camardella, L., Cancedda, R., and Pietrangelo, A., Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects, *Matrix Biol* 21 (5), 449-59, 2002.

[38.] Tare, R. S., Howard, D., Pound, J. C., Roach, H. I., and Oreffo, R. O. C., Tissue engineering strategies for cartilage generation-Micromass and three dimensional cultures using human

chondrocytes and a continuous cell line, *Biochemical and Biophysical Research Communications* 333 (2), 609-621, 2005.

[39.] Kaps, C., Frauenschuh, S., Endres, M., Ringe, J., Haisch, A., Lauber, J., Buer, J., Krenn, V., Haupl, T., Burmester, G.-R., and Sittinger, M., Gene expression profiling of human articular cartilage grafts generated by tissue engineering, *Biomaterials* 27 (19), 3617-3630, 2006.

[40.] Loty, S., Foll, C., Forest, N., and Sautier, J.-M., Association of enhanced expression of gap junctions with *in vitro* chondrogenic differentiation of rat nasal septal cartilage-released cells following their dedifferentiation and redifferentiation, *Archives of Oral Biology* 45 (10), 843-856, 2000.

[41.] Li, J., Kim, K. S., Park, J. S., Elmer, W. A., Hutton, W. C., and Yoon, S. T., BMP-2 and CDMP-2: Stimulation of chondrocyte production of proteoglycan, *Journal of Orthopaedic Science* 8 (6), 829-835, 2003.

SECTION 3.

CHAPTER VI.

Gellan Gum: A New Biomaterial for Cartilage Tissue Engineering Applications

CHAPTER VI.

Gellan Gum: A New Biomaterial for Cartilage Tissue Engineering Applications*

ABSTRACT

Gellan gum is a polysaccharide manufactured by microbial fermentation of the *Sphingomonas paucimobilis* microorganism, being commonly used in the food and pharmaceutical industry. It can be dissolved in water, and when heated and mixed with mono or divalent cations, forms a gel upon lowering the temperature under mild conditions.

In this work, gellan gum hydrogels were analysed as cells supports in the context of cartilage regeneration. Gellan gum hydrogel discs were characterised in terms of mechanical and structural properties. Transmission electron microscopy (TEM) revealed a quite homogeneous chain arrangement within the hydrogels matrix, and dynamic mechanical analysis (DMA) allowed to characterize the hydrogels discs viscoelastic properties upon compression solicitation, being the compressive storage and loss modulus of approximately 40 kPa and 3 kPa, respectively, at a frequency of 1 Hz. Rheological measurements determined the sol-gel transition started to occur at approximately 36 ºC, exhibiting a gelation time of approximately 11 seconds. Evaluation of the gellan gum hydrogels biological performance was performed using a standard MTS cytotoxicity test, which showed that the leachables released are not deleterious to the cells and hence were non cytotoxic. Gellan gum hydrogels were afterwards used to encapsulate human nasal chondrocytes ($1x10^6$cells/ml) and culture them for total periods of 2 weeks. Cells viability was confirmed using confocal calcein AM staining. Histological observations revealed normal chondrocytes morphology and the obtained data supports the claim that this new biomaterial has the potential to serve as a cell support in the field of cartilage regeneration.

*** This chapter is based on the following publication:**

JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, RL Reis. Gellan Gum: A New Biomaterial for Cartilage Tissue Engineering Applications (2008) (*submitted*).

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1. INTRODUCTION

Tissue engineering has been proposed as a new method to address problems such as organ failure and tissue regeneration, being widely studied nowadays as a tool to tackle problems in a diverse range of tissues.[1-4] Such conditions pose serious health problems, being responsible for a decrease in people quality of life. Cartilage is one of the most studied tissues in this field giving the importance it has on mobility and locomotion. Due to its limited capacity for self repair, cartilage becomes an enormous constraint to normal everyday life once degenerated or traumatised. Structures that can provide support for specific cells to develop and generate a functional cartilaginous tissue are an important subject of study. Different types of natural and synthetic biomaterials have been processed using different techniques for this purpose. We are proposing in this work a new biomaterial - Gellan gum - to be used in the engineering of cartilaginous tissues, even though its application may not be restricted to this tissue only, as it will be shown by the different 3D structures that can be obtained. Recent work performed by Smith AM et al has also suggested the use of this biomaterial for tissue engineering applications.[5]

Gellan gum is a linear anionic polysaccharide composed of tetrasaccharide (1,3-β-D-glucose, 1,4-β–D-glucuronic acid, 1,4-β-D-glucose, 1,4-α-L-rhamnose) repeating units, containing one carboxyl side group, and was initially described by Moorhouse et al.[6,7] This material has a broad use in the food industry and biomedical fields, mostly due to its processing into transparent gels that are resistant to heat and acid stress. Two Gellan gum forms exist, acetylated and deacetylated, being the latter the most common and commercially available form. Both form thermoreversible gels, varying in their mechanical properties from soft and elastic for the acetylated form to hard and brittle for the fully deacetylated polysaccharide.[8,9] Gellan gum can form gels in the following way: at high temperatures, Gellan gum is in the coil form; upon temperature decrease, a thermally-reversible coil to double-helix transition occurs, which is a prerequisite for gel formation. Afterwards, a structure composed of anti-parallel double helices self assembled to form oriented bundles, called junction zones, is formed. Untwined regions of polysaccharide chains, in the form of extended helical chains, link the junction zones, leading to the formation of a three dimensional network, that creates the gel.[10] These structural changes occurring to gellan gum molecules have been shown by different techniques. During the cooling process, for example, rheological and differential scanning calorimetry (DSC) studies revealed a first step increase of loss modulus that corresponds to the coil-helix transition, and a second step increase of loss modulus due to sol-gel transition.[11] The gelation of gellan gum solutions is strongly influenced by the chemical nature and quantity of cations present in the solution. The

presence of cations is critical when a structurally stable gel is to be prepared.[10,12,13] In fact, at low Gellan gum concentrations, the helix formation and its partial aggregation may form an ordered structure, but this does not lead to gel formation because the number of helical aggregates does not give rise to a continuous network in the whole volume.[11] The main barrier are the carboxyl side groups that repulse each other by electrostatic interaction, therefore hindering the tight binding of helices and their cohesive aggregation.[9,14-16] The introduction of cations shields the electrostatic repulsion and thereby allows the tight binding and aggregation of helices.[11,17,18]

The gelation properties of Gellan gum are also influenced by the nature of the cations employed, in which divalent cations promote the gelation much more strongly than monovalent cations.[11,12] In monovalent cations, the gelation is mainly a result of the screening of the electrostatic repulsion between the ionized carboxylate groups on the Gellan gum chains. In the case of divalent cations, the gelation and aggregation of Gellan occurs via a chemical bonding between divalent cations and two carboxylate groups belonging to glucuronic acid molecules in the Gellan chains, in adittion to the screening effect.[19] It was also suggested that different types of mono or divalent cations also influenced the viscoelastic behaviour of Gellan gum solutions. K^+ was more remarkable than Na^+, and Ca^{2+} more than Mg^{2+}.[11] Gellan gum structures have excellent heat resistance properties since the formed junctions upon gelation can be only be unzipped on heating at 120ºC.[11] In the initial state, a junction zone in Gellan gum is estimated to be four double helices wide and five repeat units long, its length being increased to seven repeat units upon annealing.[10] In the solid state, the double helix structure adopted by Gellan gum has a similar arrangement to the double helix structure of iota carragenan.[20] Previous studies indicate that solutions of deacetylated gellan gum behave as a pseudoplastic liquids, as evidenced by creep testing, and have little thixotropy.[13] Gellan gum advantageous use in the context of biomedical applications includes its lack of toxicity, processing under mild conditions, the ability to used as an injectable system in a minimally invasive manner, and also the structural similarity it presents with native cartilage glycosaminoglycans by the presence of glucuronic acid residues in their repeating unit.[21,22] The presence of this carbohydrate residue, which contains carboxylic groups, may confer added functions to this material. Some intellectual property associated with the application of this material in the medical field has already been disclosed, as its use for ophthalmologic purposes.[23,24]

This work tested for the first time gellan gum as a new biomaterial to be used in cartilage regeneration approaches. As shown here, gellan gum hydrogels are quite versatile in terms of processing and its materials properties reveal good prospects for their use as a cell encapsulating agents. Biological evaluation of their cytotoxicity and *in vitro* culturing of human nasal

chondrocytes generated interesting results indicating that this new biomaterial may play a potential role in cartilage regeneration approaches.

2. MATERIALS AND METHODS

2.1. VERSATILITY OF GELLAN GUM: PROCESSING INTO DIFFERENT STRUCTURES (DISCS, MEMBRANES, FIBRES, PARTICLES, SCAFFOLDS)

Note: Unless otherwise stated the reagents were purchased from Sigma-Aldrich Co.

Gellan gum (G1910, Sigma, St. Louis, MO, USA) was processed in different ways giving rise to various structures, therefore evidencing the versatility of this natural biomaterial. The processing involved temperature-dependent and pH-dependent reactions. Regarding gellan gum discs and membranes production the following methodology was used. Gellan gum powder was mixed with distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90ºC, under which complete and homogeneous dispersion of the material was obtained. The solution was kept at this temperature during 20-30 minutes. Afterwards, $CaCl_2$ (Merck, DE) was added to obtain a final concentration of 0.03% (w/v) in the gellan gum solution and the temperature was progressively decreased to 50ºC. Gellan discs were produced by casting the solution into cylindrical moulds and allowing it to rest at room temperature for 2-5 minutes and form a solid gel. The discs were then cut using a borer for final discs dimensions of Ø 6±0.01 mm x 5.5±0.46 mm height. Gellan gum membranes were produced by casting the solution into Petri dishes and allowing it to stand at room temperature for 2-5 minutes and form a solid gel. The Petri dishes were kept in an oven at 37ºC for 90 minutes. Concerning the production of Gellan gum fibres and particles the methodology was as follows. Gellan gum powder was mixed with a NaOH 0.10 M solution and stirred at room temperature with a final concentration of 4% (w/v). Gellan gum fibres were produced by extruding the gellan gum solution into a L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.2 ml/min, using a 21G needle. The gellan gum fibres formed were then washed in distilled water, pressed into cylindrical moulds, and dried overnight at 37ºC. Gellan gum particles were produced by extruding the Gellan gum 4% (w/v) solution dropwise to an L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.8 ml/min, using a 21G needle. Gellan gum scaffolds were produced by immersing gellan gum 0.7% (w/v) (Ø 6±0.01 mm x 5.5±0.46 mm height) discs in liquid nitrogen for 1-2 minutes and quickly transferring them to a lyophilizator (Telstar Cryodos-80, Telstar,

Spain) where they were lyophilized during 2 days. Lyophilized gellan gum 0.7% discs were further analysed under micro-computed tomography (μ-CT) using a high-resolution μ-CT Skyscan 1072 scanner (Skyscan, Kontich, Belgium) using a resolution of 6.76 μm pixel size and integration time of 1.7 ms. The x-ray source was set at 70keV of energy and 142 μA of current. Approximately 500 projections were acquired over a rotation range of 180° and a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was a 500 serial of 1024x1024 bitmap images. Representative data sets of 150 slices were segmented into binary images (CT Analyser, v1.5.1.5, SkyScan) with a dynamic threshold of 70-255 (grey values) that was applied to build the 3D models. 3D virtual models (height 1mm x 3mm) of representative regions in the bulk of the hydrogels were created, visualized and registered using image processing software (CT Analyser, v1.5.1.5 and ANT 3D creator, v2.4, both from SkyScan).

2.2. TRANSMISSION ELECTRON MICROSCOPY

Gellan gum discs were prepared for transmission microscopy analysis in the following way. Briefly, sections of $1mm^3$ were fixed in formalin-glutaraldehyde-osmium tetroxide for 2 h at room temperature and then washed three times in PBS. Semithin sections (1μm) were cut from epon-embedded blocks and stained with toluidine blue. Ultrathin sections (600 Å) were cut in a ultratome (Reichert Ultranova Leica), mounted onto copper grids, stained with uranyl acetate (7min) and lead citrate (5 min) and observed on a Zeiss 902A (50 Kv) electron microscope.

2.3. DYNAMIC MECHANICAL ANALYSIS

Dynamic mechanical analysis (DMA) was conducted to characterize the mechanical behaviour of Gellan gum hydrogel discs. Gellan gum 0.7% (w/v) discs (Ø 6±0.01 mm x 5.5±0.46 mm height) discs were subjected to compression cycles of increasing frequencies ranging from 0.1-10 Hz with constant amplitude displacements of 0.1 mm using a Tritec 2000 DMA (Triton Technology, UK). Storage and loss modulus were measured and experiments were conducted at room temperature. The total number of discs per assay were n=3. The described values for the compression modulus were collected at a frequency of 1 Hz. Statistical analysis was performed using confidence intervals based on the experimental results, with a confidence level of 99%.

2.4. RHEOLOGICAL STUDIES

Cone-Plate rheometry was conducted for gellan gum hydrogels in order to assess their rheological behaviour dependence of temperature and time. For this purpose, gellan gum powder was mixed at room temperature with distilled water at a concentration of 0.7% (w/v) under constant stirring. The solution was heated to 90ºC and kept at this temperature for 30 minutes. Afterwards, $CaCl_2$ was added to the Gellan gum solution at concentration of 0.03% (w/v) and rheological measurements were performed using a controlled stress cone-plate rheometer (Reometer Reologica, StressTech, Sweden). For each measurement, a volume of 2 ml of the Gellan gum solution was placed in the bottom plate of the rheometer and held at a constant temperature of 70ºC. The polymer solution was allowed to rest for 1 minute before starting the experiments. Measurements were performed by cooling each sample from 70ºC to 25ºC (at a cooling rate of -6ºC/min) applying a constant shear stress of 0.1 Pa. Temperature, time, shear rate and viscosity were constantly measured. The total number of repeats was n=3 and confidence intervals were estimated, with a confidence level of 99%.

2.5. CYTOTOXICITY EVALUATION

To assess the possible cytotoxicity of the processed gellan gum hydrogels, MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium) test was used according to ISO/EN 10993 part 5 guidelines, which determines whether cells are metabolically active.[25] This cytotoxicity test is based on the bioreduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) (Cell Titer 96® Aq_{ueous} Solution Cell Proliferation Assay, Promega, USA), into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. Latex rubber was used as positive control for cell death, due to its high cytotoxicity to cells, and culture medium was used as a negative control. A rat lung fibroblasts cell line – L929, acquired from the European Collection of Cell Cultures (ECACC), was used for the studies. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany; Heat Inactivated) and 1% of antibiotic-antimycotic mixture.

The gellan gum hydrogel discs were incubated in culture medium for 24h at 37ºC with constant shaking, as well as latex. Cultured L929 cells were trypsinised using trypsin-EDTA (Gibco, Invitrogen Corporation) and plated at a density of $6.6x10^4$ cells/well into 96-well micrometer plates (200 µl/well). The plates were incubated for 24 hours at 37ºC in a humidified atmosphere of 5% CO_2 in air. Afterwards, the medium was replaced by the extracts previously obtained, using culture medium as a negative control. After 72 hours, the cell culture was incubated with MTS (using culturing medium without phenol red) for further 3 hours at 37ºC in a humidified atmosphere of 5% CO_2 in air. Culture medium with MTS was then transferred to new wells. The optical density (OD) which is directly proportional to the cellular activity, being a measure of mitochondrial acitivity, was read on a multiwell microplate reader (Synergy HT, Bio-TeK Instruments, US) at 490 nm. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3.

2.6. ISOLATION AND EXPANSION OF HUMAN NASAL CHONDROCYTES

Nasal cartilage was harvested from the nasal septum of adult patients (40-65 years) undergoing reconstructive surgery. This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and always sampled upon patient informed consent. The human nasal septum cartilage free from all surrounding tissue was placed in a Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2-3 mm. The pieces were washed in sterile PBS solution, immersed in 20 ml of trypsin-EDTA solution, and incubated for 30 min at 37ºC on a rotator. Trypsin was removed and the pieces washed with basic DMEM. Then, 20 ml of filter sterilised collagenase type II solution (2mg/ml) in basic medium was added, and the mixture incubated for approximately 12 hours at 37ºC on a rotator. The digested tissue and cell suspension solution was centrifuged at 200xg for 7 min and the supernatant removed. The cell pellet was washed with PBS and the cells centrifuged as before. The procedure was repeated and the cells were ressuspended in PBS and counted using a hemocytometer. They were again centrifuged, the supernatant removed, and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium, containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 µg/ml streptomycin, 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human nasal

chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.[26]

2.7. HUMAN NASAL CHONDROCYTES ENCAPSULATION IN GELLAN GUM AND AGAROSE HYDROGELS

Human nasal chondrocytes were expanded until an adequate cell number was obtained for cells encapsulation. Cells were encapsulated at passage 1 in gellan gum hydrogels and in agarose type VII (A6560; Sigma, St. Louis, MO) hydrogels, the latter being used as controls.

Regarding gellan gum the procedure was the following. Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. A sterile $CaCl_2$ solution was added to obtain a final concentration of 0.03% (w/v). The temperature was progressively decreased to 40ºC and stabilised at this stage always under constant stirring. Human nasal chondrocytes were detached by trypsinisation, mixed with expansion medium, and centrifuged at 200xg for 7 min. The supernatant was removed and the cells were ressuspended in warm sterile PBS solution, counted using and hemocytometer, and finally centrifuged at 200xg for 7 min. The supernatant was discarded and the cells pellet kept at the bottom of the falcon tube. The gellan gum 0.7% (w/v) with $CaCl_2$ 0.03% (w/v) solution was added to the cells pellet and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of 1 $x10^6$ cells/ml. Gellan discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 1-2 minutes to form a solid gel, and then discs of Ø 6±0.01 mm x 5.5±0.46 mm height were cut using a borer.

Regarding the agarose hydrogels, the procedure is detailed elsewhere.[27] Briefly, a sterile agarose type VII low Tm 4% (w/v) solution prepared in sterile PBS was heated to 70ºC for 30 seconds, until complete dissolution. The solution was added to a human nasal chondrocytes pellet prepared as described for the gellan gum encapsulation and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of $1x10^6$ cells/ml. Agarose discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 20 minutes to form a solid gel, and then discs were cut using a borer. Both gellan gum and agarose hydrogels with encapsulated cells were cultured for two weeks with expansion medium under

orbital rotation (50 rpm). Afterwards, expansion medium was replaced for six weeks by differentiation medium to promote the formation of a chondrogenic phenotype. This medium presents the same composition as the expansion medium except for the bFGF which is replaced with 1 µg/ml of insulin and 50 µg/ml of L-ascorbic acid. The cells-hydrogel systems were returned to the orbital shaker and the culture medium was replaced every 2-3 days. The experiments were repeated 3 times independently.

2.8. HUMAN NASAL CHONDROCYTES ENCAPSULATED IN GELLAN GUM AND AGAROSE HYDROGELS: CELL VIABILITY TESTS AND HISTOLOGICAL ANALYSIS

Human nasal chondrocytes morphology in the two hydrogels used, gellan gum and agarose, was observed at 2 weeks of culture under optical microscopy. One representative sample of each type of support was observed at different magnifications using an optical microscope (Axiovert 40 CFL, Zeiss).

Cells viability at 2 weeks of culturing was assessed using calcein AM staining. Calcein AM (C3099, Invitrogen Corp.) is a fluorescence-based method for assaying cell viability and cytotoxicity in which the reagent is retained in cells that have intact membrane. Briefly, a calcein AM solution of 1/1000 was prepared in culture medium. One disc of each type of hydrogel with encapsulated human nasal chondrocytes was collected from the culturing plates and incubated in the calcein AM solution for 15-30 min at 37ºC and afterwards washed in sterile PBS. The samples were observed under fluorescent microscopy (Zeiss HAL 100/HBO 100; Axiocam MRc5 (Zeiss).

Concerning the histological analysis, hematoxylin-eosin staining was performed on 8 µm thickness sections of gellan gum and agarose discs collected at 2 weeks of culture. The discs were fixated by immersion for 30-40 minutes in glutaraldehyde 2.5% (v/v) at 4ºC, and washed in PBS. Histological processing was performed using Tecnhovit 7100® (Heraeus Kulzer GmbH, DE) and the technical details and procedure can be found in the commercial package. Sections were cut using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH).

3. RESULTS AND DISCUSSION

3.1. VERSATILITY OF GELLAN GUM: PROCESSING INTO DIFFERENT STRUCTURES (DISCS, MEMBRANES, FIBRES, PARTICLES, SCAFFOLDS)

Gellan gum was processed into different shapes as shown in Figure 1. By using simple processing methodologies, involving temperature-dependent gelation (discs, membranes, scaffolds) and pH-dependent gelation (fibres and particles), all those structures were produced. This shows the versatility of this material to obtain different geometrical forms that can be used in a broad range of tissue engineering and drug delivery applications. Gellan gum hydrogels can be used to encapsulate cells and serve as supports for their development. Gellan gum can also be processed into fibres and generate a 3D structure onto which cells can be seeded and stimulated to proliferate, an approach that may also be explored by using membranes. A gellan gum solution, in which a specific drug is dispersed, can be processed into particles such as those shown in Figure 1.D and employed as a carrier for drug delivery applications. In fact, gellan gum has been used previously as an ocular drug delivery system.[28-30] Different parameters such as temperature, pH, polymer concentration, and ions nature can be adjusted to possibly improve the biological performance or confer certain functionality. The control of the sol-gel transition at physiological temperature and pH[31-33] renders this material the possibility to be used as an injectable system, which is a highly recommended approach in several situations.[34] The most relevant factor in the hydrogel forming ability of gellan gum is the presence of D-glucuronic acid molecules in the tetrasaccharide repeating unit of the polysaccharide. These monosaccharides possess carboxylic groups in their structure that form internal hydrogen bonds and stabilise the double helices. Nevertheless, carboxyl side groups that repulse each other by electrostatic interaction, hinder the tight binding of helices and their cohesive aggregation, affecting the formation of stable gels. The mono or divalent ions present in the solution play a key role in this matter. Their presence diminishes the repulsive energy between the carboxylic groups allowing the hydrogels to be formed. The variation in pH also affects the solubility of the material being this the main factor in the processing of gellan gum fibres and particles (Fig. 1.C and 1.D). At a basic pH, such as the NaOH solution used in the experiments, the carboxylic groups present in each D-glucuronic acid residue should be in the anionic form, COO^-, and therefore soluble in solution. Once the pH is lowered, as upon extrusion into an L-ascorbic acid solution, the carboxylic groups become protonated, COOH, and the material turns insoluble.

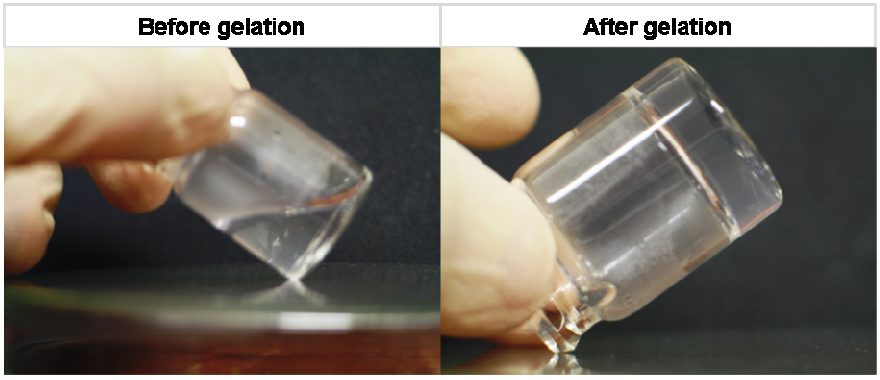

Figure VI.1. Sol-gel transition occurring in a Gellan gum solution containing $CaCl_2$.

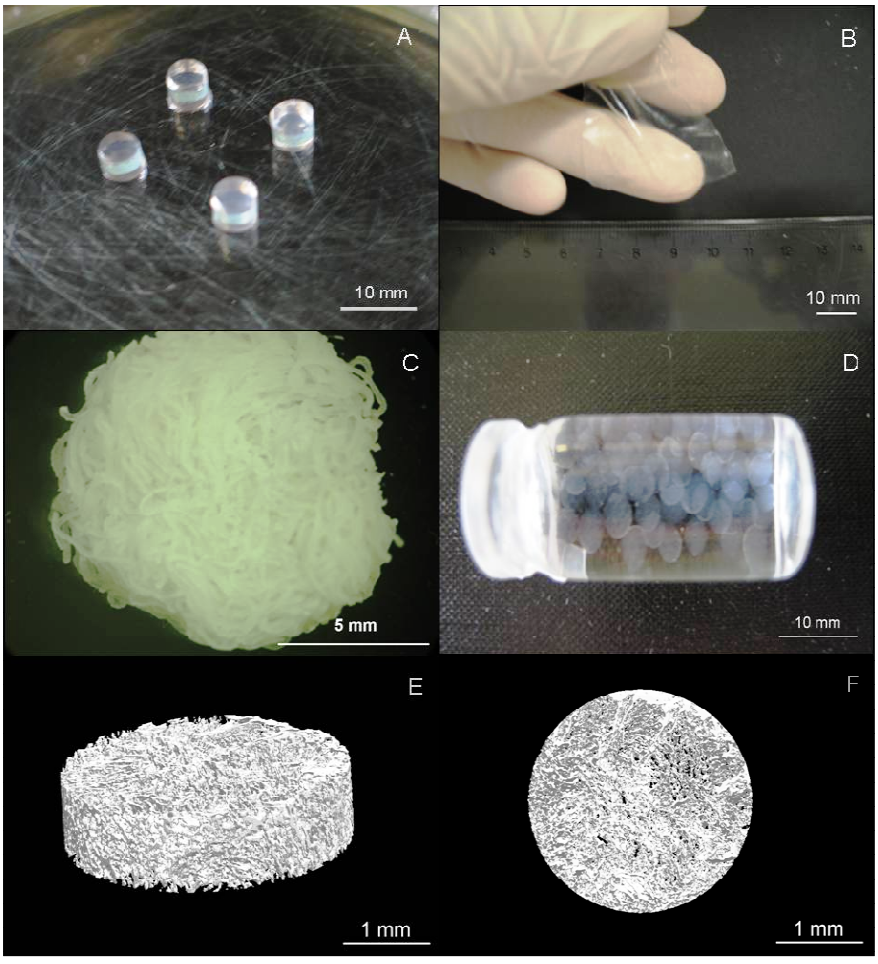

Figure VI.2. The versatility of Gellan gum structures that can be formed using simple polymer processing technologies: (A) discs; (B) membranes; (C) fibres; (D) particles; (E) and (F) 3D lyophilised scaffolds.

3.2. TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) was performed to have an insight on the ultrastructural morphology of the gellan gum hydrogels (Figure 2). As described before for the gel state, gellan gum hydrogels constitute a matrix where double helices that originated from the coil form rearrangement in solution are widely present and distributed in a rather homogeneous fashion.[35] These give rise to junction zones by linking to a neighbour double helical molecule. The overall stability of the hydrogel network derives from the loose ends within the double helical molecules. These, together with the cationic anti repulsive effect allow obtaining a stable hydrogel when the temperature is decreased below the setting point. Previous work has already used TEM as a tool to characterize the ultrastructural properties of gellan-based hydrogels.[36] The authors showed that gellan gum forms strong gels at low ionic concentrations, being these highly homogeneous and constituted by a dense fibrous network structure. The work presented here confirmed this, being observed that gellan gum hydrogels provide a uniform matrix at a nanoscale throughout which cells could be encapsulated in a rather homogeneous way.

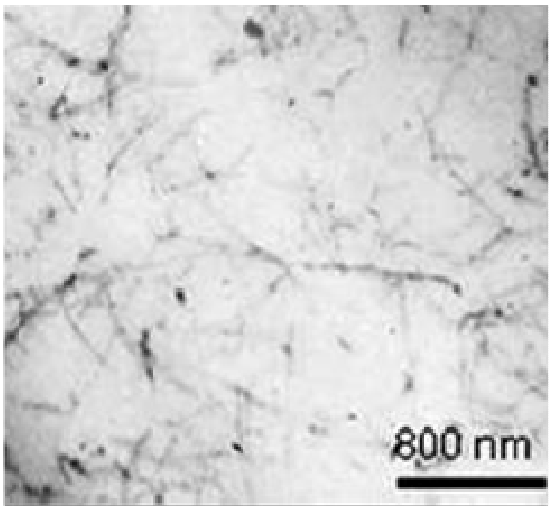

Figure VI.3. Transmission electron microscopy micrograph of a gellan gum hydrogel showing a dense and homogeneous network structure at the ultrastructural level.

3.3. DYNAMIC MECHANICAL ANALYSIS

Living tissues exhibit clear viscolelastic properties and therefore it is important to characterize the solid-state rheological features of materials that are meant to be in contact with them. Dynamic mechanical analysis (DMA) has been used in our group to assess the viscoelastic properties of biomaterials, including natural-based hydrogels or highly hydrated systems.[37-40] In this work,

gellan gum hydrogels were analysed in the wet state throughout a physiological relevant frequency range. Both the storage (elastic) and loss (viscous) components of the complex modulus are shown in Figure 4. The storage modulus (E') is about one order of magnitude higher than the loss modulus (E'') indicating a clear elastic nature of the gel. However, it possesses some damping capability that may be useful to dissipate some cyclic mechanical energy that is imposed in an implantation scenario. Although some increase in E' is observed for increasing frequencies, the elastic properties of the biomaterial are quite stable, as compared to the viscous component. In fact, a clear increase in E'' is observed between 0.4 and 10 Hz, which suggests that the material exhibits higher dissipation capability for high frequencies. At a frequency of 1 Hz, the compression modulus of the gels was estimated to be of 38.3±6.3 kPa [38.2, 38.4 $_{t(0.01,2)}$] at room temperature. Even though this value is not optimal in terms of mimicking human articular cartilage mechanical properties[41], it is higher or within the range of values found for hydrogels used in similar cartilage regenerative approaches.[42,43] The gellan gum support is conceived in this initial work to serve as a cell support due to its features, even though it may be optimized for being applied as an injectable system.[31-33] Cells encapsulation and extracellular matrix deposition may result in progressive increase of the mechanical properties of the 3D structures, as shown before for other systems.[42]

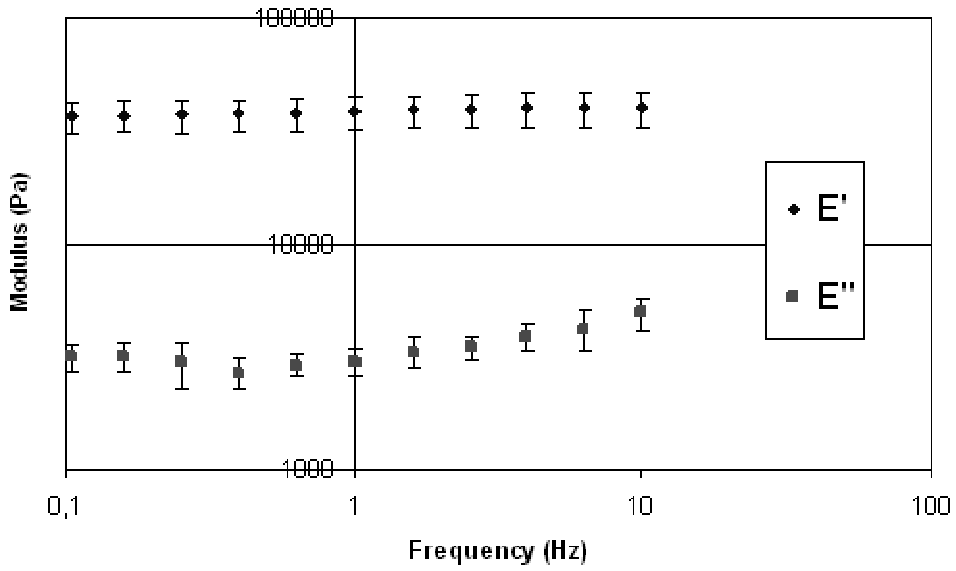

Figure VI.4. Dynamic mechanical analysis of gellan gum hydrogels showing the storage (E') and loss (E'') modulus upon compression solicitation using different frequencies.

3.4. RHEOLOGICAL STUDIES

Rheological measurements were performed to determine the temperature range at which the sol-gel transition occurred and the time-scale for gelling. Regarding gelation temperature, it is possible to state from the rheological measurements that it happens around 37ºC (36.6±0.05ºC) [36.586, 36.588 $t_{(0.01,2)}$] (Figure 5).

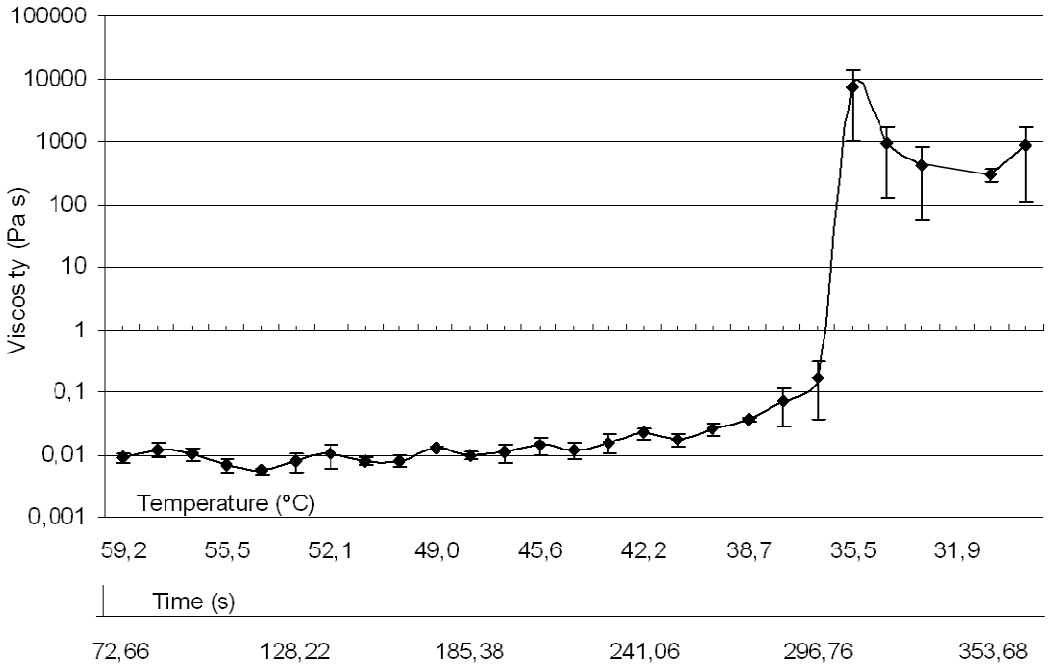

Figure VI.5. Rheological measurements of gellan gum solutions. The upper x axis shows the relation between temperature and viscosity, while the bottom x axis shows the relation between time-length and viscosity.

Concerning the time-scale for gelling, it is possible to observe from the graph on Figure 5 that it is of approximately 11 seconds (11.27±0.40 sec) [11.258, 11.275 $_{t(0.01,2)}$]. The results obtained for both temperature and time of gelation provide important information concerning subsequent experiments for cells encapsulation. The temperature at which the sol-gel transition occurs, and the overall residence time, is similar to other hydrogels used for the same purpose.[27,44] Gellan gum hydrogels allowed for a homogeneous cell suspension to be prepared at a temperature above the setting temperature of the gels. At such temperatures, the viscosity of the solution presents values near to zero, which enable it to be mixed with the cells, ressuspended to generate a uniform cells distribution, and then lower the temperature to allow gel formation and cells entrapment within the newly formed matrix. The quick gelling time may be useful in the use of Gellan gum as an injectable system that could deliver cells through a minimally invasive procedure, although these kinetics can be modified.

3.5. CYTOTOXICITY EVALUATION

The MTS cytotoxicity test results showed that the gellan gum hydrogels did not cause any deleterious alteration to the metabolic activity of L929 cells and thus, were considered as non cytotoxic (Figure 6). The results were comparable to those obtained with tissue culture polystyrene (TCPS), which was used as the negative control for cell death (differences are not statistically significant). The toxic effect of the positive control for cell death (latex) was clear, given the severe decrease on cell viability shown on the graph (Figure 6).

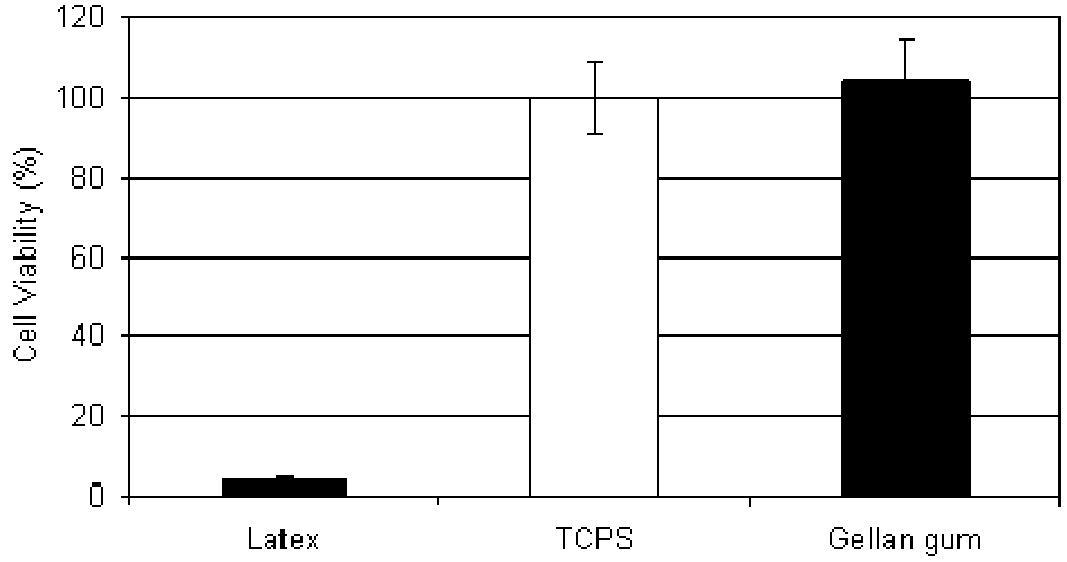

Figure VI.6. MTS cytotoxicity test performed to evaluate the possible cytotoxic effects of the leachables released by the gellan gum hydrogels. Results show the gels are non-cytotoxic.

3.6. HUMAN NASAL CHONDROCYTES ENCAPSULATED IN GELLAN GUM AND AGAROSE HYDROGELS: CELL VIABILITY TESTS AND HISTOLOGICAL ANALYSIS

Figures 7A and 7B show the human nasal chondrocytes efficiently encapsulated in the gels. This result is extremely important if the aim of the gellan gum supports is their use as cells encapsulating agents to be employed in cartilage regeneration approaches. In fact, it is known that anchorage independent cells like chondrocytes exhibit good cell viability within hydrophilic scaffolds like hydrogels, and studies using human nasal chondrocytes revealed that this hydrophilicity facilitated the re-differentiation of de-differentiated chondrocytes.[34] This evidence opens interesting prospects for the performance of these new supports in cartilage regeneration

along with the rather homogeneous distribution of the chondrocytes throughout the gellan gum hydrogels matrix (Figure 7), which showed a round-shaped morphology typically present in the native human cartilaginous tissue. An interesting result was observed in gellan gum but not in agarose. In the first the formation of chondrocytes clusters was frequently observed near 2 weeks of culture (Figure 7A), a feature that was not noticed on the early periods of culture. Such structures may be indicative of cell proliferation in these clusters which may give a positive contribution towards the production of a hyaline-like cartilage matrix[45-47] The cells may use the gellan gum as a source of carbohydrates due to its polysaccharidic nature, a fact that may be even more interesting to study in an in vivo scenario. The scenario of cells using the matrix as a source of energy is possible although such hypothesis demand proper validation testing. Also, the formation of the chondrocytes clusters has been previously described in the literature as osteoarthritis related events.[47,48] This seems not to be the case in these experiments since any hypertrophic cells, pre-osteoarthritic cells were observed.

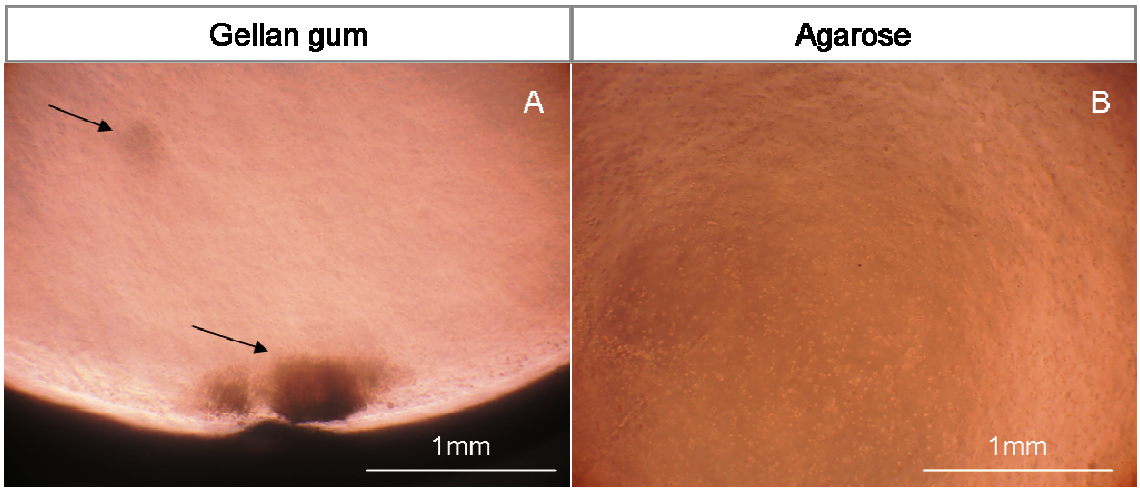

Figure VI.7. Optical microscopy images of human nasal chondrocytes encapsulated in gellan gum (A) and agarose (B) at 2 weeks of culture. The formation of human chondrocytes clusters was observed in gellan gum hydrogels (A, arrows).

Calcein AM fluorescence-based method was conducted to confirm chondrocytes viability. Results from samples collected after 2 weeks of culture are presented on Figure 8 showing the cells were viable in both hydrogels. These are also indicative of the adequacy of gellan gum for cartilage regeneration, since no apparent difference is noticeable when compared to agarose hydrogels.

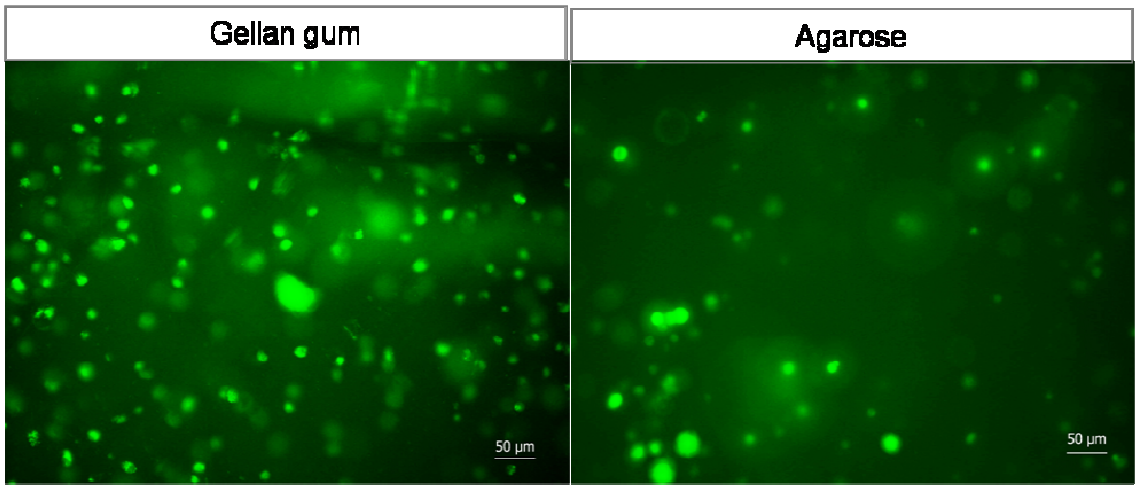

Figure VI.8. Calcein AM viability test of human nasal chondrocytes encapsulated in gellan gum (A) and agarose (B) hydrogels at 2 weeks of culture.

Concerning the histological analysis, it is clear from the images that cells distribution within the two supports are similar, presenting uniform distribution and active states of division (arrows). This indicates that gellan gum allowed adequate chondrocytes encapsulation while its network matrix permits cells to encompass active division. The data collected so far with gellan gum hydrogels showed that they possess suitable materials properties to be used as supports for chondrocytes development, such as the gelling at physiological conditions and their tested non cytotoxicity. Furthermore, they were able to efficiently encapsulate human nasal chondrocytes with a homogeneous distribution and maintain their viability for at least 2 weeks of culture. The overall data analysis suggests that this new biomaterial can have a high potential application in cartilage regeneration approaches and work is ongoing to further corroborate this hypothesis. Another aspect to look into is their potential use for other types of tissues or strategies, given its versatility in terms of processing and materials properties.

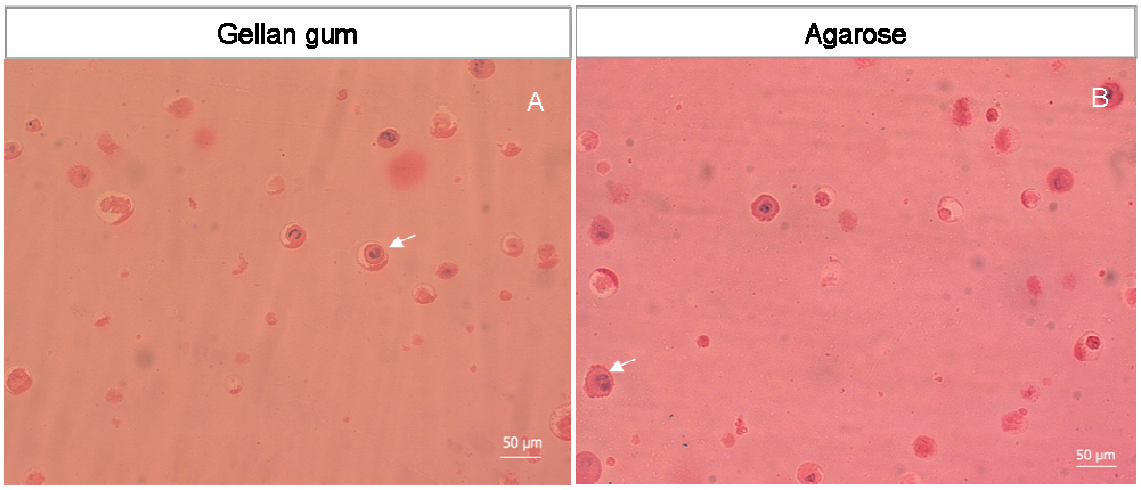

Figure VI.9. Hematoxylin-eosin staining of histological sections of gellan gum (A) and agarose (B) hydrogels at 2 weeks of culture. Human nasal chondrocytes present a typical round-shaped morphology and active cell division can be observed in both supports (arrows).

4. CONCLUSIONS

In the present work, gellan gum has been presented as a new biomaterial for cartilage tissue engineering approaches. Gellan gum was shown to be very versatile in terms of processing, which can be controlled by both temperature and pH, forming structures with different shapes using simple polymer processing technologies. Discs, membranes, fibres, particles, and scaffolds, were produced demonstrating the range of possible applications for this biomaterial. These may range from cell encapsulation technologies to drug delivery strategies, for example. Gellan gum has been used previously as an ocular drug delivery system[28-30], but to our knowledge this is the first time that it is proposed and tested for cartilage tissue engineering applications.

An extensive characterization of Gellan gum discs to be used as cell supports indicated that they are suitable for fulfilling such functions, since a solution combining non harsh reagents (gellan gum, calcium chloride and water) can be prepared, mixed uniformly with human nasal chondrocytes, and gelled near the body temperature in few seconds, enabling a high cell entrapment yield and homogeneous distribution. Gellan gum hydrogels presented viscoelastic properties within the range of other hydrogels used for cells encapsulation[42,43] and were shown to be non-cytotoxic. Calcein AM staining showed cells were viable during the time of the experiments and hematoxylin-eosin revealed that active cells division was occurring. Moreover, chondrocytes clusters were detected that may increase the potential of gellan gum hydrogels in

the generation of a hyaline-like cartilaginous matrix. Another advantage of this new biomaterial is its chemical composition that may be used to confer improved functionalities to it and thereby enhance its biological potential.

REFERENCES

1. Salgado, A. J., Gomes, M. E., Chou, A., Coutinho, O. P., Reis, R. L., and Hutmacher, D. W., Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 20 (1-2), 27-33, 2002.

2. Chen, G., Sato, T., Ohgushi, H., Ushida, T., Tateishi, T., and Tanaka, J., Culturing of skin fibroblasts in a thin PLGA-collagen hybrid mesh, *Biomaterials* 26 (15), 2559-66, 2005.

3. Holmes, T. C., de Lacalle, S., Su, X., Liu, G., Rich, A., and Zhang, S., Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds, *Proc Natl Acad Sci U S A* 97 (12), 6728-33, 2000.

4. Langer, R. and Vacanti, J. P., Tissue engineering, *Science* 260 (5110), 920-6, 1993.

5. Smith, A. M., Shelton, R. M., Perrie, Y., and Harris, J. J., An initial evaluation of gellan gum as a material for tissue engineering applications, *Journal of Biomaterials Applications* 22 (3), 241-254, 2007.

6. Jansson, P.-E., Lindberg, B., and Sandford, P. A., Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea, *Carbohydrate Research* 124 (1), 135-139, 1983.

7. Moorhouse R., C. G. T., Sandford P.A., Baird J.K. and Kang K.S., *PS-60: a new gel-forming polysaccharide* D.A. Brandt, Washington DC, 1981.

8. Kang, K. S., Colegrove T. & Veeder, G.T., USA, 1982.

9. Grasdalen, H. and Smidsrod, O., Gelation of gellan gum, *Carbohydrate Polymers* 7 (5), 371-393, 1987.

10. Quinn, F. X., Hatakeyama, T., Yoshida, H., Takahashi, M., and Hatakeyama, H., The conformational properties of gellan gum hydrogels, *Polymer Gels and Networks* 1 (2), 93-114, 1993.

11. Miyoshi, E., Takaya, T., and Nishinari, K., Rheological and thermal studies of gel-sol transition in gellan gum aqueous solutions, *Carbohydrate Polymers* 30 (2-3), 109-119, 1996.

12. Ogawa, E., Takahashi, R., Yajima, H., and Nishinari, K., Effects of molar mass on the coil to helix transition of sodium-type gellan gums in aqueous solutions, *Food Hydrocolloids* 20 (2-3), 378-385, 2006.

13. Deasy, P. B. and Quigley, K. J., Rheological evaluation of deacetylated gellan gum (Gelrite) for pharmaceutical use, *International Journal of Pharmaceutics* 73 (2), 117-123, 1991.

[14.] Crescenzi, V., Dentini, M., Coviello, T., and Rizzo, R., Comparative analysis of the behavior of gellan gum (S-60) and welan gum (S-130) in dilute aqueous solution, *Carbohydrate Research* 149 (2), 425-432, 1986.

[15.] Dentini, M., Coviello, T., Burchard, W., and Crescenzi, V., Solution properties of exocellular microbial polysaccharides. 3. Light scattering from Gellan and from the exocellular polysaccharide of Rhizobium trifolii (strain TA-1) in the ordered state, *Macromolecules* 21 (11), 3312-3320, 1988.

[16.] Milas, M., Shi, X., and Rinaudo, M., On the physicochemical properties of gellan gum, *Biopolymers* 30 (3-4), 451-464, 1990.

[17.] Morris, E. R., Rees, D. A., and Robinson, G., Cation-specific aggregation of carrageenan helices: Domain model of polymer gel structure, *Journal of Molecular Biology* 138 (2), 349-362, 1980.

[18.] Nakajima, K., Ikehara, T., and Nishi, T., Observation of gellan gum by scanning tunneling microscopy, *Carbohydrate Polymers* 30 (2-3), 77-81, 1996.

[19.] Singh, B. N. and Kim, K. H., Effects of divalent cations on drug encapsulation efficiency of deacylated gellan gum, Informa Healthcare, 2005, pp. 761 - 771.

[20.] Arnott, S., Scott, W. E., Rees, D. A., and McNab, C. G. A., i-Carrageenan: Molecular structure and packing of polysaccharide double helices in oriented fibres of divalent cation salts, *Journal of Molecular Biology* 90 (2), 253-256, 1974.

[21.] Ruoslahti, E., Proteoglycans in cell regulation, 1989, pp. 13369-13372.

[22.] Jen, A. C., Wake, M. C., and Mikos, A. G., Review: Hydrogels for cell immobilization, *Biotechnology and Bioengineering* 50 (4), 357-364, 1996.

[23.] Claude Mazuel, M.-C. F., 4861760, 1986, Ophthalmological composition of the type which undergoes liquid-gel phase transition.

[24.] Tacey X. Viegas, L. E. R., Raymond L. Henry, 5587175, 1993, Medical uses of in situ formed gels

[25.] ISO10993, d., Biological compatibility of medical devices. 5. Test for citotoxicity: *In vitro* methods, December 1992.

[26.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[27.] Mauck, R. L., Yuan, X., and Tuan, R. S., Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture, *Osteoarthritis and Cartilage* 14 (2), 179-189, 2006.

[28.] Mundorf, T. K., Ogawa, T., Naka, H., Novack, G. D., and Stephens Crockett, R., A 12-month, multicenter, randomized, double-masked, parallel-group comparison of timolol-LA once daily and timolol maleate ophthalmic solution twice daily in the treatment of adults with glaucoma or ocular hypertension, *Clinical Therapeutics* 26 (4), 541-551, 2004.

[29.] Shedden, A., Laurence, J., and Tipping, R., Efficacy and tolerability of timolol maleate ophthalmic gel-forming solution versus timolol ophthalmic solution in adults with open-angle glaucoma or ocular hypertension: a six-month, double-masked, multicenter study, *Clinical Therapeutics* 23 (3), 440-450, 2001.

[30.] Rozier, A., Mazuel, C., Grove, J., and Plazonnet, B., Gelrite(R): A novel, ion-activated, in-situ gelling polymer for ophthalmic vehicles. Effect on bioavailability of timolol, *International Journal of Pharmaceutics* 57 (2), 163-168, 1989.

[31.] Kang, K. S., Veeder, G. T., Mirrasoul, P. J., Kaneko, T., and Cottrell, I. W., Agar-Like Polysaccharide Produced by a Pseudomonas Species - Production and Basic Properties, *Applied and Environmental Microbiology* 43 (5), 1086-1091, 1982.

[32.] Ogawa E, M. H., Iwahashi M, Sagara Y, Shioya T, and T, K., Effects of metal ions on the conformational change
of gellan gumin aqueous solutions., *Trans Mater Res Soc*
Jpn 26, 613 -6, 2001.

[33.] Ogawa E, F. T., Matsuzawa H, Iwahashi M, Effects of sugars on the conformational change of gellan gum in aqueous solutions., *Trans Mater Res Soc Jpn* 26, 617 - 20, 2001.

[34.] Varghese, S. and Elisseeff, J. H., Hydrogels for musculoskeletal tissue engineering, *Polymers for Regenerative Medicine*, 95-144, 2006.

[35.] Nickerson, M. T., Paulson, A. T., and Speers, R. A., Rheological properties of gellan solutions: effect of calcium ions and temperature on pre-gel formation, *Food Hydrocolloids* 17 (5), 577-583, 2003.

[36.] Amici, E., Clark, A. H., Normand, V., and Johnson, N. B., Interpenetrating network formation in agarose-sodium gellan gel composites, *Carbohydrate Polymers* 46 (4), 383-391, 2001.

[37.] Elvira, C., Mano, J. F., San Roman, J., and Reis, R. L., Starch-based biodegradable hydrogels with potential biomedical applications as drug delivery systems, *Biomaterials* 23 (9), 1955-1966, 2002.

[38.] Boesel, L. F., Mano, J. F., and Reis, R. L., Optimization of the formulation and mechanical properties of starch based partially degradable bone cements, *Journal of Materials Science-Materials in Medicine* 15 (1), 73-83, 2004.

[39.] Tuzlakoglu, K., Alves, C. M., Mano, J. F., and Reis, R. L., Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications, *Macromolecular Bioscience* 4 (8), 811-819, 2004.

[40.] Silva, R. M., Silva, G. A., Coutinho, O. P., Mano, J. F., and Reis, R. L., Preparation and characterisation in simulated body conditions of glutaraldehyde crosslinked chitosan membranes, *Journal of Materials Science-Materials in Medicine* 15 (10), 1105-1112, 2004.

[41.] Froimson, M. I., Ratcliffe, A., Gardner, T. R., and Mow, V. C., Differences in patellofemoral joint cartilage material properties and their significance to the etiology of cartilage surface fibrillation, *Osteoarthritis and Cartilage* 5 (6), 377-386, 1997.

[42.] Kisiday, J., Jin, M., Kurz, B., Hung, H., Semino, C., Zhang, S., and Grodzinsky, A. J., Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair, *PNAS* 99 (15), 9996-10001, 2002.

[43.] Nettles, D. L., Vail, T. P., Morgan, M. T., Grinstaff, M. W., and Setton, L. A., Photocrosslinkable hyaluronan as a scaffold for articular cartilage repair, *Ann Biomed Eng* 32 (3), 391-7, 2004.

[44.] Hu, J. C. and Athanasiou, K. A., Low-density cultures of bovine chondrocytes: effects of scaffold material and culture system, *Biomaterials* 26 (14), 2001-2012, 2005.

[45.] Schulze, T., Schulze-Tanzil, G., de, S., Souza, P. d., Villegas, C., Castrejon, H. V., John, John, T., Merker, Merker, H. J., Scheid, Scheid, A., Shakibaei, and Shakibaei, M., Redifferentiation of dedifferentiated human chondrocytes in high-density cultures, *Cell and Tissue Research* 308 (3), 371-379, 2002.

[46.] Kuettner, K. E., Pauli, B. U., Gall, G., Memoli, V. A., and Schenk, R. K., Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology, 1982, pp. 743-750.

[47.] Quintavalla, J., Uziel-Fusi, S., Yin, J., Boehnlein, E., Pastor, G., Blancuzzi, V., Singh, H., Kraus, K., Byrne, E., and Pellas, T., Fluorescently labeled mesenchymal stem cells (MSCs) maintain multilineage potential and can be detected following implantation into articular cartilage defects, *Biomaterials* 23 (1), 109, 2002.

[48.] Mankin, H. J., Dorfman, H., Lippiello, L., and Zarins, A., Biochemical and Metabolic Abnormalities in Articular Cartilage from Osteo-Arthritic Human Hips: II. Correlation of morphology with biochemical and metabolic data, *J Bone Joint Surg Am* 53 (3), 523-537, 1971.

SECTION 3.

CHAPTER VII.

Gellan Gum Injectable Hydrogels for Cartilage Tissue Engineering Applications: *In vitro* Studies and Preliminary *In vivo* Evaluation

CHAPTER VII.

Gellan Gum Injectable Hydrogels for Cartilage Tissue Engineering Applications: *In vitro* Studies and Preliminary *In vivo* Evaluation*

ABSTRACT

Gellan gum is a polysaccharide manufactured by microbial fermentation of the *Sphingomonas paucimobilis* microorganism, being commonly used in the food and pharmaceutical industry. We have previously proposed it for tissue engineering applications due to its intrinsic features that include its ability to form a stable gel when heated and mixed with mono or divalent cations. Gelation can be tailored to occur at different temperatures and kinetics, enabling the use of gellan gum as an injectable system in minimally invasive surgical procedures. Moreover, it has been shown to be quite versatile in terms of processing and is generally quite biocompatible.

In this work, gellan gum hydrogels were tested for their ability to be used as injectable systems for delivering and maintaining chondrocytes by *in situ* gelation, as well as for supporting cell viability and production of extracellular matrix. Rheological measurements were performed to determine the temperature and time of gelation, and furthermore to evaluate the suitability of these systems for cell delivery *in situ* and their potential for being used in injectable applications. The sol-gel transition occurred near the normal body temperature at 39ºC, upon temperature decrease, and the time length for gelation was determined to occur in approximately 20 seconds. Discs of gellan gum 1% (w/v) were also characterised by dynamic mechanical analysis in order to assess their mechanical properties, showing a storage compression modulus of around 80 kPa at a frequency of 1 Hz. Human articular chondrocytes were encapsulated in the gels, cultured *in vitro* for total periods of 56 days, and analysed regarding cells viability and extracellular matrix (ECM) production. Calcein AM staining showed that cells kept viable after 14 days of culture and the histological analysis (hematoxylin-eosin, alcian blue, and safranin-O) and real-time quantitative PCR for Sox9, collagen I, collagen II, and aggrecan revealed that typical cartilage ECM was synthesised and deposited. In a final stage of the present study, the *in vivo* performance of the novel gellan gum hydrogels, in terms of induced inflammatory reaction and integration into the host tissue, was evaluated based on subcutaneous implantation in Balb/c mice up to 21 days. Histological analysis showed only a thin and residual fibrotic capsule at the end of the experiments. Additionally, the dynamic mechanical analysis showed that the gels were

stable throughout the time course of the experiment while evidencing a tendency for decreasing mechanical properties. This result is consistent with weight measurements that were also performed and with results from histology that showed gellan gum resorption by phagocytic cells. Altogether, the results show the adequacy of gellan gum hydrogels for non-invasive injectable applications and *in situ* cell delivery for cartilage regeneration, supporting human articular chondrocytes viability and ECM production in long term cultures *in vitro*. The *in vivo* evaluation reinforced these assumptions and corroborated the hypothesis that gellan gum can be used as a well adequate support for cell delivery and extensive culture towards the formation of a functional tissue engineered construct for cartilage tissue engineering applications.

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1. INTRODUCTION

Cartilage is a tissue with relatively low or no turnover, both at cellular and molecular level, which leads to difficulties in its repair and regeneration once traumatised. The extracellular matrix that is responsible for the functionality of the tissue has to be maintained by scarcely distributed chondrocytes, demanding a great anabolic capacity of these cells. Various therapeutic approaches that have no active biologics involved such as arthroscopy, debridement, laser abrasion, drilling, and microfracture.(1-3) They involve mostly mechanical techniques that remove the affected tissue and seek to stimulate the formation of healthy tissue. Since the outcomes of these interventions are frequently not satisfactory, namely in terms of functional tissue recovery, alternative solutions for these problems are being explored.(1, 4, 5) Brittberg et al.(6) performed autologous chondrocyte transplantation in 1994 by injecting expanded chondrocytes into cartilage defects subsequently covered with a sutured periosteal flap. Although the results were successful in some cases, the percentage of failure led the researchers to pursue other lines of study, including the use of biomaterials and cells combined with bioactive agents in tissue engineering strategies. Several biomaterials, from either natural or synthetic origin, or combinations of both types, have been proposed as supports for cell development and tissue formation in cartilage tissue engineering.(7-10) These include alginate, hyaluronic acid, collagen, fibrin, chitosan, polyglycolic acid, or polyethylene oxide.(11-14) Not only the type of material is important for the overall performance of the constructs but also its processing methodologies and the application routes employed. Hydrogels, and especially *in situ* injectable systems, have been gaining a wider interest for cartilage regeneration applications. They can be used in a minimally invasive manner by injection into the defect area, normally involve non harsh methods and reagents, possess the ability to adopt the shape of the defect thereby facilitating integration, and may be optimised for sol-gel transition to occur near body temperature while encapsulating cells and/or bioactive agents of interest.(15)

Gellan gum is a new biomaterial that we have recently proposed(16, 17) for cartilage tissue engineering applications; in addition to the above mentioned advantages of other injectable systems, it possess other features that may place it as a potential candidate for the clinical regeneration of cartilaginous tissues. Gellan gum is a linear anionic polysaccharide composed of repeating units of glucose, glucuronic acid, and rhamnose.(18, 19) It exists in the acetylated form, which is the initial raw material, and the deacetylated form, which is the most commonly used.(20, 21) Both form thermoreversible gels with differences in mechanical properties from soft and elastic for the acetylated form, to hard and brittle for the fully deacetylated polysaccharide which

opens interesting prospects for the generation of multifunctional structures.(21, 22) Gellan gum is non cytotoxic and can be easily processed without the use of harsh reagents into transparent gels that are resistant to heat and acid stress.(16, 23) Its gelation is ionotropic as in other polysaccharides, like alginate or carrageenan, and therefore the presence of cations is necessary for the formation of a stable hydrogel structure. Upon temperature decrease of a gellan gum solution, the transition of a thermally-reversible coil to a double-helix chain occurs and is followed by a self assembling mechanism that forms oriented bundles. These link themselves through untwined polysaccharide chain regions culminating in the formation of a stable gel.(24) The quantity and chemical nature of cations present in solution greatly affects gellan gum gelation; divalent cations promote a more efficient gelation than monovalent cations.(24-26) Gellan gum gels are commonly used in the food industry as thickening agents or stabilizers.(27) In the biomedical field, most applications are suggested for drug delivery approaches.(28-30) Its use in cartilage tissue engineering was pioneered by our group and the application for other soft tissues is currently under study.

In this work, gellan gum hydrogels were used as encapsulation agents and tissue engineering supports for human articular chondrocytes. Gellan gum properties were optimised and characterised so that they could be used as injectable systems in minimally invasive procedures. Gellan gum with encapsulated human articular chondrocytes systems were tested *in vitro* for periods of up to 56 days of culture. Cell viability and extracellular matrix formation and deposition were evaluated by molecular and histological techniques. A preliminary *in vivo* evaluation of the gellan gum hydrogels was also performed upon subcutaneous implantation in Balb/c mice for total periods of up to 21 days. The obtained results showed the suitability of gellan gum hydrogels for injectable applications and *in situ* cell delivery towards the formation of functional tissue engineered cartilage.

2. MATERIALS AND METHODS

2.1. RHEOLOGICAL STUDIES

Cone-Plate rheometry was conducted for gellan gum hydrogels in order to assess their rheological behaviour as function of temperature and time. For this purpose, gellan gum (G1910,

Sigma-Aldrich Co, USA) powder was mixed at room temperature with distilled water at a concentration of 1% (w/v) under constant stirring. The solution was heated to 90ºC and kept at this temperature for 30 minutes. Afterwards, calcium chloride ($CaCl_2$) was added to the gellan gum solution at a concentration of 0.03% (w/v) and rheological measurements were performed using a controlled stress cone-plate rheometer (Reometer Reologica, StressTech, Sweden). For each measurement, a volume of 2 ml of the Gellan gum solution was placed in the bottom plate of the rheometer and kept at a constant temperature of 70ºC. The polymer solution was allowed to stabilise during 1 minute before starting the experiments. Measurements were performed by decreasing the temperature from 70ºC to 25ºC (at a cooling rate of -6ºC/min) and applying a constant shear stress of 0.1 Pa. Temperature, time, shear rate and viscosity were constantly measured. The total number of repeats was five (n=5) and confidence intervals were estimated with a confidence level of 99%.

2.2. DYNAMIC MECHANICAL ANALYSIS

Dynamic mechanical analysis (DMA) was conducted to characterize the mechanical behaviour of gellan gum hydrogel discs. The samples were prepared using a methodology previously described elsewhere.(16) Gellan gum 1% (w/v) discs (Ø 6±0.01 mm x 5.5±0.46 mm height) were subjected to compression cycles of increasing frequencies ranging from 0.1 to 10 Hz with constant amplitude displacements of 0.1 mm using a Tritec 2000 DMA (Triton Technology, UK). Storage and loss modulus were measured and experiments were conducted at room temperature. The total number of discs per assay were five (n=5). The described values for the compression modulus were collected at a frequency of 1 Hz. Statistical analysis was performed using confidence intervals based on the experimental results, with a confidence level of 99%.

2.3. HUMAN ARTICULAR CHONDROCYTES ISOLATION AND EXPANSION

Human articular cartilage was harvested from the femoral head and condyles of adult patients (40-65 years) undergoing knee arthroplasty surgery. This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and upon patient informed consent. Chondrocytes were isolated by enzymatic digestion; in detail, the human articular cartilage, free from all surrounding tissue, was placed in a

Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2-3 mm. The pieces were washed in sterile PBS solution, immersed in 20 ml of trypsin-EDTA solution, and incubated for 30 min at 37ºC under agitation. Trypsin was removed and the tissue pieces washed with basic Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Co, USA). Then, 20 ml of sterile collagenase type II solution (2mg/ml) (Sigma-Aldrich Co, USA) in basic medium was added, and the mixture incubated for approximately 12 hours at 37ºC under agitation. The digested tissue and cell suspension solution was centrifuged at 200g for 7 min and the supernatant discarded. The cell pellet was washed with PBS and centrifuged again under the same conditions. Cells were again centrifuged, the supernatant removed, and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Co, USA), containing 10 mM HEPES buffer pH 7.4, 10000 units/ml penicillin/10000 µg/ml streptomycin, 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human nasal chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.(31)

2.4. ENCAPSULATION OF HUMAN ARTICULAR CHONDROCYTES IN GELLAN GUM HYDROGELS AND *IN VITRO* CULTURING

Human articular chondrocytes were expanded and encapsulated at passage 1 in gellan gum hydrogels using the following procedure. Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. A sterile $CaCl_2$ solution was added to obtain a final concentration of 0.03% (w/v). The temperature was progressively decreased to 42ºC and stabilised at this value for posterior use always under constant stirring. Human articular chondrocytes were detached by trypsinisation, mixed with expansion medium, and centrifuged at 200g for 7 min. The supernatant was removed and the cells were ressuspended in warm sterile PBS solution, counted using an hemocytometer, and finally centrifuged at 200g for 7 min. The supernatant was discarded and the cells pellet kept at the bottom of the falcon tube. The gellan gum 1% (w/v) with $CaCl_2$ 0.03% (w/v) solution was added to the pellet of cells and the mixture ressuspended for complete homogenization of the cells within the matrix. Gellan gum discs containing $8x10^6$ cells/ml human articular chondrocytes

were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 1-2 minutes to form a solid gel. Discs of Ø 6±0.01 mm x 5.5±0.46 mm height were cut using a borer. The discs were cultured in expansion medium for 7 days which was afterwards replaced by differentiation medium for 49 days. The differentiation medium was prepared by replacing the bFGF in the expansion medium with insulin (1µg/ml) and ascorbic acid (50 µg/ml) (Sigma-Aldrich Co, USA). The culture medium was changed every 3-4 days.

2.5. CELL VIABILITY: CALCEIN AM STAINING

Chondrocytes viability after 14 days of culture was assessed using calcein AM staining. Calcein AM (C3099, Invitrogen Corporation, USA) assay is a fluorescence-based method for assessing cell viability and cytotoxicity in which the reagent is retained in cells that have an intact cell membrane. Briefly, a calcein AM solution of 1/1000 was prepared in culture medium. After the end of each time point, one disc of gellan gum with encapsulated human articular chondrocytes was collected from the culturing plates and incubated in the calcein AM solution for 15-30 min at 37ºC and afterwards washed in sterile PBS. DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich Co, USA), a fluorescent stain that binds strongly to DNA was used for counterstaining. The samples were observed under fluorescent microscopy (Zeiss HAL 100/HBO 100; Axiocam MRc5).

2.6. HISTOLOGY (HEMATOXYLIN-EOSIN, ALCIAN BLUE, SAFRANIN-O)

Histological analysis was performed with hematoxylin-eosin (H&E), alcian blue and safranin-O staining on 4 µm thickness sections of the cell-scaffold constructs collected at different periods of culture. The samples were fixed in glutaraldehyde 2.5% (v/v), for 30-40 minutes at 4ºC and washed in PBS. Histological processing was performed using Tecnhovit 7100® (Heraeus Kulzer GmbH, Germany) following the commercial procedure. Sections were cut using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH, Germany). H&E staining was performed using automatic processor (Leica Auto Stainer XL) according to in-house methodology (Leica TP1020-1, Leica MicroSystems GmbH, Germany). Histological staining with alcian blue and safranin-O was performed using standard histological methods. The slides were afterwards washed 3 times in distilled water, quickly dehydrated through 95% and 100% ethanol and then

cleared in Histoclear® (National Diagnostics) and mounted using Microscopy Entellan® (Merck) for observation.

2.7. REALTIME PCR (SOX9, COL I, COL II, AGGRECAN)

Samples were collected at defined time periods, quickly frozen in liquid nitrogen, and stored at −80°C until the analysis was performed. RNA was extracted using TRIzol® (Invitrogen Corporation, USA) according to the provided technical datasheet. Briefly, 3 samples of each condition were grinded and mechanically homogenized with a mortar and pestle in TRIzol reagent, being each condition performed in duplicate. Afterwards, chloroform was added and the samples centrifuged to establish a three-phase composition in the tube. The aqueous phase was collected and put in a new tube where isopropanol was added. The samples were centrifuged, the supernatant discarded and the pellet washed with 75% ethanol. After a final centrifugation the samples were allowed to air-dry, and suspended in ultrapure water for posterior analysis. The amount of isolated RNA and A260/280 ratio was determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies). After these determinations, 1µg of RNA of each sample was reverse transcribed into cDNA using the IScript™ cDNA synthesis kit (Biorad) in a MJ Mini™ Personal Thermal Cycler (Biorad). Cartilage related markers were chosen to evaluate the chondrogenic phenotype of the cultured systems. These included Sox9, collagen type I, collagen type II, and aggrecan, using GAPDH as the housekeeping gene for normalization. The expression of each gene was normalized to the GAPDH value in that sample. The relative gene expression quantification was performed using the $2^{-\Delta\Delta Ct}$ (Livak) method,(32) considering that:

$$2^{-\Delta\Delta Ct} = \text{Normalized expression ratio}$$

All the primer sequences were generated using Primer3™ software(33) and acquired from MWG Biotech™, Germany. More details can be found in Table 1. Real-time PCR was performed using an MJ Mini™ Personal Thermal Cycler (Biorad Laboratories, USA) machine and SYBR Green IQ™ Supermix (Biorad Laboratories, USA) to detect amplification variations. The analysis of the results was performed with MJ Opticon Monitor 3.1 software (Biorad Laboratories, USA).

Table VII.1. Primers used for realtime PCR evaluation of human articular chondrocytes gene expression.

Gene	Accession number	Left primer	Right primer
Sox9	NM_000346	TTGAGCCTTAAAACGGTGCT	CTGGTGTTCTGAGAGGCACA
Collagen type I	NM_000089	CTGCAAGAACAGCATTGCAT	GGCGTGATGGCTTATTTGTT
Collagen type II	NM_001844	TCACGTACACTGCCCTGAAG	TGCAACGGATTGTGTTGTTT
Aggrecan	NM_001135	ACAGCTGGGGACATTAGTGG	GTGGAATGCAGAGGTGGTTT
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

2.8. SUBCUTANEOUS IMPLANTATION IN MICE – HISTOLOGY AND DYNAMIC MECHANICAL ANALYSIS

Gellan gum discs were prepared under sterile conditions following a methodology previously described.(16) Gellan gum 1% (w/v) (Ø 6±0.01 mm x 5.5±0.46 mm height) discs were subcutaneously implanted in the dorsal part of Balb/c mice (2-3 months with an average weight of 20 g) during periods of up to 21 days. Six female mice were used (2 for each period of implantation). Each animal was anaesthetized with a mixture of 5:1 Imalgene® 1000 (Merial Toulouse, France) and Domitor® (Orion Corporation, Finland) (1.25 mg/mouse, and 25 µg/mouse, respectively) prepared in physiological serum. Under surgical sterile conditions, 2 medial and ventral incisions (approximately 0.7 cm) containing the subcutis and the *Panniculus Carnosus* (skin smooth muscle) were performed in the dorsum of the mice. Craniolateral oriented pockets (2 per incision) were subcutaneously created by blunt dissection. Into these pockets, the Gellan gum discs were inserted (4 discs per animal) and the *Panniculus carnosus* and the skin were carefully sutured. The animals were kept with food and water *ad libitum* during all time of implantation. After predetermined time periods, each test animal was sacrificed and the implanted

scaffolds and respective surrounding tissue were explanted from each animal. These were subjected to macroscopic observation and processed for histological analysis to evaluate the induced inflammatory response and integration into the host tissue. Briefly, the explanted samples were fixed with formalin solution, dehydrated through ethanol solutions and embedded in paraffin. Sections with 4 μm thickness of the gellan gum structure and surrounding tissue interface were stained with haematoxylin-eosin using automatic colouring equipment, as previously described in part 6 of Materials & Methods. Dynamic mechanical analysis was also performed using the procedure previously described in part 2 of the present Materials & Methods section.

3. RESULTS

3.1. RHEOLOGICAL STUDIES

Rheological measurements were performed mainly to determine the temperature range at which the sol-gel transition occurred and the time-scale for gelling as these are important parameters to look into when the material is expected to be applied as an injectable system and as a cell encapsulating agent. The sol-gel transition temperature was observed around 39ºC (39.4±0.16ºC) (Figure 1). This enables the solution to be injected into the organism and gelate at the defect site once it stabilises at the body temperature. Moreover, the low viscosity of the solution before gelation allows cells to be efficiently mixed and homogeneously dispersed within the carrier before the application. Regarding the time-scale for gelling, it is possible to observe from the graph on Figure 2 that it is approximately 20 seconds. The results obtained for both temperature and time of gelation are critical to demonstrate the concept of using gellan gum hydrogels as injectable systems for minimally invasive surgical procedures *in vivo*. A gellan gum solution can be kept above the sol-gel transition maintaining a low viscosity that enables cells or bioactive agents of interest to be homogeneously dispersed. Upon lowering the temperature, the gel forms entrapping the cells in its matrix in a rather efficient way.

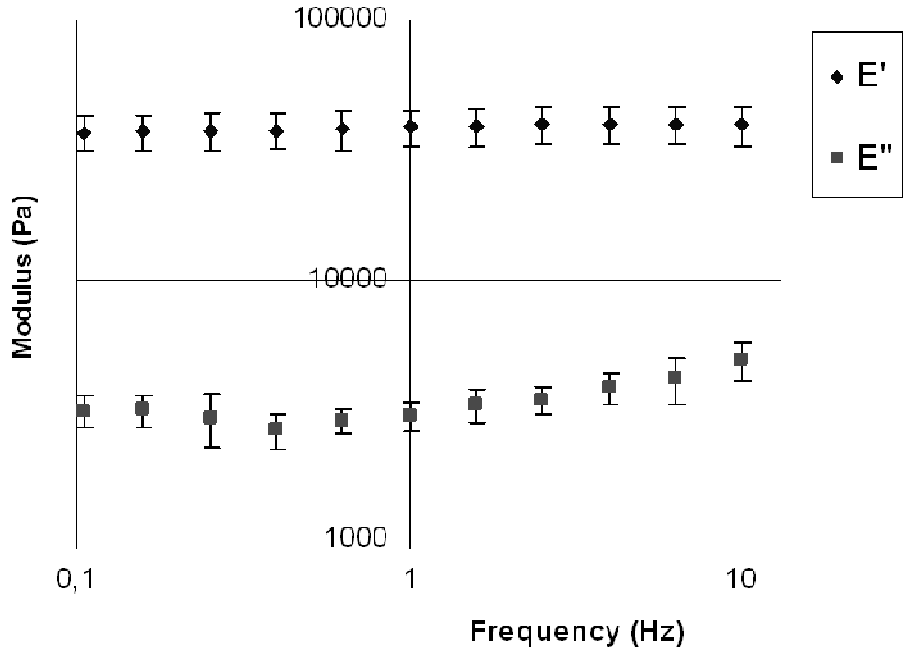

Figure VII.1. Rheological measurements of gellan gum solutions showing the relation temperature-viscosity and time-viscosity. The sol-gel transition is noticeable by the change in viscosity present in the graph around 39-38ºC with an extent of time for gelation of approximately 11 seconds.

3.2. DYNAMIC MECHANICAL ANALYSIS

The viscoelastic characterization of the gellan gum hydrogels was performed using dynamic mechanical analysis (DMA). This technique also gives important information regarding the mechanical performance that these hydrogels may have once implanted *in vivo*. Gellan gum hydrogels were analysed in the wet state throughout a physiological relevant frequency range. Storage (elastic) and loss (viscous) components of the complex modulus were determined and are shown in Figure 2. The storage modulus (E') is about one order of magnitude higher than the loss modulus (E'') which clearly reveals the elastic nature of these gels. It also possesses some damping capability that may be useful to dissipate cyclic mechanical energy that is imposed in an implantation scenario. Although some increase in E' is observed for increasing frequencies, the elastic properties of the hydrogel biomaterial, as compared to the viscous component, are quite stable. An increase in E'' is observed from 0.6 Hz to 10 Hz, suggesting a higher dissipation

capability of gellan gum hydrogels at higher frequencies. At a frequency of 1 Hz, the compression modulus of the gels was estimated to be of 78.6±2.3 kPa. In fact, although this value does not mimic the mechanical properties found in human articular cartilage,(34) it is higher or within the range of values found for other hydrogels used in similar cartilage regenerative approaches.(8, 35)

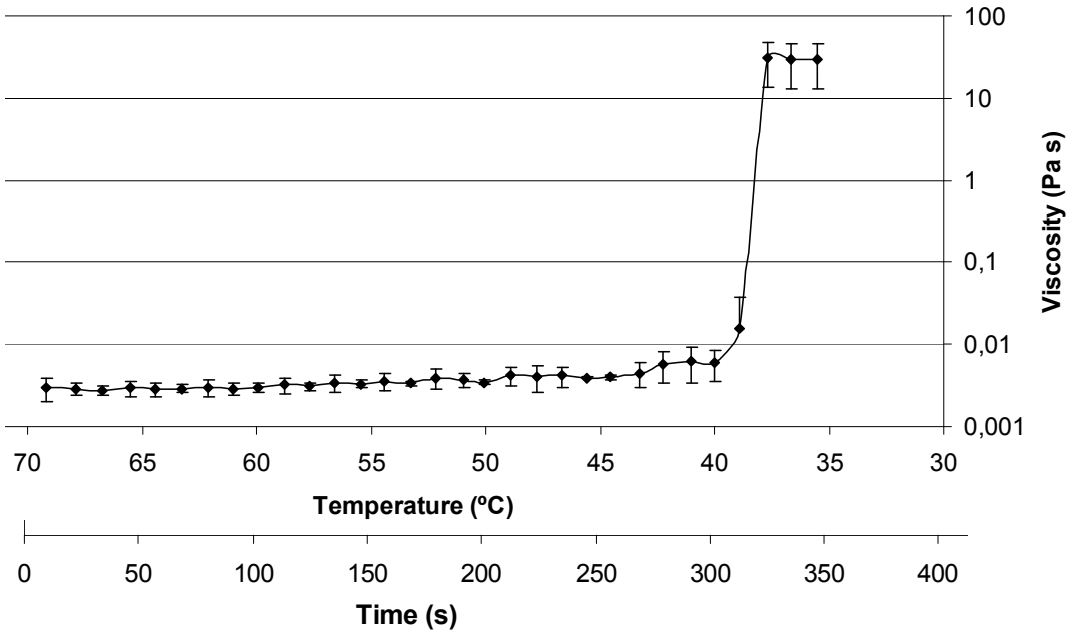

Figure VII.2. Mechanical properties evaluation of gellan gum discs using dynamic mechanical analysis. The tests were performed in intervals of 0.1-15 Hz in hydrated samples.

3.3. CELL VIABILITY AND HISTOLOGICAL ANALYSIS

Calcein AM coupled with DAPI fluorescence staining was conducted to confirm the chondrocytes viability. Calcein AM penetrates the cell membrane of living cells, being subsequently hydrolyzed to a cell membrane-impermeable green-fluorescent calcein by esterases present in viable cells. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to DNA. By coupling these two agents, viable cells are presented with green cytoplasm and blue nuclei. Results from samples collected after 14 days of culture are presented on Figure 3 showing viable human chondrocytes inside the hydrogels. The majority of the cells were positive for calcein and DAPI which indicates a dominance of viable cells. These results show that both the gellan gum

hydrogel is not cytotoxic and that the temperature cycle used to promote the sol-gel transition does not affect cell viability.

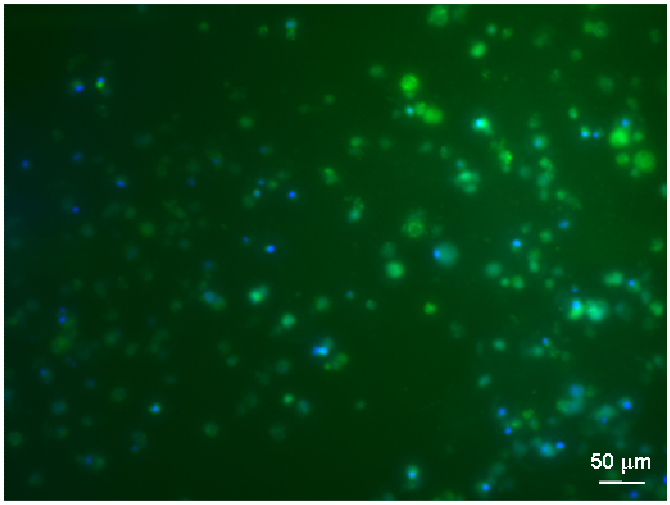

Figure VII.3. Calcein AM/DAPI viability staining of human articular chondrocytes encapsulated in gellan gum hydrogels at 14 days of culture.

Histological analysis of samples taken after 56 days of culture was performed using hematoxylin-eosin for regular morphological cellular analysis, alcian blue and safranin-O which are commonly used for staining extracellular matrix proteoglycans (glycosaminoglycans). Hematoxylin-eosin stained slides (Figure 4.A-B) show an homogeneous chondrocyte distribution throughout the gellan gum hydrogel matrix. Representative images of the deposition of proteoglycans (glycosaminoglycans) within the gellan gum matrix, commonly found in native articular cartilage extracellular matrix, can be found in Figure 4.C-D. A positive staining in some pericellular areas of newly formed cell clusters was observed. An improvement in the nature of the staining that evolved from a more orthochromatic in the initial periods of culture (data not shown) to a more pronounced metachromatic staining in the latter periods was also noticed. This effect was more evident in the regions where cell clusters formed in comparison to individual chondrocytes.

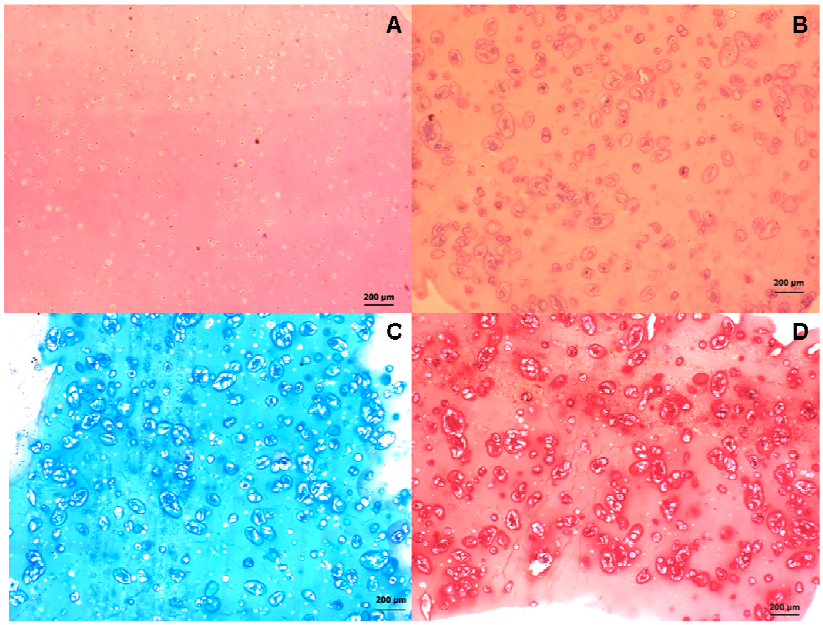

Figure VII.4. Histological analysis of sections of gellan gum with human articular chondrocytes after culturing. Hematoxylin-eosin (A,B – after 2 and 56 days of culture, respectively) was used for general cell morphology and distribution while alcian blue (C), and safranin-O (D) were performed for proteoglycans (glycosaminoglycans) detection.

3.4. REALTIME PCR (SOX9, COLLAGEN TYPE I, COLLAGEN TYPE II, AGGRECAN)

Real-time PCR is frequently used to amplify and simultaneously quantify a specific sequence in a DNA sample. This technique was used to assess the expression profile of different molecules associated with hyaline cartilage ECM and thereby evaluate the nature and type of matrix that is being formed. Quantitative data may be obtained on gene expression and variations that are occurring (upregulation or downregulation) in comparison to a housekeeping gene. Sox9, collagens type I and II, and aggrecan are common ECM constituents and their expression pattern from 14 to 56 days of culture was assessed. GAPDH was chosen as the reference housekeeping gene since most of the studies presented on chondrocytes gene expression use this standard and therefore the results can be analysed in a comparative way. The Ct (cycle threshold) value for each sample was determined only when the exponential phase of amplification was reached. In each sample, the Ct value was normalised to the Ct value of the reference gene (GAPDH). Collagen type II and aggrecan are considered to be the two major and most important constituents of hyaline cartilage ECM since the functionality of this tissue relies mostly on the presence of these components. Both collagen type II and aggrecan experienced a significant

increase of approximately 400-fold and 35-fold, respectively, from 14 to 56 days of culture in the gellan gum with encapsulated human chondrocytes hybrid systems. Collagen type I was not detected after 56 days and Sox9 was downregulated to values close to zero from 14 to 56 days.

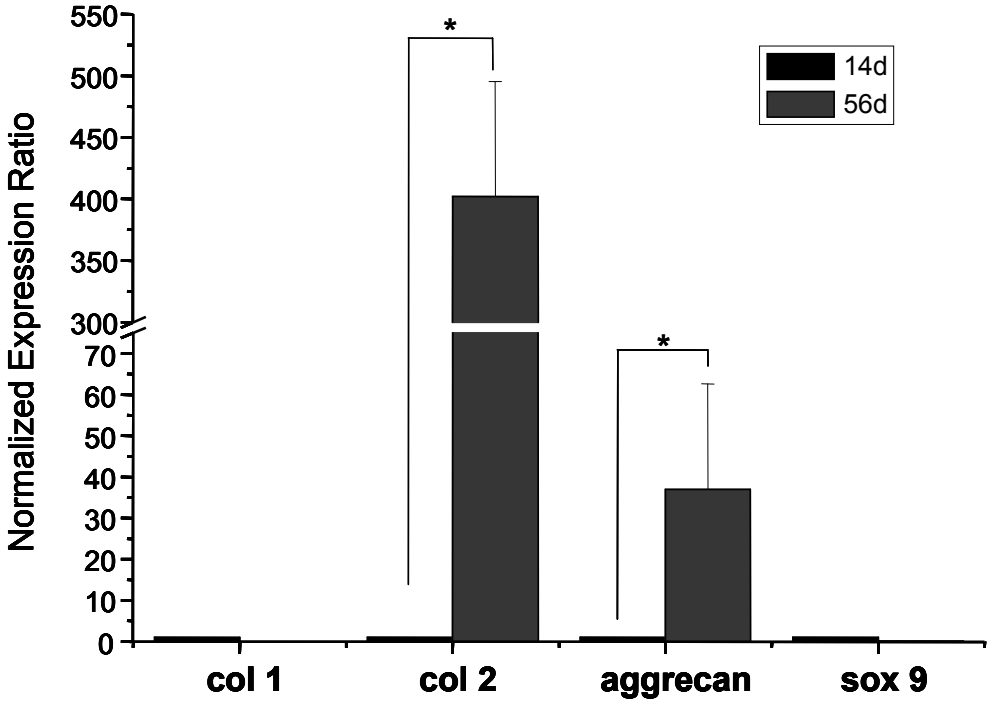

Figure VII.5. Graphical representation of the realtime semiquantitative PCR analysis for collagen type I, collagen type II, aggrecan and Sox9 based on the mRNA produced by the encapsulated human articular chondrocytes after 14 days and 56 days of culture.

3.5. *IN VIVO* TESTS: HISTOLOGY, MECHANICAL ANALYSIS AND WEIGHT MEASUREMENTS

The initial response of a living organism to any kind of implanted biomaterial is absolutely necessary before proceeding to further tissue engineering studies. For this, the subcutaneous implantation of a biomaterial and the subsequent evaluation of the extent of the provoked inflammatory response is a common first screening methodology. As a way to confirm the adequacy of the developed hydrogels to be put through further screening, gellan gum discs were subcutaneously implanted in the back of Balb/c mice for periods of up to 21 days. Upon

explantation, no evident macroscopic changes of the discs and surrounding tissues were observed after this period and the discs maintained its structural integrity. Histological analysis of the explanted samples, stained with hematoxylin-eosin showed good integration within the surrounding host tissue. In terms of inflammatory response associated cells, the normal presence of neutrophils was observed at 7 days of implantation (Figure 6A, 6B). This cell population revealed a progressive decrease along the implantation time, almost inexistent at 14 days (Figure 6C, 6D). Additionally, a thin and residual fibrotic capsule (Figure 6E, 6F) and the infiltration of some phagocytic cells (Figure 6F, arrow) were evident by the end of 21 days. Dynamic mechanical analysis performed on the hydrogel samples at different periods of explantation revealed no statistically significant differences between the samples, although a slight decrease tendency in the compressive modulus was noticed (Figure 7). In terms of weight measurements, which can be an indicator of biodegradation of gellan gum hydrogels, a statistical difference was observed after 21 days of implantation. Although the weights of the samples at different time points did not present relevant alterations, a statistically significant decrease was noticed after 21 days when compared to non implanted gellan gum gel discs and gel discs with 14 days of implantation (Figure 8).

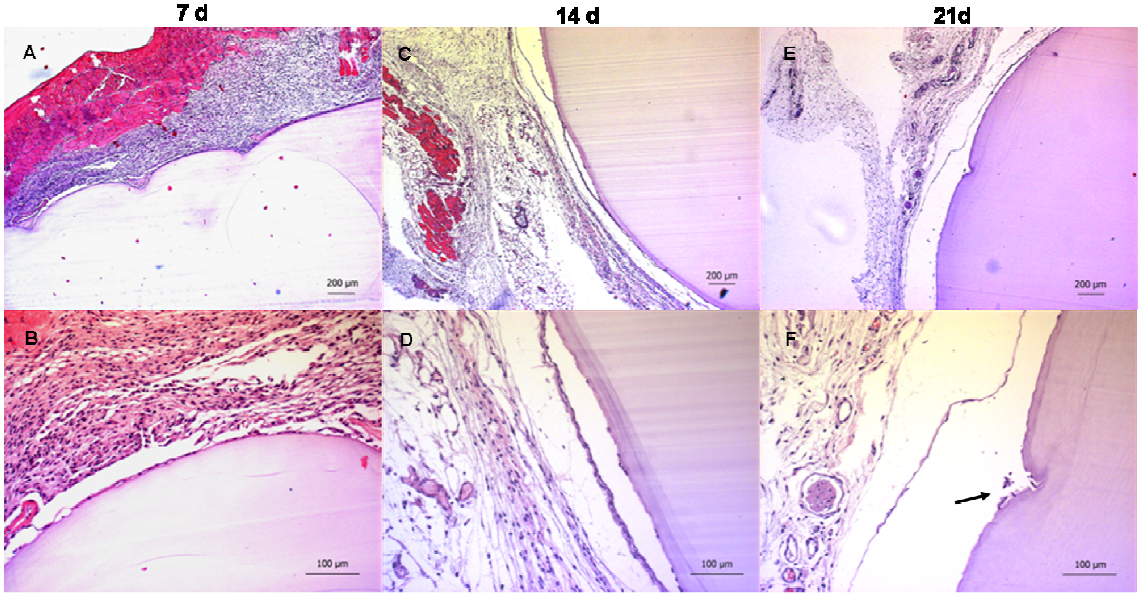

Figure VII.6. Histological analysis showing two different magnifications of sections of gellan gum after 7, 14 and 21 days of implantation. The images evidenced a good integration with the surrounding tissue and the progressive reduction of the fibrotic capsule that was almost unnoticed after 21 days.

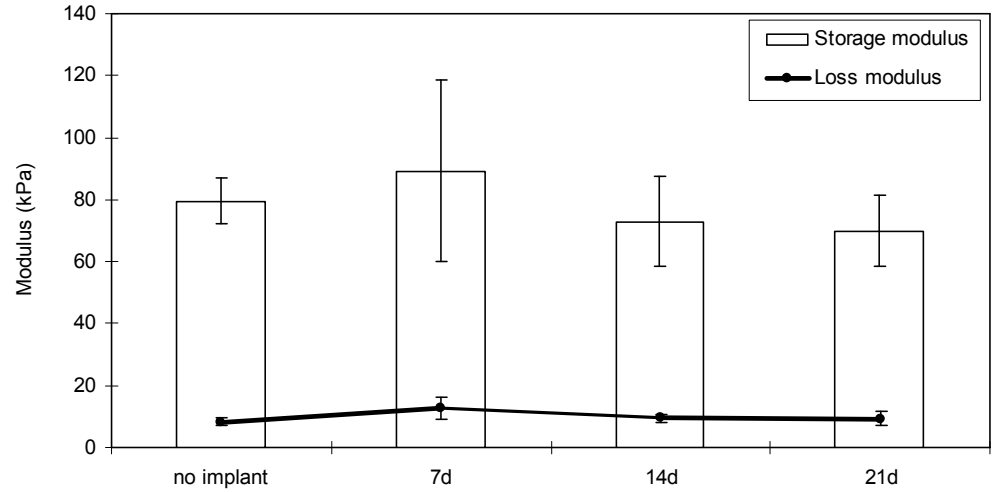

Figure VII.7. Storage and loss modulus at 1 Hz obtained from compression dynamic mechanical tests of gellan gum discs after subcutaneous implantation in Balb/c mice. Statistical analysis revealed no difference through the various time points ($p>0.05$) and were performed using a two-sample t-test assuming unequal variances for $n=5$, $\alpha=0.05$.

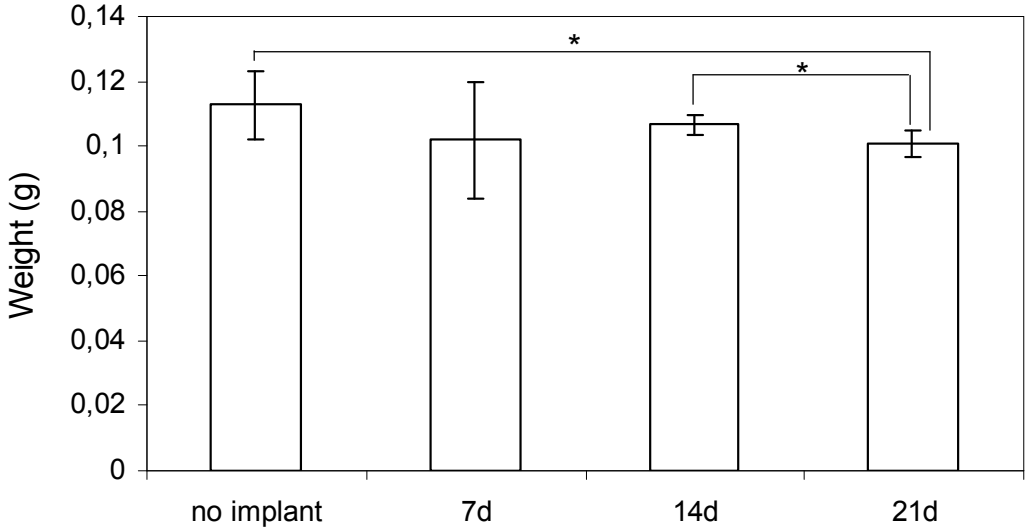

Figure VII.8. Weight measurements of gellan gum discs after subcutaneous implantation in Balb/c mice. Statistical analysis revealed a significant difference in gellan discs implanted for 21 days when compared to no implant and 14 days of implantation. No Statistical analysis through the various time points ($p>0.05$) was performed using a two-sample t-test assuming unequal variances for $n=5$, $\alpha=0.05$.

4. DISCUSSION

The use of hydrogels in cartilage regeneration is considered to be a promising strategy due to some intrinsic features that they frequently exhibit. Most of them can be applied as minimally invasive systems that are able to deliver cells and/or bioactive agents of interest *in situ* and to keep them at the implantation site. The processing under mild conditions coupled to the use of non harsh reagents is another positive aspect. In addition, the gelation *in situ* enables the formed hydrogel to quickly set its volume and easily adapt to the shape of the defect site, establishing an efficient integration with the host tissue.(36) Gellan gum is a new biomaterial proposed for cartilage regeneration that exhibits those features. It can be prepared by simple methods using non aggressive reagents and can be combined with cells or bioactive agents of interest at the body temperature and physiological pH. It can be injected under a minimally invasive surgical intervention and gelate upon temperature stabilization at body temperature giving rise to an efficient defect filling and cell entrapment system. Another potentially interesting aspect is the structural unit of gellan gum that includes glucuronic acid in its composition.(18) This monosaccharide residue possesses a functional carboxylic group that may be modified to confer improved functionalities to this biomaterial. One final aspect is the fact that this biomaterial is already used in humans in ophthalmic applications(37) which may clearly enable a fast approval by the regulatory bodies. In this work, gellan gum hydrogels were tested for their ability to be used as injectable systems in cartilage repair applications. The injectability of this biomaterial was analysed and confirmed by rheological measurements. Cone-plate rheology was used to determine the temperature range and time duration of the sol-gel transition in gellan gum hydrogels. The gellan gum solution revealed a consistent increase in viscosity around 40ºC that stabilised at approximately 39ºC, being the temperature at which the gelation process is finalised. This analysis also provided data on the time during which this process occurred which was shown to be of approximately 20 seconds. The interpretation of both these parameters demonstrates the potential of these hydrogels to be used as minimally invasive injectable systems that are able to deliver and encapsulate cells and/or bioactive agents to a defect site in the human body. In addition, the injectability of gellan gum hydrogels is good since their viscosity before the onset of gelation is low, enabling the overall mixing and application of the gel to be easily conducted. Mechanical properties tested using DMA retrieved a wet state compression modulus of approximately 80 kPa at a frequency of 1 Hz analysed in the wet state using a physiologically relevant frequency range. The storage modulus (E') is about one order of magnitude higher than the loss modulus (E'') which indicates an elastic nature of gellan gum. Some damping capability

was noticed when analysing tan δ results, which may be considered an advantage for dissipating some cyclic mechanical energy that is generated *in vivo*. This value is fairly good when compared to other hydrogels frequently described in the literature for cartilage regenerative approaches.(8, 35) Nonetheless, human cartilage exhibits higher compressive modulus of approximately one order of magnitude.(34) This difference is expected to be compensated by ECM deposition during the formation of a cartilaginous structure.(8) *In vitro* tests showed that chondrocytes were viable and homogeneously distributed inside the hydrogels as observed under fluorescence microscopy using calcein AM combined with DAPI staining, and hematoxylin-eosin staining. The chondrocytes typical round shaped morphology was confirmed at both initial and long-term culture periods. Cell dispersion throughout the gellan gum matrix was quite homogeneous and the cells maintained normal morphological features and active division to some extent. Using the calcein AM-DAPI staining it was possible to observe that the chondrocytes are viable inside the matrix, although cell division state may somehow be limited due to physical restrictions of the hydrogel network which is a typical feature in similar systems.(38) Nonetheless, it may be that the carbohydrate nature of gellan gum, which contains high glucose content, can be used as a source of energy by the cells, thereby progressively giving rise to spaces within the hydrogel matrix and the consequent chondrocytes proliferation. Ongoing work performed by our group on the subcutaneous implantation of gellan gum discs through extensive periods has gathered consistent information that corroborates this hypothesis. Other mechanical events associated with cells proliferation may include the formation of microcracks and the propagation of fracture creating additional spaces for cell growth. Besides cells viability and distribution, the formation of an adequate extracellular matrix is of utmost importance to the performance of the tissue engineered construct. Active collagen type II and aggrecan deposition contributes to the formation of a functional hyaline-like cartilage engineered tissue. These are in fact the two major ECM molecules responsible for the articular cartilage mechanical properties; collagen type II confers tensile strength and aggrecan is responsible for the compressive resistance.(39) Alcian blue and safranin-O performed after 56 days of culturing on sections of gellan gum with encapsulated human articular chondrocytes evidenced glycosaminoglycans deposition in the pericellular regions of most cell clusters. Real-time PCR analysis for Sox9, collagen I, collagen II, and aggrecan strengthened these findings confirming the increased levels of expression of transcripts from collagen type II and aggrecan from 14 to 56 days. Collagen type II was upregulated approximately 400 fold and aggrecan approximately 35 fold; no statistically significant variation in Sox9 was encountered and no collagen type I was detected. Such results clearly show that human articular chondrocytes encapsulated in gellan gum hydrogels were able

to deposit a hyaline-like extracellular matrix therefore contributing to the formation of a functional tissue engineered construct. However, the low expression of Sox9 seems to diverge from this pattern since this factor is apparently associated with the chondrocyte phenotype maintenance and collagen type II expression, although this is not consensual.(40, 41) Chondrocytes from osteoarthritis cartilage present low levels of Sox9(41) and the cell source may be a possible explanation for this occurrence. Also, the de-differentiation process occurring during in vitro expansion(42) or the existence of some associated hypertrophy of the chondrocytic cells may be occurring.(43)

The following step in this study was to evaluate the response of a host to a first contact with this biomaterial. Gellan gum discs were subcutaneously implanted in the back of Balb/c mice and histological analysis was conducted to assess the inflammatory processes associated to the implantation, as well as the integration into the host tissue. A negligent, thin and residual fibrotic capsule was observed with no evident polymorphonuclear and mononuclear cells present, as well as a good integration with the involving tissues. It should be mentioned that some phagocytic cell infiltration (Figure 6.F, arrow) into the hydrogel matrix was observed after 21 days indicating that this might be a probable scenario in further *in vivo* studies. One might speculate that such an event will enable gellan gum with encapsulated cell systems to efficiently integrate with the host tissues and establish a functional transition zone at the interface area, although this must be shown in further studies.

Dynamic mechanical analysis performed on the explants after the defined time periods revealed no statistically significant differences among the various samples. Weight measurements showed that although no relevant alterations were detected, a significant decrease was noticed after 21 days. This may correlate with some cell infiltration into the gellan gum hydrogel matrix and possible resorption, as observed in the histological analysis. In an *in vivo* scenario where the gellan gum carries encapsulated cells intended to form a tissue, the tendency may be the deposition of a cartilage-like ECM along with the possible degradation of the hydrogel. Further studies on this respect will be conducted to understand how gellan gum will degrade or will be replaced at the implantation site by the newly formed tissue. As a summary, this work demonstrates the potential of gellan gum hydrogels to be used as injectable systems in minimally invasive surgical procedures for cartilage regeneration. The properties of this biomaterial were characterised and shown to be compatible with the application envisaged. The *in vitro* studies with clinically relevant cells showed successful human articular chondrocytes viability and ECM formation, culminating with the *in vivo* reaction evaluation upon implantation of the gels in an ectopic site. The overall data analysis shows that this system has all the requirements to be used

for cartilage tissue engineering and its potential should continue to be assessed by further *in vivo* studies.

5. CONCLUSIONS

Gellan gum hydrogels were proposed and used in the herein presented studies as injectable systems for cell delivery and support, aimed at cartilage regeneration. Gellan gum ability to be applied as a minimally invasive system upon injection and *in situ* gelation was supported by rheological measurements. The gels exhibit a low viscosity in the range of 42-41ºC enabling an efficient and homogeneous mixing of the cells, and can afterwards by injected forming a stable gel with entrapped cells when reaching the body temperature. Gelation occurs around 39ºC in about 20 seconds. The evaluation of the mechanical properties of the hydrogels showed that these are within the normal range of other hydrogels used in these applications and are should be able to adapt to the cartilage environment. *In vitro* tests performed with human articular chondrocytes encapsulated in gellan gum hydrogels revealed that the cells remained viable and produced hyaline-like extracellular matrix as observed by the significant increase of collagen type II and aggrecan after 56 days of culture. Subcutaneous implantation of these materials in the back of mice revealed highly satisfactory results since the histology evidenced integration of the gels within the host tissue with no persistent inflammatory response. The dynamic mechanical analysis showed that the gels maintained their mechanical stability and weight measurements by the end of the experiments reinforced these observations. Taking together all this data, it can be concluded that gellan gum hydrogels are adequate for injectable applications and *in situ* cell delivery in cartilage regeneration approaches. This new biomaterial has generated interesting and promising results that justify its use in further *in vivo* studies highly benefiting from their ease of processing performed under non harmful conditions to the cells.

REFERENCES

1. Hunt SA, Jazrawi LM, Sherman OH. Arthroscopic management of osteoarthritis of the knee. The Journal of the American Academy of Orthopaedic Surgeons. 2002 Sep-Oct;10(5):356-63.

2. Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S, Goldberg VM. Principles of cartilage repair and regeneration. Clin Orthop. 1997 Sep(342):254-69.

3. Cancedda R, Dozin B, Giannoni P, Quarto R. Tissue engineering and cell therapy of cartilage and bone. Matrix Biol. 2003 Mar;22(1):81-91.

4. Ge Z, Hu Y, Heng BC, Yang Z, Ouyang H, Lee EH, et al. Osteoarthritis and therapy. Arthritis and rheumatism. 2006 Jun 15;55(3):493-500.

5. J. F. Mano RLR. Osteochondral defects: present situation and tissue engineering approaches. Journal of Tissue Engineering and Regenerative Medicine. 2007;1(4):261-73.

6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med. 1994 Oct 6;331(14):889-95.

7. Malafaya PB, Santos TC, van Griensven M, Reis RL. Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures. Biomaterials. 2008;29(29):3914-26.

8. Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, et al. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair. PNAS. 2002 July 23, 2002;99(15):9996-10001.

9. Wan-Ju Li HC, Tzong-Fu Kuo, Hsuan-Shu Lee, Ching-Chuan Jiang, Rocky S. Tuan. Evaluation of articular cartilage repair using biodegradable nanofibrous scaffolds in a swine model: a pilot study. Journal of Tissue Engineering and Regenerative Medicine. 2009;3(1):1-10.

10. Richardson SM, Curran JM, Chen R, Vaughan-Thomas A, Hunt JA, Freemont AJ, et al. The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-l-lactic acid (PLLA) scaffolds. Biomaterials. 2006;27(22):4069-78.

11. Risbud MV, Sittinger M. Tissue engineering: advances in in vitro cartilage generation. Trends Biotechnol. 2002 Aug;20(8):351-6.

12. Tuzlakoglu K, Alves CM, Mano JF, Reis RL. Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications. Macromolecular Bioscience. 2004 AUG 9;4(8):811-9.

13. Lee CR, Breinan HA, Nehrer S, Spector M. Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior in vitro. Tissue Eng. 2000 Oct;6(5):555-65.

14. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials. 2000 2000/12/15;21(24):2529-43.

15. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. 2003 2003/11;24(24):4337-51.

16. JT Oliveira LM, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, RL Reis. Gellan gum: A new biomaterial for cartilage tissue engineering applications submitted. 2008.

17. JT Oliveira RS, RL Reis, inventor Gellan gum based hydrogels for use in regenerative medicine and tissue engineering, its system, and processing devices (patent pending). 2008.

18. Jansson P-E, Lindberg B, Sandford PA. Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea. Carbohydrate Research. 1983;124(1):135-9.

19. Moorhouse R. CGT, Sandford P.A., Baird J.K. and Kang K.S. PS-60: a new gel-forming polysaccharide. Washington DC: D.A. Brandt; 1981.

20. Rozier A, Mazuel C, Grove J, Plazonnet B. Gelrite(R): A novel, ion-activated, in-situ gelling polymer for ophthalmic vehicles. Effect on bioavailability of timolol. International Journal of Pharmaceutics. 1989;57(2):163-8.

21. Grasdalen H, Smidsrod O. Gelation of gellan gum. Carbohydrate Polymers. 1987;7(5):371-93.

22. Kang KS, Colegrove T. & Veeder, G.T., inventor. USA. 1982.

23. Ogawa E, Takahashi R, Yajima H, Nishinari K. Effects of molar mass on the coil to helix transition of sodium-type gellan gums in aqueous solutions. Food Hydrocolloids. 2006;20(2-3):378-85.

24. Quinn FX, Hatakeyama T, Yoshida H, Takahashi M, Hatakeyama H. The conformational properties of gellan gum hydrogels. Polymer Gels and Networks. 1993;1(2):93-114.

25. Ogawa E, Matsuzawa H, Iwahashi M. Conformational transition of gellan gum of sodium, lithium, and potassium types in aqueous solutions. Food Hydrocolloids. 2002;16(1):1-9.

26. Miyoshi E, Takaya T, Nishinari K. Rheological and thermal studies of gel-sol transition in gellan gum aqueous solutions. Carbohydrate Polymers. 1996;30(2-3):109-19.

27. Mao R, Tang J, Swanson BG. Texture properties of high and low acyl mixed gellan gels. Carbohydrate Polymers. 2000;41(4):331-8.

28. Coviello T, Dentini M, Rambone G, Desideri P, Carafa M, Murtas E, et al. A novel co-crosslinked polysaccharide: studies for a controlled delivery matrix. Journal of Controlled Release. 1998;55(1):57-66.

29. Kubo W, Miyazaki S, Attwood D. Oral sustained delivery of paracetamol from in situ-gelling gellan and sodium alginate formulations. International Journal of Pharmaceutics. 2003;258(1-2):55-64.

30. Sanzgiri YD, Maschi S, Crescenzi V, Callegaro L, Topp EM, Stella VJ. Gellan-based systems for ophthalmic sustained delivery of methylprednisolone. Journal of Controlled Release. 1993;26(3):195-201.

31. Crawford A, Dickinson, S.C. Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering. Hollander AP, Hatton, P.V., editor. Totowa, NJ: Humana Press Inc., US; 2004.

32. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. Methods. 2001;25(4):402-8.

33. http://frodo.wi.mit.edu/. [cited]; Available from.

34. Froimson MI, Ratcliffe A, Gardner TR, Mow VC. Differences in patellofemoral joint cartilage material properties and their significance to the etiology of cartilage surface fibrillation. Osteoarthritis and Cartilage. 1997;5(6):377-86.

35. Nettles DL, Vail TP, Morgan MT, Grinstaff MW, Setton LA. Photocrosslinkable hyaluronan as a scaffold for articular cartilage repair. Ann Biomed Eng. 2004 Mar;32(3):391-7.

36. Hoffman AS. Hydrogels for biomedical applications. Adv Drug Deliv Rev. 2002 Jan 17;54(1):3-12.

37. Dickstein K, Hapnes R, Aarsland T. Comparison of aqueous and gellan ophthalmic timolol with placebo on the 24-hour heart rate response in patients on treatment for glaucoma. Am J Ophthalmol. 2001 Nov;132(5):626-32.

38. Weber M, Steinert A, Jork A, Dimmler A, Thürmer F, Schütze N, et al. Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates. Biomaterials. 2002;23(9):2003-13.

39. Knudson CB, Knudson W. Cartilage proteoglycans. Seminars in Cell & Developmental Biology. 2001;12(2):69-78.

40. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Pöschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biology. 2003;22(4):363-72.

41. Tew SR, Clegg PD, Brew CJ, Redmond CM, Hardingham TE. SOX9 transduction of a human chondrocytic cell line identifies novel genes regulated in primary human chondrocytes and in osteoarthritis. Arthritis Res Ther. 2007;9(5):R107.

42. Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. Embo J. 1998 Oct 1;17(19):5718-33.

43. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 2002 Nov 1;16(21):2813-28.

SECTION 3.

CHAPTER VIII.

Performance Of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes For Cartilage Regeneration When Subcutaneously Implanted In Nude Mice

CHAPTER VIII.

Performance of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes for Cartilage Regeneration When Subcutaneously Implanted In Nude Mice*

ABSTRACT

Gellan gum is a natural biomaterial that has been recently proposed by our group for cartilage tissue engineering applications. It is a polysaccharide produced by microbial fermentation and it is commonly used in the food and pharmaceutical industry. Gellan gum exhibits rather promising properties that make it a strong candidate for cartilage regeneration in a clinical scenario. Gellan gum has the ability to easily form stable gels under extremely mild processing and without requiring the use of harsh reagents that sometimes compromise the application of other systems. One of its most relevant characteristics is that it can function as an injectable system in minimally invasive procedures, being applied as a liquid but gelling within body *in situ* under physiological conditions and efficiently adapting to the defect site. A previous work performed by our group has shown that gellan gum hydrogels were able to support the growth of human articular chondrocytes and enable the deposition of a hyaline-like extracellular matrix.

In this work, gellan gum hydrogels were combined with human articular chondrocytes (hAC) and were subcutaneously implanted in the back of nude mice for total periods of up to 4 weeks. The implants were recovered at defined time points for histological, biochemical, molecular, and immunological analyses. The morphology and distribution of human articular chondrocytes within the gellan matrix was then observed by hematoxylin-eosin staining of histological sections. Proteoglycans synthesis and quantification was performed using alcian blue staining of histological sections and the dimethylmethylene blue (GAG) assay for the tissue engineered constructs, respectively. Real-time PCR analyses were conducted to quantify collagen type I, collagen type II, aggrecan, and collagen type X levels during the course of the experiments. Immunolocalisation of collagen type I and collagen type II was performed on histology sections of the constructs. Results showed a homogeneous cell distribution and the typical round shape morphology of the chondrocytes within the matrix upon implantation. Proteoglycans synthesis was detected in the histological sections by the presence of a metachromatic alcian blue staining and a statistically significant increase of proteoglycans content in gellan gum-human articular chondrocytes tissue engineered constructs was measured with the GAG assay quantified from 1

to 4 weeks of implantation. Real-time PCR analyses showed a statistically significant upregulation of collagen type II and aggrecan levels in the same periods. The immunological assays suggest deposition of collagen type II along with some collagen type I. The overall data shows that gellan gum hydrogels adequately support the growth and ECM deposition of human articular chondrocytes when implanted subcutaneously in nude mice. Taking into consideration the *in vivo* performance of these systems, further studies should be performed towards the development of a fully functional cartilage tissue engineered construct.

*** This chapter is based on the following publication:**

JT Oliveira, TC Santos, L Martins, MA Silva, AP Marques, AG Castro, NM Neves, RL Reis. Performance Of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes For Cartilage Regeneration When Subcutaneously Implanted In Nude Mice. (2008) *(submitted)*.

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1. INTRODUCTION

Cartilage is a supporting connective tissue made broadly of proteins, polysaccharides and chondrocytes that develops an important role in maintaining mobility and a smooth gliding surface of joints in the skeleton. This function is mainly assured by the extracellular matrix that surrounds the chondrocytes which is able to withstand physical deformation and facilitate tissue function. Chondrocytes synthesize and maintain this extracellular matrix, producing especially collagen type II and agggrecan that confer articular cartilage its tensile and compressive resistance, respectively. Cartilage has a low metabolism that may constitute a serious barrier to normal locomotion when the tissue is traumatised or degenerated. As healthy cartilage is only synthesized to a short extent, and frequently this neocartilage presents a much more pronounced fibrocartilaginous nature than hyaline, pathological scenarios are triggered and life quality is highly diminished.[1-3] Various ways to address these problems have been suggested such as debridement or drilling but the outcome is still not satisfactory.[4,5] Mosaicplasty,[6,7] although being a widely widespread surgical technique to treat a cartilage lesion, creates a problem of reconstruction of the cartilage collection site, while repairing the original defect. Among the different alternatives proposed, the tissue engineering of cartilage tissues was proposed[8-12] using biomaterials, cells and/or bioactive agents. One of the first definitions of tissue engineering was proposed by Langer and Vacanti in Science in 1993.[13] Since then, tissue engineering concepts have evolved and progressed through several lines of study that range from nanotechnology inspired systems to rapid prototyping methods aimed to produce hybrid structures.[14-18] Also the types of biodegradable materials chosen passed from those already employed in clinical procedures (in applications such as sutures, stents, etc) to others used in environmental applications,[19] and food industry.[20] Biomaterials used in regenerative medicine are frequently divided in terms of their natural or synthetic origin. Examples of the first include alginate,[21] hyaluronic acid,[22] chitosan,[23] and starch,[24] and of the second, polyglycolic acid,[25] polylactic acid,[26] and polyethylene oxide.[27] Gellan gum is a biomaterial of natural origin recently proposed for applications in the cartilage regeneration field.[28,29] It is a polysaccharide produced by bacterial fermentation and its basic structural unit is composed of glucose, rhamnose and glucuronic acid residues. Gellan gum is able to form gels with differences in mechanical properties from soft and elastic, to hard and brittle through an ionotropic gelation mechanism.[30,31] Other interesting characteristics of the material include its heat and acid resistance, gel formation under mild conditions without using harsh reagents and its non cytotoxic behaviour.[32] Our group has suggested[29,32] the use of gellan gum as an encapsulating and support agent of different cells

towards the formation of a functional cartilaginous tissue. Human articular cartilage chondrocytes were encapsulated and cultured *in vitro* in optimised injectable gellan gum systems for total periods of 8 weeks, maintaining their viability and synthesising hyaline-like extracellular matrix components, mainly collagen type II and aggrecan.

In this work, gellan gum hydrogels were combined with human articular chondrocytes acting as encapsulating agents and supports for their development. Gellan gum discs were formed and subcutaneously implanted in the back of nude mice for 4 weeks periods. The *in vivo* results showing the formation of a cartilage tissue of hyaline nature suggest that these systems may be potentially used in the repair of cartilage lesions.

2. MATERIALS AND METHODS

2.1. HUMAN ARTICULAR CHONDROCYTES ISOLATION AND EXPANSION

Articular cartilage was harvested from the femoral head and condyles macroscopically healthy parts of adult patients (40-65 years) undergoing replacement surgery based on a protocol previously described by Crawford et al.[33] This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and always sampled upon patient informed consent. Human chondrocytes were isolated by enzymatic digestion with posterior collection. The human articular cartilage free from all surrounding tissue was placed in a Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2-3 mm. The pieces were washed in sterile PBS solution, immersed in 20 ml of trypsin-EDTA solution, and incubated for 30 min at 37ºC on a rotator. Trypsin was removed and the pieces washed with basic DMEM (Sigma-Aldrich Co. USA). Then, 20 ml of filter sterilised collagenase type II (Sigma-Aldrich Co. USA) solution (2mg/ml) in basic medium was added, and the mixture incubated for approximately 10 hours at 37ºC on a rotator. The digested tissue and cell suspension solution was centrifuged at 1200 rpm for 8 min and the supernatant removed. The cell pellet was washed and centrifuged twice with PBS and the cells counted using a hemocytometer. Chondrocytes were then collected by centrifugation and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Co. USA), containing 10 mM HEPES buffer pH 7.4, 1% antibiotic (antibiotic-antimycotic Gibco 15240), 20 mM L-alanyl glutamine, 1x MEM non-essential amino

acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human articular chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.

2.2. HUMAN ARTICULAR CHONDROCYTES ENCAPSULATION IN GELLAN GUM HYDROGELS

Human articular chondrocytes were expanded and encapsulated at passage 2 in gellan gum hydrogels using the following procedure. Briefly, gellan gum powder (G1910, Sigma-Aldrich Co. USA) was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1.25% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. The temperature was progressively decreased to 42ºC and stabilised always under constant stirring. Human articular chondrocytes were detached by trypsinisation, mixed with culture medium, and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cells were ressuspended in sterile PBS solution, counted using and hemocytometer, and finally centrifuged as before. The cell number was calculated so that the final concentration after encapsulation was of $5x10^6$ cells/ml. The supernatant was discarded and the cells pellet kept at the bottom of the tube were ressuspended in PBS. The gellan gum 1.25% (w/v) solution was extensively mixed with the chondrocytes suspension for complete homogeneous dispersion within the gel. Gellan gum with the encapsulated cells was allowed to gel in a cylindrical mould for 2-3 minutes. Discs of Ø 3 mm x 3 mm height were cut using a sterile blade and kept in sterile PBS before the implantation procedure. Gellan gum discs with no cells encapsulated were also prepared using the same procedure and used as controls.

2.3. *IN VIVO* SUBCUTANEOUS IMPLANTATION IN NUDE MICE

Six 4-week-old female Balb/C nude mice (Charles River Laboratories Inc. USA) female with an average weight of 20 g were anaesthetized with a mixture of ketamine (1.2 mg/mouse s.c., Imalgene® 1000, Merial, Lyon, France) and medetomidine (20 µg/mouse s.c., Domitor®, Orion Corporation, Finland) prepared in physiological serum. After the confirmation of analgesia/anaesthesia two incisions were performed (reaching a maximum of 1.5 cm each) being

one in the intrascapular region and another in the lumbar region. With the help of a forceps two side pockets were created through each of the incisions and gellan gum discs with encapsulated chondrocytes and with no encapsulated cells (control) were subcutaneously implanted. Four discs were implanted per animal, being two on the anterior region and other two on the posterior region. The incision sites were sutured and the mice transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. After 1 week and 4 weeks post implantation, mice were euthanized (n=3 for each time point) by exposure to a saturated carbon dioxide environment and the gellan gum discs were surgically recovered and processed for histological analysis, biochemical, and molecular analyses.

2.4. HISTOLOGY

Common histological analysis was performed on 4 μm thickness sections of the explants collected at different periods of culture. Hematoxylin-eosin (H&E) was conducted to observe general cell morphology and overall distribution, and alcian blue was performed to evaluate extracellular matrix components deposition, namely proteoglycans (glycosaminoglycans). Briefly, the constructs were carefully dissected from the subcutaneous tissue of nude mice and collected in eppendorf tubes. They were immediately fixated in formalin for 30-40 minutes and washed in PBS. Histological processing was conducted by dehydrating the samples in increasing ethanol concentrations, embedding them in paraffin and cutting sections for posterior analysis using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH, Germany). H&E staining was performed using an automatic processor according to in-house methodology (Leica TP1020-1, Leica MicroSystems GmbH, Germany) and alcian blue staining was performed using standard histological methods. The slides were washed afterwards in distilled water, dehydrated through increasing ethanol concentrations, and finally cleared in xylene substitute and mounted using Microscopy Entellan® (Merck & Co., Inc., USA) for observation.

2.5. QUANTIFICATION OF PROTEOGLYCAN CONTENT

Proteoglycans were determined by measuring the level of sulfated glycosaminoglycans (GAGs) using 1,9-dimethylmethylene blue (DMB) metachromatic assay as previously described by

Kafienah et al.[34] GAG levels can be quantified in solution using DMB since the mechanical entanglement of this reagent with GAGs generates a peak shift at $A_{525-530}$ that can be measured spectrophotometrically. Briefly, the constructs were carefully dissected from the subcutaneous tissue of nude mice and collected in eppendorf tubes. The samples were grinded with a mortar and pestle and immersed in a digestion solution with papain and N-acetyl cysteine at 60ºC for approximately 3 hours. The tubes were centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected for biochemical analysis. A chondroitin sulfate standard solution was prepared in water and kept at 4ºC. The samples and the chondroitin sulfate standards were placed in a 96 well round-bottomed plate, DMB solution was added to each well, and the optical density was measured using a microplate reader at 530 nm. Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3.

2.6. REAL-TIME PCR (COLLAGEN TYPE I, COLLAGEN TYPE II, AGGRECAN, COLLAGEN TYPE X)

The constructs were carefully collected upon dissection of the subcutaneous tissue of nude mice, immersed in TRIzol® (Invitrogen, USA), and quickly and stored at −80°C until the analysis was performed. RNA was extracted using TRIzol® and more details can be found in the technical datasheet provided. Briefly, triplicates of each condition were grinded and mechanically homogenized with a mortar and pestle in TRIzol® reagent. Chloroform was then added and the samples centrifuged to establish a three-phase composition in the tube and the aqueous phase was collected to a new tube and mixed with isopropanol. The samples were once again centrifuged, the supernatant discarded and the pellet washed with 75% ethanol. The samples were again centrifuged, let to air-dry, and suspended in ultrapure water for posterior analysis. The amounts of isolated RNA and A260/280 ratio were determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). After these determinations, RNA from each sample was reverse transcribed into cDNA using the IScript™ cDNA synthesis kit (Bio-rad Laboratories, USA) in a BioRad CFX96 real-time PCR detection system (Bio-rad Laboratories, USA). Cartilage related markers were chosen to evaluate the chondrogenic phenotype of the cultured systems. These included collagen type I, collagen type II, aggrecan, and collagen type X using GAPDH as the housekeeping gene for normalization. The expression of each gene was normalized to the GAPDH value in that sample. The relative gene expression quantification was performed using the $2^{-\Delta\Delta Ct}$ (Livak) method, considering that:

$2^{-\Delta\Delta Ct}$ = Normalized expression ratio

All the primer sequences were generated using Primer3 software[35] and acquired from MWG Biotech™, Germany. More details can be found in Table 1. Real-time PCR was performed using a BioRad CFX96 real-time PCR detection system (Bio-rad Laboratories, USA) and SYBR Green IQ™ Supermix (Bio-rad Laboratories, USA) to detect amplification variations. The analyses of the results were performed with CFX Manager Software - version 1.0 (Bio-rad Laboratories, USA). Statistical analyses were conducted using a two-sample t-test assuming unequal variances for n=3.

Table 1. Primers used for real-time PCR evaluation of human articular chondrocytes gene expression.

Gene	Accession number	Left primer	Right primer
Collagen type I	NM_000089	CTGCAAGAACAGCATTGCAT	GGCGTGATGGCTTATTTGTT
Collagen type II	NM_001844	TCACGTACACTGCCCTGAAG	TGCAACGGATTGTGTTGTTT
Aggrecan	NM_001135	ACAGCTGGGGACATTAGTGG	GTGGAATGCAGAGGTGGTTT
Collagen type X	NM_000493	AATCCCACAGGCATAAAAG	AGGACTTCCGTAGCCTGGTT
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

2.7. IMMUNOLOCALISATION OF COLLAGEN TYPE I AND COLLAGEN TYPE II

Collagen types I and II were detected immunohistochemically with monoclonal antibodies against collagen types I and II (Southern Biotechnology, UK) using the Vectastain® Universal Elite ABC Kit PK-7200 (Vector Laboratories Ltd, UK) and DAB Substrate Kit for Peroxidase SK-4100 (Vector Laboratories Ltd, UK) according to the suppliers indications. Briefly, paraffin sections on the collected explants were deparafinized and hydrated through decreasing ethanol concentrations. The sections were treated with 3% hydrogen peroxide in 50% methanol for 5 minutes, washed in PBS buffer, and incubated in pre-diluted blocking serum. The incubation with the primary antibodies and negative control followed (collagen type I 1:100; collagen type II 1:20,

normal horse serum 1:100). The remaining protocol is as described in the Vectastain® Elite ABC Kit. The sections were further incubated with the DAB substrate at room temperature until suitable staining develops according the suppliers indications. The sections were counterstained with neutral red, dehydrated through increasing ethanol concentrations, and finally cleared in Xylene substitute ® (National Diagnostics, USA) and mounted using Microscopy Entellan® (Merck, USA) for observation.

3. RESULTS

3.1. HISTOLOGY

The explants collected after 1 and 4 weeks of implantation were analysed using histological methods. Hematoxylin-eosin staining was performed on the sections of the explants since it can provide relevant information on cell morphology and distribution within the gel matrix. It was observed that the cells had been homogeneously distributed throughout the whole hydrogel, while also exhibiting the typical round shape morphology of native articular chondrocytes. An increase in cell mass was noticed after 4 weeks of culture (Figure 1.A) with individual chondrocytes giving rise to clusters of 2-3 cells. Sulfated glycosaminoglycans are important components of the native articular cartilage extracellular matrix due to their water retention ability that highly contributes to the mechanical functionality of the tissue. Sulfated glycosaminoglycans were detected in histological sections of the explants of the implanted gellan gum systems using alcian blue staining, mostly after 4 weeks of implantation (Figure 1.B). The staining evolved from a more orthochromatic nature in the early periods to a pronounced metachromatic staining after 4 weeks of implantation (Figure 1.B). The positive staining was localised in the pericellular regions of chondrocyte clusters, being its presence and intensity quite regular throughout the gellan gum matrix. It should also be mentioned that the discs maintained their structural integrity upon microscopical observation and were well integrated with the surrounding tissues.

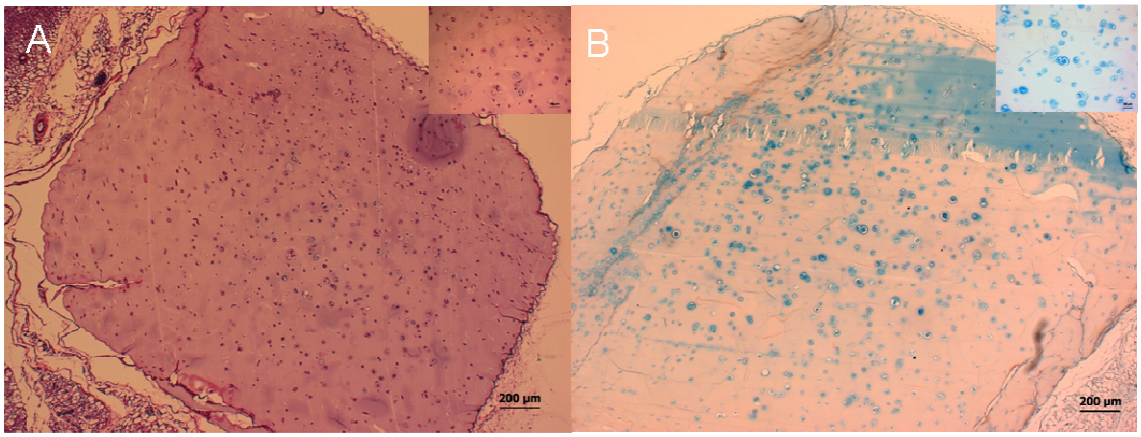

Figure VIII.1. Hematoxylin-eosin (A) and alcian blue (B) staining of histological sections of the explanted gellan gum hydrogels with human articular chondrocytes after 4 weeks of culture. The cells divided in clusters of 2-3 cells and presented the typical round shape morphology of native articular chondrocytes. A metachromatic staining, mostly in the pericellular regions, can be observed indicating the deposition of extracellular matrix proteoglycans (glycosaminoglycans) (B).

3.2. QUANTIFICATION OF PROTEOGLYCAN CONTENT

Following the qualitative analysis performed with alcian blue staining on histological sections of the gellan gum hydrogels-human articular chondrocytes tissue explants, the proteoglycan content of the constructs was quantitatively evaluated using the GAG assay. The glycosaminoglycan content was found to increase steadily from 1 to 4 weeks of implantation, being this variation statistically significant (Figure 2). An increase of approximately 2.4 fold amount was measured in gellan gum tissue engineered constructs collected after 4 weeks in comparison to 1 week of implantation. These results are in accordance and reinforce the positive identification of sulfated glycosaminoglycans with the histological analysis.

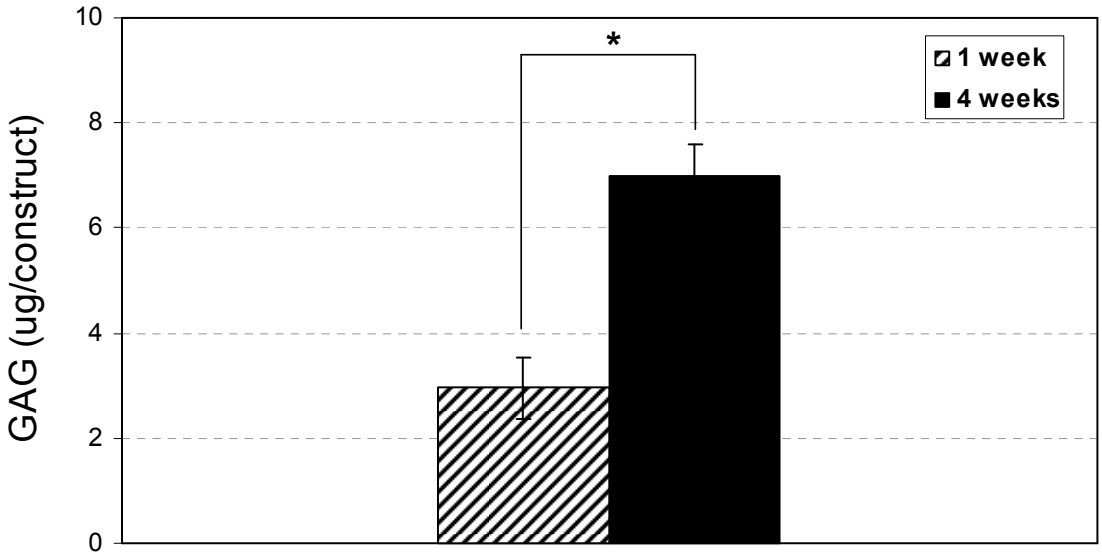

Figure VIII.2. Graphical representation of the results obtained from DMB assay for glycosaminoglycans (GAGs) quantification of the explants after 1 and 4 weeks of implantation. A statistically significant increase was observed from 1 to 4 weeks ($p<0.05$).

3.3. REAL-TIME PCR (COLLAGEN TYPE I, COLLAGEN TYPE II, AGGRECAN, COLLAGEN TYPE X)

Real-time PCR was used to quantitatively assess the upregulation and downregulation of genes typically associated with chondrocytes and cartilage tissue formation. Collagens type I, II, X, and aggrecan are common ECM constituents present at different stages of the chondrogenic process and the transcription of their genes was evaluated after 1 and 4 weeks of implantation. Among those, collagen type II and aggrecan are considered to be the two major and most important constituents of hyaline cartilage ECM since they are responsible for the mechanical functionality of the tissue. Collagen type I is associated with the dedifferentiation period that frequently occurs in 2D culturing and is a reflection of poor hyaline-nature of the formed tissue.[36,37] Collagen type X is a marker associated to hypertrophic chondrocytes and matrix mineralization, and its presence is a poor indicator towards the formation of a stable hyaline-like ECM.[38] The graphical representation of the molecular analyses data shows a statistically significant increase of both collagen type II and aggrecan of approximately 230-fold and 6-fold, respectively, from 1 to 4 weeks of implantation. Collagen type I and collagen type X were not detected until the end of the experiments.

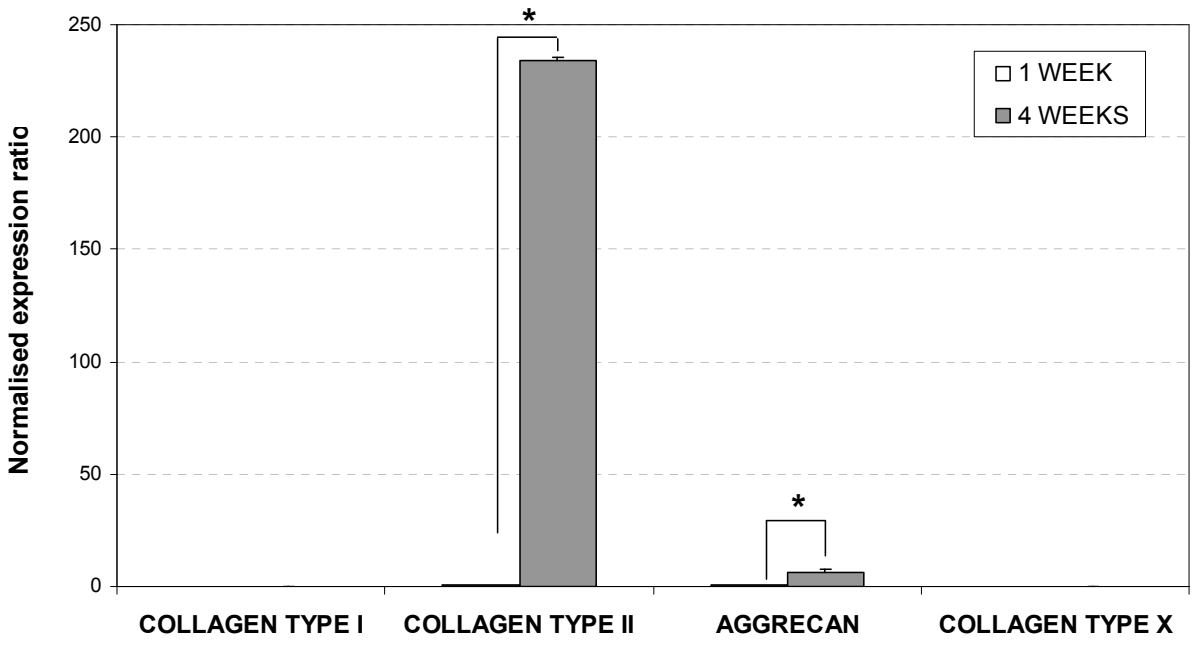

Figure VIII.3. Real-time PCR analyses results for collagen type I, collagen type II, aggrecan, and collagen type X during the 4 weeks of implantation. Collagen type II and aggrecan presented statistically significant increases from 1 to 4 weeks, indicating the hyaline-like nature of the newly formed tissue ($p<0.05$).

3.4. IMUNOLOCALISATION OF COLLAGEN TYPE I AND COLLAGEN TYPE II

As previously mentioned, collagen type II is the most important major protein produced by chondrocytes in articular cartilage, with key functions in the weight bearing ability and shock adsorbing properties of the tissue. The analysis of the immunostaining profiles indicates that both collagen type I and collagen type II are present in the explants when compared with the negative control (normal horse serum). No striking difference is encountered between collagen type I and II, although some stronger staining may be noticed for collagen type II. However, the qualitative evaluation is not simple mostly due to the background staining of the hydrogel that is also present in the negative control. The identification of collagen type I and II, with a suggested prevalence of the latter, is another evidence of the hyaline nature ECM of the tissue engineered constructs and is in agreement with previously molecular data obtained from Real-time PCR analyses that showed an upregulation of collagen type II mRNA.

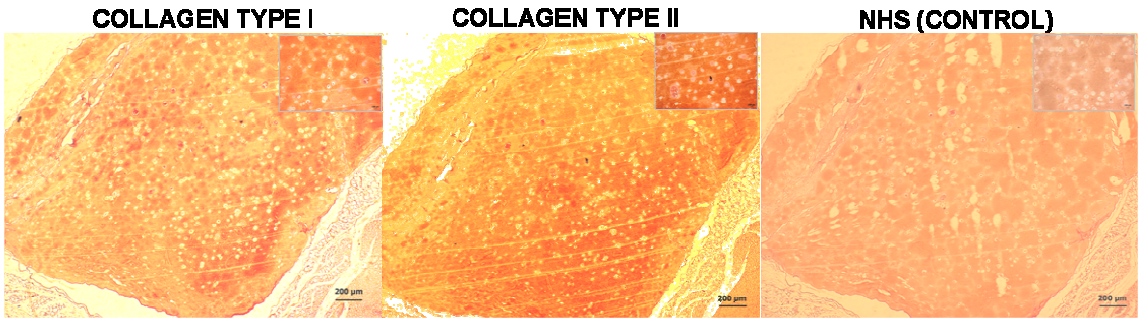

Figure VIII.4. Imunolocalisation of collagen type I and collagen type II in histology sections of the explanted gellan gum hydrogels-human articular chondrocytes systems after 4 weeks. Images show collagen type I, collagen type II, and normal horse serum (negative control) (left to right, respectively).

4. DISCUSSION

Gellan gum hydrogels have been previously suggested by our group[28,29,32] for applications in cartilage regeneration since it possesses adequate materials properties for these purposes. It can be efficiently used as a minimally invasive injectable system able to deliver and encapsulate cells, and to support their development *in vitro*. In those works, human articular chondrocytes have been encapsulated in injectable gellan gum hydrogels and cultured *in vitro* for extensive periods. The chondrocytes were viable and formed a hyaline-like ECM composed of collagen type II and aggrecan. The evaluation of the *in vivo* response upon subcutaneous implantation of these biomaterials was conducted in mice, revealing a good integration with the surrounding tissues and the presence of a residual fibrotic capsule. The preliminary data collected with these hydrogels was quite promising regarding their potential use in the treatment of cartilage pathologies and further *in vivo* studies were set up to validate this hypothesis which are presented in this work.[32]

In this work, gellan gum hydrogels were used to encapsulate human articular chondrocytes and were processed in the form of discs to be subcutaneously implanted in the back of nude mice. The main objective was to assess the formation of a hyaline-like cartilage tissue and to conclude on the adequacy of these systems to generate a functional tissue engineered construct with *in vivo* relevance in the future using histological, biochemical, and molecular characterisation techniques. Hematoxylin-eosin staining of histological sections revealed a homogeneous distribution of the chondrocytes within the gellan gum matrix with the cells exhibiting the common

round shape phenotype characteristic of native chondrocytes. During the implantation time, the chondrocytes exhibited active division (Figure 1.B) which is a good indication of their viability and growth supported by the gellan gum hydrogels. Although these are important aspects to take into account when following cartilage tissue engineering approaches, the essential feature is the formation of a stable and functional tissue, more precisely an ECM that should be similar to the native one. When referring to the biomimetization of the native articular cartilage ECM, efforts are frequently put on the creation of a matrix composed mostly of collagen type II and aggrecan. In fact, these two molecules are responsible for the important functions that cartilage has in normal mobility and joint movement. Articular cartilage has a physical role in the tissue load-bearing properties, mainly due to the interactions of water, ions, and aggrecan molecules within the collagenous meshwork. The collagen type II confers tensile strength and the aggrecan molecules provide compressive stiffness to the tissue.[2,39] The study of the deposition and quantification of proteoglycans, where aggrecan is the most abundant, was performed with alcian blue staining and the GAG assay. The staining profile improved from 1 to 4 weeks of implantation mostly located in the pericellular regions (Figure 1). The metachromatic staining was more pronounced in the last time point, indicating a higher deposition of proteoglycans at this stage, thereby implying that the production of these molecules increased during the course of the experiments. These results are in accordance with the quantitative analysis that showed a statistically significant increase from 1 to 4 weeks of implantation and reinforce the fact that proteoglycans are being synthesised by the encapsulated human articular chondrocytes. Real-time PCR analyses were performed to quantify collagen type I, collagen type II, aggrecan, and collagen type X mRNA levels throughout the time course of the experiments. It was observed a statistically significant upregulation of both collagen type II and aggrecan mRNA levels from 1 to 4 weeks of implantation. As previously referred, these are the most important components of the articular cartilage ECM and the transcription of both genes emphasises the fact that a hyaline cartilage ECM is being deposited, opening interesting prospects regarding future applications of this biomaterial. To this adds the fact that collagen type I and collagen type X are downregulated from 1 to 4 weeks. When in a 2D environment, chondrocytes dedifferentiate loosing their round shape phenotype and decreasing the production of collagen type II and aggrecan, while increasing the production of type I collagen.[37,40] The presence of collagen type I is therefore a negative indicator for hyaline like cartilage ECM formation. Collagen type X is frequently expressed by hypertrophic chondrocytes and is associated with matrix mineralization. For instances, Kirsch et al.[41] observed that collagen type X synthesis is normally linked to an increase of intracellular calcium and deposition of calcium mineral, ultimately leading to matrix calcification. Chondrocytes expressing

this marker are usually part of the deep zone of articular cartilage and its presence indicates an hyperthrophic state of the chondrocytes which is not favourable when attempting to obtain an articular cartilage like ECM.[42] The absence of both collagen type I and collagen type X suggests that chondrocytes were able to redifferentiate after the previous 2D expansion period *in vitro*, but were kept in that stage since no collagen type X typical of hyperthrophic chondrocytes could be detected. Finally, to complement the analyses, immunological localisation of collagen type I and collagen type II was performed on histological sections of the explants. The analysis of the immunostained sections appears to indicate the presence of a stronger staining with the collagen type II antibody when compared to collagen type I, which is in agreement with the real-time PCR analyses, although the concluding evidence of this is complicated by the background staining of the hydrogel. Collagen type I expression is linked to the dedifferentiation process that occurs in 2D culturing and the balance between collagen type I and collagen type II is frequently used as an indicator of the cartilage type formed. The presence of some collagen type I may relate to the redifferentiation process, where collagen type I expression starts to decrease and in turn collagen type II and aggrecan are produced. The noticed stronger staining for collagen type II suggest the deposition of a hyaline-like ECM by the human articular chondrocytes encapsulated in gellan gum hydrogels after implantation in the back of nude mice.

Taken together, the results from this work have shown that gellan gum hydrogels are adequate supports for the growth and differentiation of human articular chondrocytes when implanted subcutaneously in nude mice giving rise to the formation of a hyaline like extracellular matrix. Some aspects should however be considered when optimising this system for further *in vivo* applications. It is likely that the new cartilage tissue formed would benefit from higher cell concentrations and higher implantation periods in order to achieve the formation of a tissue with improved potential. The mechanical properties of these systems should also be more thoroughly considered when approaching a load bearing *in vivo* scenario. However, the synthesis profiles of collagen type II and aggrecan indicate that the mechanical support that is lacking at an initial stage may be in part assured by the newly formed ECM. Finally, it can be referred that tests with alternative cell sources such as stem cells should also be pursued. Given the scarcity of cartilage samples with potential use in patients and the immunological adversities that may arise from the use of allogenous material, such type of improvement might increase the potential of gellan gum hydrogels as compared to other systems proposed for these applications.

5. CONCLUSIONS

In this work, gellan gum hydrogels were used to encapsulate and support human chondrocytes development upon *in vivo* subcutaneous implantation in nude mice. The results were quite promising in terms of the generation of a functional cartilage tissue engineered construct. The human chondrocytes proliferated during the 4 weeks of the experiments and deposited a hyaline like extracellular matrix, typical of native articular cartilage. Collagen type II and aggrecan showed increasing profiles being the results coherent in the analyses performed. The maintenance of the hyaline cartilage phenotype was suggested by the absence or decrease of collagen type I and collagen type X. The *in vivo* performance of these systems so far, along with previous data, suggests their further study in larger animals and the testing of different parameters towards the development of a fully functional cartilage tissue engineered construct to be applied clinically.

REFERENCES

1. Temenoff, J. S. and Mikos, A. G., Review: tissue engineering for regeneration of articular cartilage, *Biomaterials* 21 (5), 431-40, 2000.

2. Ge, Z., Hu, Y., Heng, B. C., Yang, Z., Ouyang, H., Lee, E. H., and Cao, T., Osteoarthritis and therapy, *Arthritis Rheum* 55 (3), 493-500, 2006.

3. J. F. Mano, R. L. R., Osteochondral defects: present situation and tissue engineering approaches, *Journal of Tissue Engineering and Regenerative Medicine* 1 (4), 261-273, 2007.

4. Hunt, S. A., Jazrawi, L. M., and Sherman, O. H., Arthroscopic management of osteoarthritis of the knee, *J Am Acad Orthop Surg* 10 (5), 356-63, 2002.

5. Jackson, R. W. and Dieterichs, C., The results of arthroscopic lavage and debridement of osteoarthritic knees based on the severity of degeneration, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 19 (1), 13-20, 2003.

6. Hangody, L. and Fules, P., Autologous Osteochondral Mosaicplasty for the Treatment of Full-Thickness Defects of Weight-Bearing Joints: Ten Years of Experimental and Clinical Experience, *J Bone Joint Surg Am* 85 (suppl_2), 25-32, 2003.

7. Jakob, R. P., Franz, T., Gautier, E., and Mainil-Varlet, P., Autologous osteochondral grafting in the knee: indication, results, and reflections, *Clin Orthop Relat Res* (401), 170-84, 2002.

8. Freed, L. E., Vunjak-Novakovic, G., and Langer, R., Cultivation of cell-polymer cartilage implants in bioreactors, *J Cell Biochem* 51 (3), 257-64, 1993.

9. Vacanti, C. A. and Vacanti, J. P., BONE AND CARTILAGE RECONSTRUCTION WITH TISSUE ENGINEERING APPROACHES, *Otolaryngologic Clinics of North America* 27 (1), 263-276, 1994.

10. Sittinger, M., Bujia, J., Minuth, W. W., Hammer, C., and Burmester, G. R., Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture, *Biomaterials* 15 (6), 451-6, 1994.

11. Oliveira, J. M., Rodrigues, M. T., Silva, S. S., Malafaya, P. B., Gomes, M. E., Viegas, C. A., Dias, I. R., Azevedo, J. T., Mano, J. F., and Reis, R. L., Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells, *Biomaterials* 27 (36), 6123-6137, 2006.

12. Oliveira, J. T., Correlo, V. M., Sol, P. C., Costa-Pinto, A. R., Malafaya, P. B., Salgado, A. J., Bhattacharya, M., Charbord, P., Neves, N. M., and Reis, R. L., Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach, *Tissue Eng Part A* 14 (10), 1651-61, 2008.

13. Langer, R. and Vacanti, J. P., Tissue engineering, *Science* 260 (5110), 920-6, 1993.

14. Capito, R. M., Azevedo, H. S., Velichko, Y. S., Mata, A., and Stupp, S. I., Self-Assembly of Large and Small Molecules into Hierarchically Ordered Sacs and Membranes, *Science* 319 (5871), 1812-1816, 2008.

15. Moroni, L., de Wijn, J. R., and van Blitterswijk, C. A., 3D fiber-deposited scaffolds for tissue engineering: Influence of pores geometry and architecture on dynamic mechanical properties, *Biomaterials* 27 (7), 974-985, 2006.

16. Hendriks, J., Riesle, J., and van Blitterswijk, C. A., Co-culture in cartilage tissue engineering, *J Tissue Eng Regen Med* 1 (3), 170-8, 2007.

17. Tuzlakoglu, K., Bolgen, N., Salgado, A. J., Gomes, M. E., Piskin, E., and Reis, R. L., Nano- and micro-fiber combined scaffolds: A new architecture for bone tissue engineering, *Journal of Materials Science-Materials in Medicine* 16 (12), 1099-1104, 2005.

18. Andersson, H. and van den Berg, A., Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities, *Lab on a Chip* 4 (2), 98-103, 2004.

19. Videki, B., Klebert, S., and Pukanszky, B., Grafting of caprolacton to cellulose acetate by reactive processing, *European Polymer Journal* 41 (8), 1699-1707, 2005.

20. Mao, R., Tang, J., and Swanson, B. G., Texture properties of high and low acyl mixed gellan gels, *Carbohydrate Polymers* 41 (4), 331-338, 2000.

21. Rowley, J. A., Madlambayan, G., and Mooney, D. J., Alginate hydrogels as synthetic extracellular matrix materials, *Biomaterials* 20 (1), 45, 1999.

22. Cortivo, R., Brun, P., Rastrelli, A., and Abatangelo, G., In vitro studies on biocompatibility of hyaluronic acid esters, *Biomaterials* 12 (8), 727-30, 1991.

23. Malafaya, P. B., Santos, T. C., van Griensven, M., and Reis, R. L., Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures, *Biomaterials* 29 (29), 3914-3926, 2008.

24. Gomes, M. E., Azevedo, H. S., Moreira, A. R., Ella, V., Kellomaki, M., and Reis, R. L., Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: structure, mechanical properties and degradation behaviour, *J Tissue Eng Regen Med* 2 (5), 243-52, 2008.

25. Mooney, D. J., Mazzoni, C. L., Breuer, C., McNamara, K., Hern, D., Vacanti, J. P., and Langer, R., Stabilized polyglycolic acid fibre-based tubes for tissue engineering, *Biomaterials* 17 (2), 115-124, 1996.

26. Cao, Y., Vacanti, J. P., Paige, K. T., Upton, J., and Vacanti, C. A., Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear, *Plast Reconstr Surg* 100 (2), 297-302, 1997.

[27.] Hubbell, J. A., Massia, S. P., Desai, N. P., and Drumheller, P. D., Endothelial Cell-Selective Materials for Tissue Engineering in the Vascular Graft Via a New Receptor, *Nat Biotech* 9 (6), 568-572, 1991.

[28.] JT Oliveira, RA Sousa, and RL Reis, 2008, Gellan gum based hydrogels for use in regenerative medicine and tissue engineering, its system, and processing devices (patent pending).

[29.] JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, and RL Reis, Gellan gum: A new biomaterial for cartilage tissue engineering applications *submitted*, 2008.

[30.] Kang, K. S., Veeder, G. T., Mirrasoul, P. J., Kaneko, T., and Cottrell, I. W., Agar-Like Polysaccharide Produced by a Pseudomonas Species - Production and Basic Properties, *Applied and Environmental Microbiology* 43 (5), 1086-1091, 1982.

[31.] Grasdalen, H. and Smidsrod, O., Gelation of gellan gum, *Carbohydrate Polymers* 7 (5), 371-393, 1987.

[32.] J. T. Oliveira, T. C. Santos, L. Martins, R. Picciochi, A. P. Marques, A. G. Castro, N. M. Neves, J. F. Mano, and R. L. Reis, Gellan Gum Injectable Hydrogels For Cartilage Tissue Engineering Applications: In Vitro Studies And Preliminary In Vivo Evaluation, *submitted*, 2008.

[33.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[34.] Kafienah, W., Sims, T.J., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[35.] http://frodo.wi.mit.edu/.

[36.] Benya, P. D. and Shaffer, J. D., Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels, *Cell* 30 (1), 215-24, 1982.

[37.] Cancedda, R., Descalzi Cancedda, F., and Castagnola, P., Chondrocyte differentiation, *Int Rev Cytol* 159, 265-358, 1995.

[38.] von der Mark, K., Kirsch, T., Nerlich, A., Kuss, A., Weseloh, G., Gluckert, K., and Stoss, H., Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy, *Arthritis Rheum* 35 (7), 806-11, 1992.

[39.] Knudson, C. B. and Knudson, W., Cartilage proteoglycans, *Seminars in Cell & Developmental Biology* 12 (2), 69-78, 2001.

[40.] Vunjak-Novakovic, G. and Freed, L. E., Culture of organized cell communities, *Adv Drug Deliv Rev* 33 (1-2), 15-30, 1998.

41. Thorsten Kirsch, B. S. K. M., Ascorbate independent differentiation of human chondrocytes in vitro: simultaneous expression of types I and × collagen and matrix mineralization, *Differentiation* 52 (1), 89-100, 1992.

42. Girkontaite, I., Frischholz, S., Lammi, P., Wagner, K., Swoboda, B., Aigner, T., and von der Mark, K., Immunolocalization of type X collagen in normal fetal and adult osteoarthritic cartilage with monoclonal antibodies, *Matrix Biology* 15 (4), 231-238, 1996.

SECTION 3.

CHAPTER IX.

Rabbit Articular Cartilage Full-Thickness Size Defects Treated With Novel Gellan Gum Injectable Hydrogels and Autologous Adipose Stem Cells

CHAPTER IX.

Rabbit Articular Cartilage Full-Thickness Size Defects Treated With Novel Gellan Gum Injectable Hydrogels and Autologous Adipose Stem Cells*

ABSTRACT

Gellan gum is a natural origin biomaterial, originally produced by *Sphingomonas paucimobilis* that can easily form gels in the presence of cations enabling the entrapment of cells inside its matrix. Furthermore, its sol-gel transition can be tailored to occur within physiological values of temperature and pH enabling gellan gum to be applied as a minimally invasive injectable system. Due to these interesting properties, gellan gum may have potential application in the field of tissue engineering, particularly in the regeneration of cartilage defects.

In this work, gellan gum hydrogels were combined with adipose stem cells (ASC) and injected in rabbit full-thickness articular cartilage defects in order to evaluate their regenerative potential. Five study groups were defined for this work: a) gellan gum with encapsulated chondrogenic pre-differentiated [transforming growth factor beta1 (TGF-β1) and bone morphogenetic protein 2 (BMP-2)] rabbit adipose stem cells (ASC + GF); b) gellan gum with encapsulated non chondrogenic pre-differentiated rabbit adipose stem cells (ASC); c) gellan gum with encapsulated rabbit articular chondrocytes (AC) (standard control); d) gellan gum alone (control); e) empty defect (control). Twelve New Zealand white rabbits were used in these experiments under an autologous approach, meaning that both adipose stem cells and articular chondrocytes were isolated from the same animals where they were later on implanted. Full-thickness articular cartilage defects were created in the medial septum of rabbit femoral condyles where the various gellan gum systems were injected. The implants were left for total periods of up to 8 weeks and the explants were collected at defined time points for analysis. The macroscopic aspect of the explants showed a progressive increase of similarity with the lateral native cartilage, changing from an initial semi-transparent to a white opaque structure at the end of the experiments. The implants were well integrated and stable at the defect site, exhibiting a smooth transition zone with the lateral cartilage. This feature was much more pronounced in the cell loaded system in comparison to gellan gum alone and empty defects. Cell morphology and organization presented similar results for ASC + GF and ASC, with the systems containing AC having a slightly different phenotype. Histological scoring provided semi-quantitative information on the tissue quality and showed that ASC + GF exhibited the best results in terms of tissue quality progression. Finally,

ECM formation analyses were assessed by alcian blue histological staining and real-time semiquantitative PCR analyses. Alcian blue retrieved similar results in staining profiles with a better outcome for the cell loaded systems. Regarding real-time PCR analyses, ASC + GF had the best progression of markers production with collagen type II and aggrecan steadily increasing, accompanied by a downregulation of collagen type I. In ASC systems, the profile was the same with the exception of aggrecan which showed no variation from 4 to 8 weeks of implantation. Articular chondrocytes had the highest values after 4 weeks for collagen type II and aggrecan, which decreased in both situations after 8 weeks of implantation. Gellan gum alone and the empty defects showed the worst performances regarding the formation of a hyaline nature ECM. The overall data shows that gellan gum hydrogels in combination with adipose stem cells constitute a promising approach for articular cartilage defects treatment, being a possible candidate for future clinical applications in an autologous context.

*** This chapter is based on the following publication:**

JT Oliveira, L Gardel, T Rada, L Martins, ME Gomes, RL Reis. Rabbit Articular Cartilage Full-Thickness Size Defects Treated With Novel Gellan Gum Injectable Hydrogels And Autologous Adipose Stem Cells (2008) *(submitted)*.

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1. INTRODUCTION

Cartilage is a connective tissue that supports the embryonic skeletal development and redefines its location in the adult organism to specific areas. In terms of structure and organization, cartilage consists of an extracellular matrix in which chondrocytes are dispersed. Unlike in the early embryonic development, in adulthood cartilage is avascular and presents a low metabolic rate. The nutrient supply to assure chondrocytes viability is dependent upon diffusion of nutrients, wastes, ions, and gases through the intercellular substance from adjacent capillaries. Due to its function in mobility and locomotion, articular cartilage, a subtype of hyaline cartilage, is considered to be the most important type. Articular cartilage forms smooth gliding surfaces in the joints areas (e.g. knee, hip), thereby enabling normal movement and limb articulation. Due to the tissues' intrinsic features such as low metabolic turnover and absence of vascularisation, trauma and degenerative conditions (e.g. osteoarthritis, rheumatoid arthritis) associated to it frequently create severe disability states leading to pain and decreased life quality.[1-3] Several therapeutic approaches have been attempted but its outcomes are not fully satisfactory, which set the basis for other lines of research to be put forward.[4-6] The use of biomaterials, cells and bioactive agents, either alone or combined, has been proposed[7-10] as an alternative way to solve the problem of overall tissue regeneration and this new field of research was defined as Tissue Engineering.[11,12] Biomaterials from both natural and synthetic origin have been studied as supports for cells to develop and form a functional cartilaginous tissue.[13-16] The use of hydrogels in the form of injectable systems that can be applied in a minimally invasive manner gelling *in situ* under physiological conditions has gathered considerable attention in cartilage tissue engineering approaches.[17-19] Avoiding open surgeries and methods that are not harmful to the surrounding tissue counterparts is a desirable clinical scenario. In addition, injectable hydrogels can easily adapt to the defect site contributing to integration, and efficiently delivering and retaining encapsulated cells within the cartilage defect. Gellan gum is a polysaccharide that forms thermoreversible gels with different mechanical and rheological characteristics, being in different tested situations non cytotoxic and resistant to heat and acid stress.[20-23] It is commonly applied in the food industry as a thickening agent or stabilizer,[24] and it has been previously used in the biomedical field for drug delivery approaches.[25-27] Gellan gum has been originally proposed by our group[28-30] as a new biomaterial for cartilage tissue engineering applications and by another group for general tissue engineering uses.[31] This new biomaterial has been used as an encapsulation and support agent for human nasal chondrocytes and as a minimally invasive injectable system with human articular chondrocytes *in vitro*. Moreover, it has been tested *in vivo*

subcutaneously implanted in nude mice with human articular chondrocytes for cartilage tissue engineering applications.[28-30] The results obtained so far are promising and prompted the study of this biomaterial to move forward and closer to a real clinical scenario.

The common stages in the study of a biomaterial for medical applications frequently make use of animal models in clinical like conditions before moving onto clinical trials. Evaluating the clinical potential of a determined biomaterial should then encompass experimental work with a relevant cell type in a simulated clinical scenario. Adipose stem cells (ASCs) are a feasible and valid cell source that can be applied in these situations. Adipose tissue is routinely available in high quantities from liposuction surgeries, yielding a considerable number of cells after expansion. ASCs are multipotent cells with the ability to express diverse phenotypes under the adequate conditions, among which of chondrocytes. Their shown potential in previous works[32-34] makes these cells good candidates to be coupled with gellan gum hydrogels for the treatment of cartilage defects.

In this work, adipose stem cells were mixed with gellan gum and injected in rabbit knee full thickness size defects under an autologous approach, using articular chondrocytes as a standard control. The results show that gellan gum systems are able to efficiently regenerate cartilage tissue in the created defects, having the most promising results been obtained with the combination of adipose stem cells subjected to prior chondrogenic differentiation.

2. MATERIALS AND METHODS

Five groups were defined for this work: a) gellan gum with encapsulated chondrogenic pre-differentiated [transforming growth factor beta1 (TGF-β1) and bone morphogenetic protein 2 (BMP-2)] rabbit adipose stem cells (ASC + GF); b) gellan gum with encapsulated non chondrogenic pre-differentiated rabbit adipose stem cells (ASC); c) gellan gum with encapsulated rabbit articular chondrocytes (AC) (standard control); d) gellan gum alone (GELLAN) (control); e) empty defect (EMPTY) (control). The experiments with the New Zealand White rabbit model (n=12) involved an autologous approach, meaning that the cells extracted from a specific rabbit were implanted in the same rabbit.

2.1. RABBIT ADIPOSE STEM CELLS ISOLATION AND EXPANSION

Rabbit adipose tissue was obtained from the intrascapular region of 10-11 weeks old /2.4-2.6 Kg female New Zealand White rabbits. Briefly, the rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corporation, Finland). After the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the intrascapular region and disinfected with povidone iodide. An incision was performed (reaching a maximum of 2 cm) in the intrascapular region and adipose tissue from this region was collected to a falcon tube containing sterile phosphate-buffered saline solution (PBS) with 10% antibiotic (antibiotic-antimycotic 15240, Initrogen Corporation, USA). The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. The collected tissue was washed in sterile PBS in order to remove contaminating debris and red blood cells. The adipose samples were then incubated in a 1 mg/ml collagenase type II (Sigma-Aldrich Co. USA) solution prepared in PBS for 60-90 minutes at 37°C with constant agitation. The processed adipose tissue was afterwards filtered and the released cells collected in a falcon tube and centrifuged at 1200 rpm for 10 minutes. The formed cell pellet was washed in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich Co. USA), 10% (v/v) foetal bovine serum (FBS, Biochrom, Berlin, Germany; Heat Inactivated), 1% antibiotic, and centrifuged as before. The obtained cell pellet was again ressuspended in culture medium, and seeded in tissue culture polystyrene flasks. Rabbit adipose stem cells (ASC) were incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion. Once the adequate cell number was reached, the cells were divided in two groups: one group subjected to a chondrogenic predifferentiation period consisting of DMEM, sodium pyruvate $1.0x10^{-3}$ M, ascorbate-2-phosphate 0.17 mM, proline 0.35 mM, ITS 1X, and supplemented with 10 ng/ml TGF-β1 (Sigma-Aldrich Co. USA) for 2 days followed by 100 ng/ml BMP-2 (R&D BioSystems, USA) for 3 days prior to *in vivo* implantation (ASC + GF); another group cultured with the same medium but without TGF-β1 and BMP-2 (ASC).

2.2. RABBIT ARTICULAR CHONDROCYTES ISOLATION AND EXPANSION

The chondrocytes isolation and expansion protocol was based on previous reports from Crawford *et al*[35] Rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corporation, Finland). After

the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the joint area of the left posterior leg and disinfected with povidone iodide. Incisions were performed through the skin and muscle to access the articular capsule. Articular cartilage was harvested from the femoral condyles of the rabbits and washed in sterile phosphate buffered saline (PBS) with 1% antibiotic (antibiotic-antimycotic 15240, Initrogen Corporation, USA). The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. The cartilage pieces were immersed in trypsin-EDTA solution for 15-20 min at 37ºC under constant agitation. Trypsin was removed, the pieces were washed with DMEM and sterile collagenase type II solution (2mg/ml) prepared in basic medium was added. The mixture was kept for 8-10 hours at 37ºC under constant agitation. The digested mixture was filtered using a 100 µm filter and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cell pellet washed with PBS and the cells counted using a hemocytometer. Cells were then collected by centrifugation and ressuspended in expansion medium consisting of DMEM, containing 10 mM HEPES buffer pH 7.4, 1% antibiotic, 20 mM L-alanyl glutamine, 1x MEM non-essential amino acids and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, UK). Human articular chondrocytes were plated into tissue culture flasks and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for expansion.

2.3. *IN VIVO* INJECTION IN RABBIT ARTICULAR CARTILAGE DEFECTS OF GELLAN GUM-CELLS SYSTEMS

Chondrogenic pre-differentiated rabbit adipose stem cells (ASC + GF), non chondrogenic pre-differentiated rabbit adipose stem cells (ASC) and rabbit articular chondrocytes (AC) were expanded until an adequate cell number was obtained for cells encapsulation and used at passage 2 under the following procedure. Gellan gum powder (G1910, Sigma-Aldrich Co. USA) was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 1.25% (w/v). The solution was progressively heated to 90ºC and kept at this temperature for 20-30 minutes. The temperature was progressively decreased to 42ºC and stabilised always under constant stirring. Articular cartilage full-thickness defects with a diameter of 4 mm were created in the medial septum of rabbit femur condyles. A 1 mm diameter hole was drilled to the subchondral bone in order to establish a link between the implant site and the

subchondral bone marrow (Figure 1). Briefly, the rabbits were pre-anaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corporation, Finland). After the confirmation of analgesia/anaesthesia the rabbits were subjected to tricotomy in the joint area of the right posterior leg and disinfected with povidone iodide. Incisions were performed through the skin and muscle to access the articular capsule. The defects were created as schematically detailed in Figure 1. The isolated cells were detached by trypsinisation, mixed with culture medium, and centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cells were ressuspended in sterile PBS solution, counted using and hemocytometer, and finally centrifuged as before. The cell number was calculated so that the final concentration after encapsulation was $10x10^6$ cells/ml. The supernatant was discarded and the cells pellet kept at the bottom of the tube were ressuspended in a small amount of PBS. The gellan gum 1.25% (w/v) solution was mixed at a temperature of 40-41ºC with the cell suspension for complete homogeneous dispersion within the gel and the mixture was then injected into the defect. A waiting time of 2-3 minutes was given for the gels to form *in situ*. Defects were also filled with gellan gum without cells and other defects were left empty. The experiments were conducted for periods of up to 8 weeks with data collection points at 1 week, 4 weeks, and 8 weeks. The incision sites were sutured and the rabbits transferred to heating recovery compartments and when the recovery from analgesia/anaesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum* without movement restrictions. At the established time points, the animals were euthanized by injection of an overdose of pentobarbital sodium (Eutasil® Ceva Sante Animale, France) and the defect sites were surgically exposed. These were subjected to macroscopic observation and afterwards processed for histological and molecular analyses.

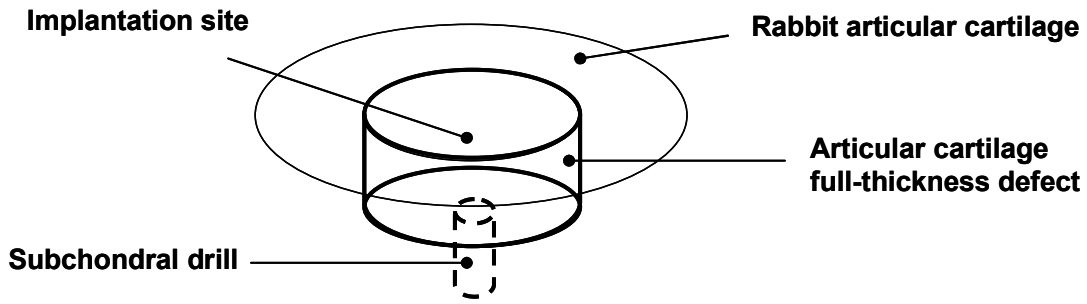

Figure IX.1. Schematic representation of the articular cartilage defect created in the rabbits femoral condyles.

2.4. HISTOLOGY

Common histological analysis was performed on 4 μm thickness sections of the samples collected after 1 week, 4 weeks, and 8 weeks of implantation. Hematoxylin-eosin (H&E) was conducted to observe general cell morphology and overall distribution, and alcian blue staining was performed to evaluate extracellular matrix components deposition, namely proteoglycans (glycosaminoglycans). The femoral condyles with the implanted systems were carefully dissected and samples for molecular analysis were collected. The femur condyles were then fixed in formalin for 30-40 minutes at room temperature, and kept in PBS at 4ºC. Histological processing was performed by dehydrating the samples in increasing ethanol concentrations, embedding them in paraffin and cutting sections for posterior analysis using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH). H&E staining was performed using an automatic processor according to in-house methodology (Leica TP1020-1, Leica MicroSystems GmbH) and alcian blue staining followed standard histological methods.

2.5. HISTOLOGICAL SCORING (PINEDA SCORING SYSTEM)

The Pineda scoring system[36] was used for histological evaluation of the explants collected after determined implantation periods. The degree and the quality of healing in all defects was assessed and scored blindly by 3 independent researchers. The graded parameters included filling of defect, reconstruction of osteochondral junction, matrix staining, and cell morphology. The maximum possible score in the Pineda scoring system is 0 points (increased regenerative potential) with a minimum of 14 points (decreased regenerative potential). More detailed information can be found in Table 1.

Table IX.1. Cartilage repair score by Pineda and co-workers.[30]

Characteristics	score
Filling of defect	
125%	1
100%	0
75%	1
50%	2
25%	3
0%	4
Reconstruction of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced staining	1
Significantly reduced staining	2
Faint staining	3
No stain	4
Cell morphology	
Normal	0
Most hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage, but mostly nonchondrocytic cells	3
Nonchondrocytic cells only	4

2.6. REAL-TIME PCR (SOX9, COL I, COL II, AGGRECAN)

Samples were collected after 1 week, 4 weeks, and 8 weeks of implantation upon exposure of the defect site area. The samples were obtained by collecting half of the implant from the implant site with the help of a scalpel. The results of this procedure can be seen on the histological analysis (Figure 2), being the reason for the observation of only one half of the implant. The collected samples were immersed in TRIzol® (Invitrogen, USA), and quickly stored at −80°C for posterior analysis. RNA was extracted using TRIzol® and more details can be found in the technical datasheet provided. Briefly, samples were grinded and mechanically homogenized with a mortar and pestle in TRIzol® reagent. Chloroform was then added and the samples centrifuged to

establish a three-phase composition in the tube and the aqueous phase was collected to a new tube and mixed with isopropanol. The samples were once again centrifuged, the supernatant discarded and the pellet washed with 75% ethanol. The samples were again centrifuged, let to air-dry, and suspended in ultrapure water for posterior analysis. The amounts of isolated RNA and A260/280 ratio were determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). After these determinations, a pre-determined amount of RNA from each sample was reverse transcribed into cDNA using the IScript™ cDNA synthesis kit (Bio-rad Laboratories, USA) in a BioRad CFX96 real-time PCR detection system (Bio-rad Laboratories, USA). Cartilage related markers were chosen to evaluate the chondrogenic phenotype of the cultured systems. These included Sox9, collagen type I, collagen type II, and aggrecan, using GAPDH as the housekeeping gene for normalization. The expression of each gene was normalized to the GAPDH value in that sample. The relative gene expression quantification was performed using the $2^{-\Delta\Delta Ct}$ (Livak) method, considering that:

$$2^{-\Delta\Delta Ct} = \text{Normalized expression ratio}$$

All the primer sequences were generated using Primer3 software[37] and acquired from MWG Biotech AG, Germany. More details can be found in Table 2. Real-time PCR was performed using a BioRad CFX96 real-time PCR detection system (Bio-rad Laboratories, USA) and SYBR Green IQ™ Supermix (Bio-rad Laboratories, USA) to detect amplification variations. The analyses of the results were performed with CFX Manager Software - version 1.0 (Bio-rad Laboratories, USA).

Table IX.2. Primers used for real-time PCR analysis of rabbit gene expression associated with cartilage extracellular matrix formation.

Gene	Accession number	Left primer	Right primer
Sox9	AY598935	TTCATGAAGATGACCGACGA	GTCCAGTCGTAGCCCTTGAG
Collagen type I	D49399	TAAGAGCTCCAAGGCCAAGA	TGTTCTGAGAGGCGTGATTG
Collagen type II	D83228	CAACAACCAGATCGAGAGCA	GCTCCACCAGTTCTTCTTGG
Aggrecan	L38480	GAGGTCGTGGTGAAAGGTGT	GTGTGGATGGGGTACCTGAC
GAPDH	NM_001082253	AGGTCATCCACGACCACTTC	GTGAGTTTCCCGTTCAGCTC

3. RESULTS

3.1. HISTOLOGY

The explants collected after 1, 4, and 8 weeks of implantation were analysed using hematoxylin-eosin and alcian blue staining. As previously referred in the Materials and Methods section 6., only half of the implants can be observed on the histological sections since the samples for RNA isolation and real-time PCR analysis were performed using the other half of the implant. Hematoxylin-eosin is a general staining for the majority of the cells that provides important information regarding their morphology, distribution and integrity. The results presented in Figure 2 show that the injected gellan gum hydrogels containing the different cell types studied kept in the defect after injection and unrestricted movement of the animals. The implants appear to be well integrated in the defect, both in the osteochondral junction and with the lateral native articular cartilage. This observation is evident in all the systems with the exception of gellan gum alone where the inclusion and integration is apparently weaker. Regarding cell morphology, similar results were found for ASC + GF and ASC, with the systems containing AC presenting a slightly different phenotype when compared to the native cartilage. Gellan gum alone and the empty defect presented the less similar morphologies, exhibiting only some focal spots of hyaline like chondrocytes, which were detected more frequently in gellan gum hydrogels alone. In terms of overall cartilage tissue structure, the newly formed tissues that mostly resembled the articular cartilage phenotype seem to be ASC + GF and ASC. Although these were quite similar with AC, the latter presents more frequently some fibrocartilage regions dispersed within the implant area. These fibrocartilage areas were detected to a greater extent in gellan gum alone, and represent the majority of the new tissue formed in the empty defects. Alcian blue staining identifies sulphated glycosaminoglycans in a histological section of a construct being a standard procedure for these purposes in cartilage tissue engineering analysis. Sulphated glycosaminoglycans are important for their water retention ability that contributes to the mechanical performance of the tissue. Sulfated glycosaminoglycans were detected in histological sections of all the explants, although with different extents (Figure 3). The staining was pronouncedly metachromatic in all the study samples and was present mostly in the areas not in close contact with the synovial capsule

where a different mechanical environment is present. Regarding the regularity of the staining profiles, gellan gum with ASC + GF and ASC were found to be the most uniform extending from the centre of the defect site to the contact with the lateral cartilage present on each side.

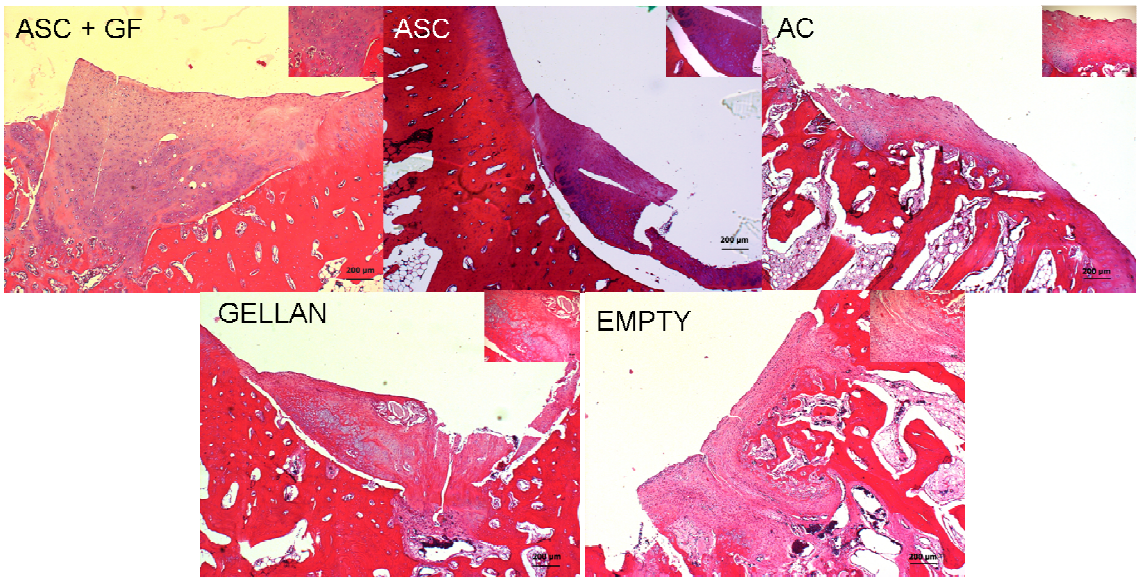

Figure IX.2. Hematoxylin-eosin staining of histological sections of the explanted gellan gum hydrogels with the different cell types after 8 weeks of culture.

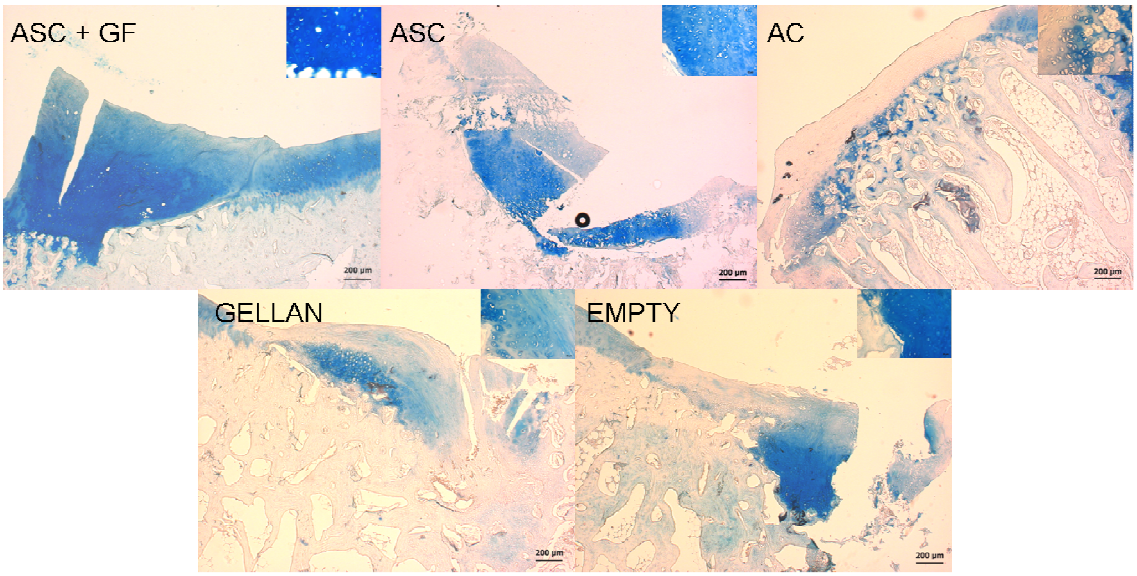

Figure IX.3. Alcian blue staining of histological sections of the explanted gellan gum hydrogels with the different cell types after 8 weeks of culture.

3.2. HISTOLOGICAL SCORING (PINEDA SCORING SYSTEM)

Histological score systems are frequently used to grade the healing process and tissue quality of cartilage defects. These semi-quantitative systems provide reliable information on the pathophysiological condition of the tissue being investigated and have good reproducibility. Among the various scoring systems proposed, the Pineda score is considered to be one of the most reliable.[38,39] The results previously presented in the histological analysis find some reflection on this semi-quantitative analysis. Among the various systems studied, ASC + GF exhibited the best results in terms of quality of the newly formed tissue. When looking to the scores for 1, 4, and 8 weeks of implantation, ASC + GF systems show a continuous increase in performance culminating at the final time point where they were ranked the best within the five groups. Gellan gum hydrogels with encapsulated ASC have demonstrated some variability throughout the implantation stages, but considerably improved after 8 weeks being ranked after ASC + GF. The variations within AC systems occurred in a smoother way presenting increasing performances from 1 to 4 weeks, and stabilising afterwards. These systems were positioned after ASC + GF and ASC in terms of cartilage tissue quality. The gellan gum hydrogels alone showed slightly decreasing qualities of the newly formed tissue, maintaining nevertheless some stability in terms of the scoring. Finally, the empty defects exhibited an improvement tendency with time more noticeable at 4 weeks of implantation. From 4 to 8 weeks, no relevant variations are encountered and this system was ranked the worse regarding the quality and performance of the newly formed tissue.

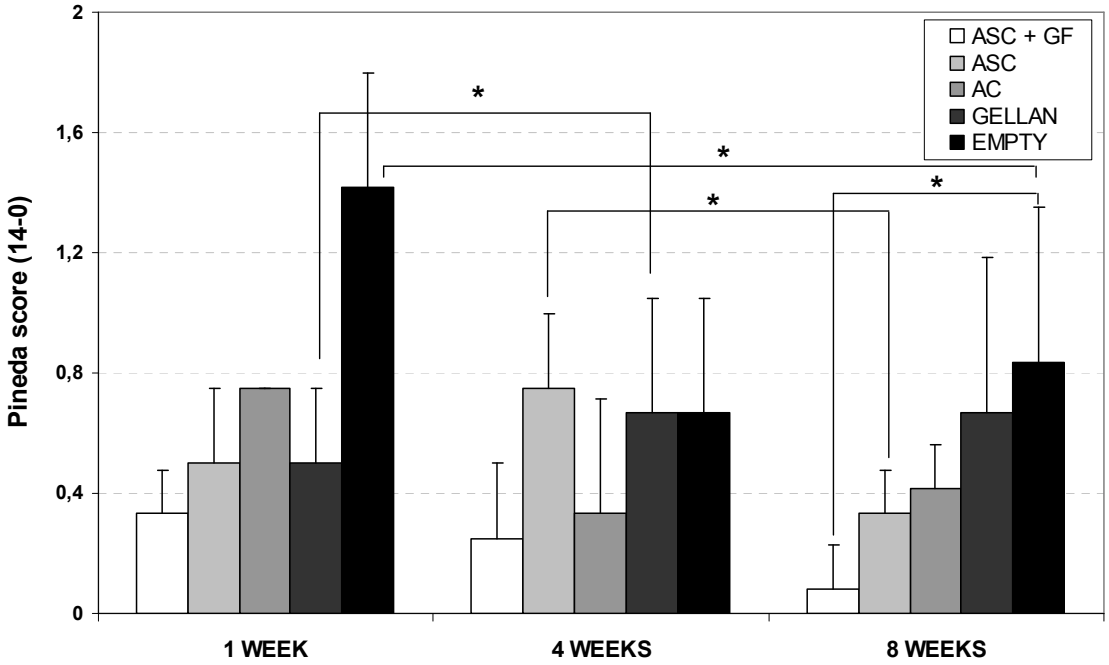

Figure IX.4. Graphical analysis of the results obtained with the Pineda scoring system on the explanted gellan gum-cell systems after 1, 4, and 8 weeks of implantation.

3.3. REAL-TIME PCR (SOX9, COLLAGEN TYPE I, COLLAGEN TYPE II, AGGRECAN)

Real-time PCR analysis is commonly applied to assess the upregulation and downregulation of specific genes related to the tissue being studied. In articular cartilage regeneration, several marker molecules are usually tested for variations through time, in order to provide a wider insight on the dynamics and nature of the tissue being formed. Sox9, collagens type I and II, and aggrecan are among those and their patterns of expression were analysed with this technique. GAPDH was chosen as the reference housekeeping gene. In each sample, the cycle threshold (Ct) value was normalised to the Ct value of the reference gene (GAPDH). In the gellan gum hydrogels with encapsulated ASC + GF, no Sox9 expression was detected. Collagen type I levels were residual at 4 weeks and were not observed after 8 weeks of implantation. Regarding collagen type II, an upregulation of approximately 30-fold was noticed from 4 to 8 weeks, and the same upregulation profile was identified for aggrecan, in this case with a 9-fold increase. ASC revealed no Sox9 expression at all time points, and collagen type I was also maintained at residual levels. Collagen type II experienced a 58-fold upregulation, and aggrecan evidenced only a slight increase from 4 to 8 weeks of implantation. Regarding the systems with articular chondrocytes, no Sox9 was again detected, and collagen type I was noticed after 4 weeks but

was downregulated approximately 4-fold after 8 weeks. The same pattern of variation was observed with collagen type II and aggrecan, which exhibited a downregulation of 2-fold and 3-fold from 4 to 8 weeks of implantation. Gellan gum alone did not present Sox9 transcripts and collagen type I levels were also residual. Collagen type II and aggrecan showed an upregulation of 13-fold and 2-fold, respectively. Finally, the empty defect showed no existence of Sox9, and evidenced a slight upregulation of collagen type I levels from 4 to 8 weeks. Collagen type II was upregulated 1.6-fold and aggrecan was also upregulated 1.8-fold. In terms of higher gene transcription levels after 8 weeks, the empty defect group showed the highest values for collagen type I; AC, followed by ASC + GF and ASC presented the highest collagen type II expression profiles, and ASC + GF followed by AC had the best results regarding aggrecan levels.

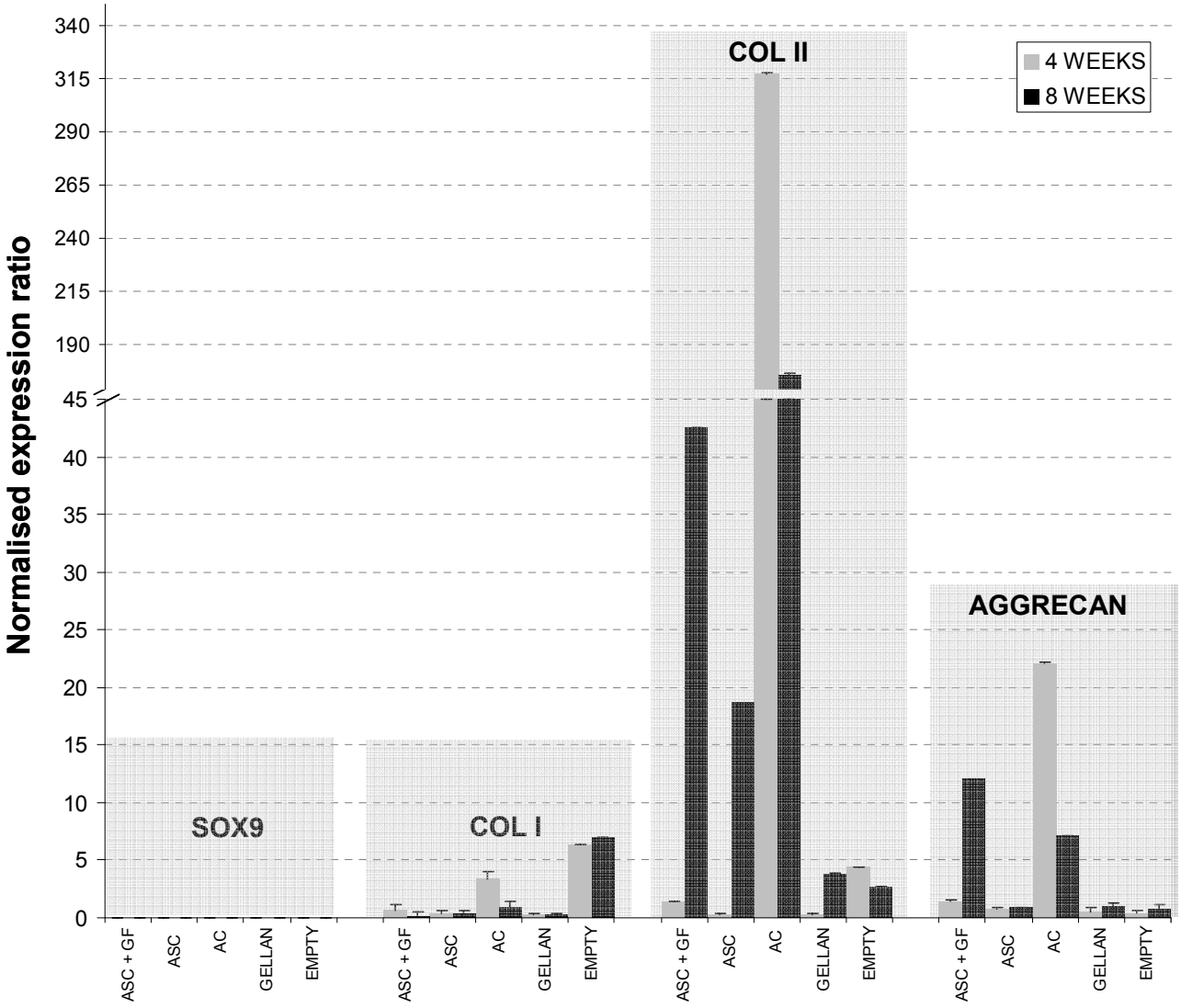

Figure IX.5. Graphical representation of the real-time semi-quantitative PCR analysis results for Sox9, collagen type I, collagen type II, and aggrecan performed on the explants collected after 1, 4, and 8 weeks of implantation.

4. DISCUSSION

The regeneration of cartilage tissues, mainly articular cartilage, is still a difficult task to achieve. The research strategies on this matter have been numerous,[4-10] and the search for an efficient alternative to the drawbacks of current therapies is still ongoing. Tissue engineering concepts are frequently suggested to address these conditions by making use of support structures, cells and cocktails of bioactive agents in controlled environments[7-10,40]. The importance of the cells used in such approaches is crucial since they are expected to form and maintain a newly formed tissue able to replace the damaged one, particularly due to the low regeneration ability of cartilage. In cartilage tissue engineering, most options rely on the use of either primary culture chondrocytes[41,42] or stem/progenitor cells.[43-45] The use of chondrocytes is limited by problems such as the scarcity of the tissue, rejection by the host, and graft-versus-host disease.[46] Stem/progenitor cells brought new possibilities to the cartilage tissue engineering field due to their potential to form different tissues, low immunogenicity, and higher cell division potential.[34,47,48] Adipose stem cells (ASCs) are considered a promising alternative to fulfil the role of chondrocytes in these applications and their potential has been previously evaluated.[49-51] Erickson *et al*[32] observed significant production of cartilage matrix molecules when examining the chondrogenic potential of human adipose tissue derived stromal cells cultured in alginate and implanted *in vivo* in nude mice.

Based on the previous considerations, this work tried to mimic a close to real clinical scenario where adipose stem cells are isolated, expanded *in vitro*, and administered with an adequate support *in vivo* in an autologous way. In this sense, the patients' own adipose stem cells are used in the treatment of the cartilage defect, surpassing the problems of cell scarcity and immunological rejection. Gellan gum is a recently proposed biomaterial that has proven adequate for these applications.[28,31] It can be prepared in a non harmful environment to cells, efficiently mixed with them, and finally injected *in vivo* in a minimally invasive manner gelating at the defect site under physiological conditions and serving as a support for cartilage tissue formation. Considering the potential of this biomaterial and the possibilities granted by adipose stem cells, five study groups were defined in order to evaluate the cartilage regeneration potential of gellan

gum combined with ASCs in the treatment of New Zealand White rabbits articular cartilage defects: a) chondrogenic pre-differentiated rabbit adipose stem cells (ASC + GF); b) rabbit adipose stem cells (ASC); c) rabbit articular chondrocytes (AC) (standard control); d) gellan gum alone (control); e) empty defect (control). The chondrogenic pre-differentiation of rabbit ASCs was performed to confer an improved performance to these cells and to compare them with non chondrogenic pre-differentiated ASCs. TGF-β1 and BMP-2 are common growth factors used in cartilage repair strategies and their use has been found to be beneficial for cartilage tissue formation in adipose and other progenitor cells.[52-57] Hanada et al[58] observed that combined treatment with TGF-β1 and BMP-2 induced time-dependent mRNA expression of aggrecan protein and type II collagen in rat periosteum-derived cells. Unlike with the use of BMP-2 alone, cell aggregates incubated with both TGF-β1 and BMP-2 showed no expression of type X collagen at later times in culture. In the present work, the animals were sacrificed after defined time periods and the defect sites were observed macroscopically as a preliminary assessment of the regenerative potential of the gellan gum systems. After 1 week, the implants were similar in appearance, exhibiting a faint opaque white colour. This occurrence was more pronounced and homogeneous in gellan gum discs with encapsulated cells (ASC + GF, ASC, AC). Even so, the gel transparency was still noticeable in all systems. After 4 weeks, the white opaque nature of the defects was even more pronounced in the cell loaded systems when compared to gellan alone and empty defects, being this pattern also observed after 8 weeks. The defects were homogeneously filled and well integrated with the surrounding cartilage presenting smooth transition zones between the implant and the native tissue in ASC + GF, ASC, AC. The results for gellan gum alone and the empty defects were more heterogeneous. The morphological appearance was much similar to the lateral native cartilage in those groups than in gellan gum alone and empty defects, particularly after 8 weeks of implantation. These results clearly demonstrate a difference between the systems with cells and the systems without cells which were also confirmed by histological analyses. Histological sections of the collected explants were stained with hematoxylin-eosin providing data on cell distribution, morphology and integration of the implants in the defect site. The implants were all kept at the implantation site, even though unrestricted movement of the animals was permitted right after the surgeries. This fact demonstrates that these gellan gum systems are able to be injected, remain, and adapt to articular cartilage defects under normal load bearing conditions. A progression of tissue formation and integration was observed in all the systems throughout the implantation periods. Both the osteochondral junction and the lateral native cartilage presented a continuous tissue bridging with the implants. The best results were observed for the cell containing gels mostly in prolonged

stages of implantation. These results were somehow predictable since the cells incorporated are expected to confer an improved advantage in tissue formation and integration due to their autologous nature and the role they can develop in tissue rebuilding. Another evidence of the advantages of cell delivery is the result obtained for cell morphology and overall cartilage tissue structure, where ASC + GF and ASC clearly presented the best results. This suggests that not only the use of a cellular counterpart improves the performance of the forming tissue engineered constructs, but also the types of cells used can dictate this outcome. A constant observation in all the implants was the formation of fibrocartilage-like tissue in the top part of the implant. Such an event is probably due to the shear stress events that are constantly occurring in the gliding surface of cartilage parts. Hu et al[59] found evidences that the regional distribution fibrocartilage in rabbit jaw joint condyles was apparently optimized for withstanding tissue-borne shear stresses, which implicates the location of this tissue in specific shear stress spots. In other studies,[60,61] fibrocartilage was reported to be the main functional adaptation in resisting shear and compressive mechanical stress in a pathological inflammatory condition. It was observed that when stress levels were elevated, the quantity of fibrocartilage increased. These occurrences may be prevented in a clinical scenario through short-term immobilization of the injured limb post-surgery, thereby reducing the aggressive shear stress effects. The semi-quantitative histological scoring system initially proposed by Pineda et al[36] reinforced some of the conclusions obtained with macroscopic and histological analysis. The ASC + GF system had the best scores calculated from the average of the individual scores of 3 independent researchers. The quality of the tissue formed throughout time had borderline statistically significant improvements ($p=0.05$) from 1 to 8 weeks, a result that was also observed in ASC from 4 to 8 weeks ($p<0.05$), and in the empty defects from 1 to 8 weeks ($p<0.05$). Gellan gum on the other hand exhibited a statistically significant deterioration of tissue quality from 1 to 4 weeks of implantation ($p<0.05$). After 8 weeks of implantation, the best score is evident for ASC + GF being statistically different from ASC and the empty defect. The comparison of both subsets of ASC studied shows in fact the importance that the chondrogenic pre-differentiation environment has in the final performance of the constructs. Indeed, although ASC + GF and ASC ranked 1st and 2nd place, respectively, the effect of the culturing methodologies prior to culturing exerted its effect on the chondrogenic potential of the newly formed tissue. When compared to AC, although without a statistically significant difference, both systems with adipose stem cells have shown to perform better in terms of the progression of the quality and integrity of the newly formed tissue. A wider insight on the molecular events taking place inside each system was made available by alcian blue staining and real-time PCR semi-quantitative analyses that provided valuable information on the nature of the

ECM produced. The first showed that, although the presence of a metachromatic staining was present in all the explants, the most similar to the native tissue were gellan gum with ASC + GF and ASC. The staining profiles were the most uniform extending from the centre of the defect site to the contact with the lateral cartilage present on each side. Real-time PCR analyses did not detect Sox9 transcripts in any of the samples during the course of the experiments.

Sox9 develops an important role in cartilage formation being involved both at the early stage of mesenchymal condensations during embryogenesis and in the regulation of chondrocyte maturation towards hypertrophy.[62,63] Previous studies have suggested its association with the chondrocyte phenotype maintenance and collagen type II expression, although this is not completely consensual.[64,65] The confirmation of the hyaline nature of the newly formed tissue does not correlate well with the absence of Sox9 in the samples and the authors couldn't agree on a justification for these observations. Further studies should be conducted for a full gene transcription profiling of the implants, influence of in vitro expansion on markers expression, and tracking of other markers of the chondrocytes cell cycle.

Collagen type I presence only in the initial periods in AC and residual for ASC + GF, ASC, and gellan is in agreement with the formation of a cartilage tissue of hyaline nature. The detection and maintenance of collagen type I levels in the empty defects indicates otherwise. In fact, the creation of this defect is similar to both drilling and microfracture surgical techniques applied to the treatment of cartilage pathologies.[66-68] The outcome of this and similar surgical procedures is frequently the formation of a fibrocartilage tissue with inferior mechanical properties, and therefore the upregulation of collagen type I levels typical of fibrocartilage was expected.[67,69] Collagen type II and aggrecan presented the highest levels in gellan gum-AC systems although exhibiting a decrease from 4 to 8 weeks of implantation. This could be associated to an eventual hypertrophy of chondrocytes, which carries a decrease in collagen type II values.[70] Nonetheless, collagen type levels are still significantly higher than for all the other systems. The same is not true for aggrecan. ASC + GF presented the best results in aggrecan expression with a steadily upregulation until the final implantation period. The same pattern of variation was observed for collagen type II which opens good prospects for the generation of a fully functional tissue engineered construct since these two molecules are the agents that confer cartilage with its load bearing functions. ASC showed the same upregulation pattern in collagen type II but not in aggrecan which may be related with the prior chondrogenic stimulation given to ASC + GF. Hanada et al[58] observed that combined treatment with TGF-β1 and BMP-2 induced time-dependent mRNA expression of aggrecan core protein and type II collagen. Altogether, these results clearly show the success of gellan gum systems combined with different cell types in the

treatment of articular cartilage defects. The results indicate that the most promising outcomes can be obtained with adipose stem cells pre conditioned to chondrogenic differentiation *in vitro*. Their performance should be further optimised by varying the culture cocktails, exploiting other cells parameters (number, stage, subpopulation), and analysing their effectiveness in real clinical conditions.

5. CONCLUSIONS

Although recent, the field of cartilage tissue engineering has gone through constant developments and the research performed nowadays is taking this area one step further close to new clinical applications. In this work, we have shown that gellan gum hydrogels are promising candidates for these applications. This newly proposed natural biomaterial possesses interesting features that prompted its study for the regeneration of cartilage tissues. It was shown here that the combination of gellan gum hydrogels with different cell types produced articular cartilage-like tissue and was highly beneficial in the treatment of rabbit full thickness articular cartilage defects. The group of gellan gum with adipose stem cells subjected to a chondrogenic pre-differentiation period originated the most promising results when compared to the other groups. The potential revealed by this system imposes further tests on its performance and may clearly place them as possible future biomedical products to be applied clinically in human patients.

REFERENCES

1. Temenoff, J. S. and Mikos, A. G., Review: tissue engineering for regeneration of articular cartilage, *Biomaterials* 21 (5), 431-40, 2000.

2. Wolfe, F., Zhao, S., and Lane, N., Preference for nonsteroidal antiinflammatory drugs over acetaminophen by rheumatic disease patients: a survey of 1,799 patients with osteoarthritis, rheumatoid arthritis, and fibromyalgia, *Arthritis Rheum* 43 (2), 378-85, 2000.

3. Newman, A. P., Articular cartilage repair, *Am J Sports Med* 26 (2), 309-24, 1998.

4. Hunt, S. A., Jazrawi, L. M., and Sherman, O. H., Arthroscopic management of osteoarthritis of the knee, *J Am Acad Orthop Surg* 10 (5), 356-63, 2002.

5. Ge, Z., Hu, Y., Heng, B. C., Yang, Z., Ouyang, H., Lee, E. H., and Cao, T., Osteoarthritis and therapy, *Arthritis Rheum* 55 (3), 493-500, 2006.

6. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L., Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, *N Engl J Med* 331 (14), 889-95, 1994.

7. Athanasiou, K. A., Agrawal, C. M., Barber, F. A., and Burkhart, S. S., Orthopaedic applications for PLA-PGA biodegradable polymers, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 14 (7), 726-737, 1998.

8. Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T., Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold, *Biochemical and Biophysical Research Communications* 322 (1), 50, 2004.

9. Santos, M. I., Tuzlakoglu, K., Fuchs, S., Gomes, M. E., Peters, K., Unger, R. E., Piskin, E., Reis, R. L., and Kirkpatrick, C. J., Endothelial cell colonization and angiogenic potential of combined nano- and micro-fibrous scaffolds for bone tissue engineering, *Biomaterials* 29 (32), 4306-13, 2008.

10. Wan-Ju Li, H. C., Tzong-Fu Kuo, Hsuan-Shu Lee, Ching-Chuan Jiang, Rocky S. Tuan, Evaluation of articular cartilage repair using biodegradable nanofibrous scaffolds in a swine model: a pilot study, *Journal of Tissue Engineering and Regenerative Medicine* 3 (1), 1-10, 2009.

11. Langer, R. and Vacanti, J. P., Tissue engineering, *Science* 260 (5110), 920-6, 1993.

12. Rotter, N., Bucheler, M., Haisch, A., Wollenberg, B., and Lang, S., Cartilage tissue engineering using resorbable scaffolds, *J Tissue Eng Regen Med* 1 (6), 411-6, 2007.

[13.] Risbud, M. V. and Sittinger, M., Tissue engineering: advances in in vitro cartilage generation, *Trends Biotechnol* 20 (8), 351-6, 2002.

[14.] Tuzlakoglu, K., Alves, C. M., Mano, J. F., and Reis, R. L., Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications, *Macromolecular Bioscience* 4 (8), 811-819, 2004.

[15.] Lee, C. R., Breinan, H. A., Nehrer, S., and Spector, M., Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior in vitro, *Tissue Eng* 6 (5), 555-65, 2000.

[16.] Hutmacher, D. W., Scaffolds in tissue engineering bone and cartilage, *Biomaterials* 21 (24), 2529-2543, 2000.

[17.] Drury, J. L. and Mooney, D. J., Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials* 24 (24), 4337-4351, 2003.

[18.] Hoemann, C. D., Sun, J., Légaré, A., McKee, M. D., and Buschmann, M. D., Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle, *Osteoarthritis and Cartilage* 13 (4), 318-329, 2005.

[19.] Elisseeff, J., Anseth, K., Sims, D., McIntosh, W., Randolph, M., Yaremchuk, M., and Langer, R., Transdermal photopolymerization of poly(ethylene oxide)-based injectable hydrogels for tissue-engineered cartilage, *Plast Reconstr Surg* 104 (4), 1014-22, 1999.

[20.] Moorhouse R., C. G. T., Sandford P.A., Baird J.K. and Kang K.S., *PS-60: a new gel-forming polysaccharide* D.A. Brandt, Washington DC, 1981.

[21.] Jansson, P.-E., Lindberg, B., and Sandford, P. A., Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea, *Carbohydrate Research* 124 (1), 135-139, 1983.

[22.] Kang, K. S., Veeder, G. T., Mirrasoul, P. J., Kaneko, T., and Cottrell, I. W., Agar-Like Polysaccharide Produced by a Pseudomonas Species - Production and Basic Properties, *Applied and Environmental Microbiology* 43 (5), 1086-1091, 1982.

[23.] Grasdalen, H. and Smidsrod, O., Gelation of gellan gum, *Carbohydrate Polymers* 7 (5), 371-393, 1987.

[24.] Mao, R., Tang, J., and Swanson, B. G., Texture properties of high and low acyl mixed gellan gels, *Carbohydrate Polymers* 41 (4), 331-338, 2000.

[25.] Coviello, T., Dentini, M., Rambone, G., Desideri, P., Carafa, M., Murtas, E., Riccieri, F. M., and Alhaique, F., A novel co-crosslinked polysaccharide: studies for a controlled delivery matrix, *Journal of Controlled Release* 55 (1), 57-66, 1998.

[26.] Kubo, W., Miyazaki, S., and Attwood, D., Oral sustained delivery of paracetamol from in situ-gelling gellan and sodium alginate formulations, *International Journal of Pharmaceutics* 258 (1-2), 55-64, 2003.

[27.] Sanzgiri, Y. D., Maschi, S., Crescenzi, V., Callegaro, L., Topp, E. M., and Stella, V. J., Gellan-Based Systems for Ophthalmic Sustained Delivery of Methylprednisolone, *Journal of Controlled Release* 26 (3), 195-201, 1993.

[28.] JT Oliveira, L Martins, R Picciochi, PB Malafaya, RA Sousa, NM Neves, JF Mano, and RL Reis, Gellan gum: A new biomaterial for cartilage tissue engineering applications *submitted*, 2008.

[29.] J. T. Oliveira, T. C. Santos, L. Martins, R. Picciochi, A. P. Marques, A. G. Castro, N. M. Neves, J. F. Mano, and R. L. Reis, Gellan Gum Injectable Hydrogels For Cartilage Tissue Engineering Applications: In Vitro Studies And Preliminary In Vivo Evaluation, *submitted*, 2008.

[30.] J. T. Oliveira, T. C. Santos, L. Martins, M. A. Silva, A. P. Marques, A. G. Castro, N. M. Neves, and R. L. Reis, Subcutaneous Implantation In Nude Mice Of Gellan Gum Hydrogels Combined With Human Articular Chondrocytes For Cartilage Regeneration, *submitted*, 2008.

[31.] Smith, A. M., Shelton, R. M., Perrie, Y., and Harris, J. J., An initial evaluation of gellan gum as a material for tissue engineering applications, *Journal of Biomaterials Applications* 22 (3), 241-254, 2007.

[32.] Erickson, G. R., Gimble, J. M., Franklin, D. M., Rice, H. E., Awad, H., and Guilak, F., Chondrogenic Potential of Adipose Tissue-Derived Stromal Cells in Vitro and in Vivo, *Biochemical and Biophysical Research Communications* 290 (2), 763-769, 2002.

[33.] Im, G.-I., Chondrogenesis from mesenchymal stem cells derived from adipose tissue on the fibrin scaffold, *Current Applied Physics* 5 (5), 438-443, 2005.

[34.] Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H., Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng* 7 (2), 211-28, 2001.

[35.] Crawford, A., Dickinson, S.C., *Methods in Molecular Biology: Biopolymer Methods in Tissue Engineering* Humana Press Inc., US, Totowa, NJ, 2004.

[36.] Pineda, S., Pollack, A., Stevenson, S., Goldberg, V., and Caplan, A., A semiquantitative scale for histologic grading of articular cartilage repair, *Acta Anat (Basel)* 143 (4), 335-40, 1992.

[37.] http://frodo.wi.mit.edu/.

[38.] Moojen, D. J. F., Saris, D. B. F., Auw Yang, K. G., Dhert, W. J. A., and Verbout, A. J., The Correlation and Reproducibility of Histological Scoring Systems in Cartilage Repair, *Tissue Engineering* 8 (4), 627-634, 2002.

[39.] Dorotka, R., Bindreiter, U., Macfelda, K., Windberger, U., and Nehrer, S., Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair, *Osteoarthritis and Cartilage* 13 (8), 655-664, 2005.

[40.] J. F. Mano, R. L. R., Osteochondral defects: present situation and tissue engineering approaches, *Journal of Tissue Engineering and Regenerative Medicine* 1 (4), 261-273, 2007.

[41.] Tare, R. S., Howard, D., Pound, J. C., Roach, H. I., and Oreffo, R. O. C., Tissue engineering strategies for cartilage generation-Micromass and three dimensional cultures using human chondrocytes and a continuous cell line, *Biochemical and Biophysical Research Communications* 333 (2), 609-621, 2005.

[42.] Wang, Y., Blasioli, D. J., Kim, H.-J., Kim, H. S., and Kaplan, D. L., Cartilage tissue engineering with silk scaffolds and human articular chondrocytes, *Biomaterials* 27 (25), 4434-4442, 2006.

[43.] Diduch, D. R., Jordan, L. C. M., Mierisch, C. M., and Balian, G., Marrow stromal cells embedded in alginate for repair of osteochondral defects, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 16 (6), 571-577, 2000.

[44.] Salgado A.J., O. J. T., Pedro A.J., Reis R.L., Stem Cells in Bone and Cartilage Tissue Engineering, *Current Stem Cell Research & Therapy,* 1, 231-238, 2006.

[45.] Bianco, P. and Robey, P. G., Stem cells in tissue engineering, *Nature* 414 (6859), 118-121, 2001.

[46.] Risbud, M. V. and Sittinger, M., Tissue engineering: advances in in vitro cartilage generation, *Trends in Biotechnology* 20 (8), 351, 2002.

[47.] Deans, R. J. and Moseley, A. B., Mesenchymal stem cells: Biology and potential clinical uses, *Experimental Hematology* 28 (8), 875, 2000.

[48.] Bianco, P., Riminucci, M., Gronthos, S., and Robey, P. G., Bone marrow stromal stem cells: nature, biology, and potential applications, *Stem Cells* 19 (3), 180-92, 2001.

[49.] Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M., and Guilak, F., Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds, *Biomaterials* 25 (16), 3211-3222, 2004.

[50.] Nathan, S., De, S. D., Thambyah, A., Fen, C., Goh, J., and Lee, E. H., Cell-Based Therapy in the Repair of Osteochondral Defects: A Novel Use for Adipose Tissue, *Tissue Engineering* 9 (4), 733-744, 2003.

[51.] Betre, H., Ong, S. R., Guilak, F., Chilkoti, A., Fermor, B., and Setton, L. A., Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide, *Biomaterials* 27 (1), 91-9, 2006.

[52.] Palmer, G. D., Steinert, A., Pascher, A., Gouze, E., Gouze, J.-N., Betz, O., Johnstone, B., Evans, C. H., and Ghivizzani, S. C., Gene-Induced Chondrogenesis of Primary Mesenchymal Stem Cells in vitro, *Molecular Therapy* 12 (2), 219, 2005.

[53.] Kurth, T., Hedbom, E., Shintani, N., Sugimoto, M., Chen, F. H., Haspl, M., Martinovic, S., and Hunziker, E. B., Chondrogenic potential of human synovial mesenchymal stem cells in alginate, *Osteoarthritis and Cartilage* 15 (10), 1178-1189, 2007.

[54.] Park, Y., Sugimoto, M., Watrin, A., Chiquet, M., and Hunziker, E. B., BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel, *Osteoarthritis and Cartilage* 13 (6), 527, 2005.

[55.] Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M., and Yoo, J. U., In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells, *Exp Cell Res* 238 (1), 265-72, 1998.

[56.] Allison A. Worster, Brent D. Brower-Toland, Lisa A. Fortier, Stephen J. Bent, Janice Williams, and Alan J. Nixon, Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix, *Journal of Orthopaedic Research* 19 (4), 738-749, 2001.

[57.] Awad, H. A., Halvorsen, Y.-D. C., Gimble, J. M., and Guilak, F., Effects of Transforming Growth Factor b1 and Dexamethasone on the Growth and Chondrogenic Differentiation of Adipose-Derived Stromal Cells, *Tissue Engineering* 9 (6), 1301-1312, 2003.

[58.] Keigo Hanada, Luis A. Solchaga, Arnold I. Caplan, Thomas M. Hering, Victor M. Goldberg, Jung U. Yoo, and Brian Johnstone, BMP-2 induction and TGF-beta1 modulation of rat periosteal cell chondrogenesis, *Journal of Cellular Biochemistry* 81 (2), 284-294, 2001.

[59.] Hu, K., Radhakrishnan, P., Patel, R. V., and Mao, J. J., Regional Structural and Viscoelastic Properties of Fibrocartilage upon Dynamic Nanoindentation of the Articular Condyle, *Journal of Structural Biology* 136 (1), 46-52, 2001.

[60.] A. Rufai, J. R. Ralphs, and M. Benjamin, Structure and histopathology of the insertional region of the human achilles tendon, *Journal of Orthopaedic Research* 13 (4), 585-593, 1995.

[61.] Ferretti, A., Ippolito, E., Mariani, P., and Puddu, G., Jumper's Knee, *Am J Sports Med* 11 (2), 58-62, 1983.

[62.] Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B., Sox9 is required for cartilage formation, *Nat Genet* 22 (1), 85-9, 1999.

[63.] Lefebvre, V., Behringer, R. R., and de Crombrugghe, B., L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway, *Osteoarthritis Cartilage* 9 Suppl A, S69-75, 2001.

[64.] Aigner, T., Gebhard, P. M., Schmid, E., Bau, B., Harley, V., and Pöschl, E., SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes, *Matrix Biology* 22 (4), 363-372, 2003.

[65.] Tew, S. R., Clegg, P. D., Brew, C. J., Redmond, C. M., and Hardingham, T. E., SOX9 transduction of a human chondrocytic cell line identifies novel genes regulated in primary human chondrocytes and in osteoarthritis, *Arthritis Res Ther* 9 (5), R107, 2007.

[66.] Takao, M., Uchio, Y., Kakimaru, H., Kumahashi, N., and Ochi, M., Arthroscopic Drilling With Debridement of Remaining Cartilage for Osteochondral Lesions of the Talar Dome in Unstable Ankles, *Am J Sports Med* 32 (2), 332-336, 2004.

[67.] Anderson, A. F., Richards, D. B., Pagnani, M. J., and Hovis, W. D., Antegrade drilling for osteochondritis dissecans of the knee, *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 13 (3), 319-324, 1997.

[68.] Steadman, J. R., Rodkey, W. G., and Rodrigo, J. J., Microfracture: surgical technique and rehabilitation to treat chondral defects, *Clin Orthop Relat Res* (391 Suppl), S362-9, 2001.

[69.] Howard A. Breinan, S. D. M. H.-P. H. M. S., Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes, *Journal of Orthopaedic Research* 18 (5), 781-789, 2000.

[70.] Karoliina Pelttari, Anja Winter, Eric Steck, Katrin Goetzke, Thea Hennig, Bjoern Gunnar Ochs, Thomas Aigner, and Wiltrud Richter, Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice, *Arthritis & Rheumatism* 54 (10), 3254-3266, 2006.

SECTION 3.

CHAPTER X.

General Conclusions and Final Remarks

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GENERAL CONCLUSIONS AND FINAL REMARKS

The main objective of the work conducted in the scope of this thesis was the production and delivery of potential therapeutic systems for cartilage regeneration having as basis the concept of tissue engineering. The problem of cartilage regeneration affects millions of people worldwide and the need for efficient solutions is constant. Due to its nature, cartilage may be severely affected by trauma events, aging related degeneration, or developmental disorders, since the effective tissue regeneration is not always accomplished. As a direct result, severe restrictions are imposed to the patients regarding normal life and wellbeing. The search for these solutions was the main drive behind this thesis. Based on the tissue engineering concept, the generation of potential tools and solutions to help in the resolution of cartilage associated pathologies was pursued. Although several surgical procedures have been applied for the same purposes, the idea of engineering tissues through the use of a biomaterial support combined with cells in specific culturing environments is challenging and has gained recognised potential among the research community. Following this line of study, different supports with potential to be applied clinically have been investigated and tested with the ultimate goal of delivering therapeutic solutions for cartilage problems, and placing the knowledge as close as possible to the fabrication of a medical product to be applied. The experimental work present in this thesis can be broadly divided into 2 parts: 1) the testing of several natural based supports for cartilage tissue engineering; 2) the choice of one among those, which was put through an extensive analytical screening in terms of materials properties, *in vitro* studies, and finally *in vivo* functional studies. This last stage involved the implementation of an autologous approach in a relevant animal model in order to sustainably prompt its application forward into clinical trials and clinical application.

In the first part, we have tested several types of natural-based support structures. The choice for these types of materials lies in the advantages they may ultimately confer to the performance of the tissue engineered construct, since natural materials frequently exhibit better integration, less cytotoxicity, and most are present or display structural affinity with the native tissue. Along with the choice of testing different biomaterials initially, the decision on the types of cells to be used followed a line of increasing relevance in our opinion. These included cell lines and primary cultures of differentiated and undifferentiated cells, namely mesenchymal progenitor cells from a

mouse progenitor cell line, primary culture chondrocytes of bovine and human origin, and primary culture adipose tissue derived progenitor cells and chondrocytes from rabbits (autologous model). The first material was constituted by a blend of chitosan and polybutylene succinate (C-PBS) and was described in chapters III and IV. In chapter III, C-PBS scaffolds processed by compression moulding and salt leaching were seeded with BMC9 cells and cultured in an environment that stimulated their chondrogenic differentiation. The cells proliferated and actively colonised the 3D structure, while showing evidences of commitment towards the chondrogenic pathway. The overall results were considered fairly positive and the work was continued with this material in chapter IV, but using different processing technologies for the scaffolds fabrication.

The new structures were formed from fibres produced by microextrusion. This 3D organization was expected to perform better due to enhanced cell penetration and colonization, and also improved mass transfer and metabolic waste removal. A different cell source was chosen in order to obtain more relevant data for the possible applications of these systems, and therefore primary culture bovine chondrocytes were used. Following the same rationale, the posterior studies within this thesis were also conducted with primary culture isolated cells. The analysis of the performance of C-PBS fibre scaffolds showed they performed, enabling cell proliferation with extensive ECM deposition that presented typical features of a cartilage-like tissue.

As a way to increase the knowledge and know-how on other possible structures and associated analytical techniques, we also looked into the use of starch and polycaprolactone (SPCL) scaffolds. These have been previously used in bone tissue engineering studies by our group retrieving promising results, which made them a potential candidate for applications also in the cartilage tissue engineering area. In a similar way as the C-PBS fibre scaffolds, the SPCL structures also exhibited good performances regarding cell proliferation, colonisation and cartilage-like ECM. In fact, both C-PBS and SPCL fibre systems were compared with polyglycolic acid (PGA) scaffolds which are frequent standards in these studies. The results have been promising for both types of natural-based scaffolds, positioning them as valid alternatives to commonly used standards.

Even though the support structures studied so far performed well and revealed good potential, it was our intention to obtain a material that could be mixed with cells and injected into the body without any need of *in vitro* culturing, a step that was frequent with the previous studied systems. This system should also be minimally invasive and gel at physiological conditions, thereby not

being harmful to cells and surrounding tissues. The achievement of such characteristics with the previously studied materials would be compromised by their intrinsic features and therefore the decision to choose another material was taken.

In the second part, gellan gum was originally proposed and tested by us for these purposes. It possesses all of the abovementioned features, and in addition presents other points of interest. Gellan gum can be prepared and mixed with cells/drugs using very simple techniques and using as reagents only water and PBS. Moreover, it possesses carboxylic groups in the glucuronic acid residues that may be modified to confer improved functions. A final advantage to mention is its prior use *in vivo* in humans for other applications which may place it closer to a medical application in this field.

Given this, we tested its materials properties including ultrastructural analysis, mechanical properties and rheological behaviour, and cytotoxicity. *In vitro* experiments with human cells using agarose as a standard for comparative analysis, and preliminary *in vivo* evaluation of the reaction of a host organism followed.

Afterwards, the work proceeded to the *in vivo* evaluation of cartilage tissue formation using human articular chondrocytes encapsulated in gellan gum hydrogels and subcutaneously implanted in the back of nude mice, being the results promising in terms of the formation of a functional cartilage tissue engineered construct.

The final stage of the study with this biomaterial involved the mimicking as far as possible of a real clinical scenario. The New Zealand white rabbit model was used due to its consensual feasibility and previous existing studies for other materials on cartilage tissue engineering. Cartilage full-thickness knee defects were created on the rabbits being these considered representatives of a patient condition. Cells for the own rabbit were isolated, cultured, and finally implanted in the defect site in order to observe the regenerative potential of the developed systems. Such an experimental setup provided valuable information on the systems behaviour and performance in a close to real clinical scenario. The interpretation of the collected data enabled to confirm the effective therapeutic potential of gellan gum systems and conclude on their possible application as medical products in the future.

One of the components of the gellan gum systems, and those that granted it its potential in these experiments, were the cells used. We investigated the potential of adipose tissue derived progenitor cells as an alternative to primary cultured chondrocytes. As it is known, obtaining chondrocytes from a patient is not always simple, and the possibility of a donor supply carries rejection episodes, graft-versus-host disease (GVhD), and is not always available due to scarcity of biological samples. The accessibility of adipose tissue and its proven added value in the formation of differentiated cells and tissues, such as cartilage, led us to study it as part of the system to be implemented in the treatment of cartilage defects. The results were good with the ASC showing high-quality performances, even when compared to healthy chondrocytes from the control groups which again reinforced the previous data on the potential of the gellan gum systems.

As final remarks, it can be stated that the work performed and included in this thesis provided interesting studies on different types of natural based supports for cartilage TE, all having shown potential for further uses in the area. In fact, standard materials such as PGA and agarose were used in different studies for comparison purposes. The herein proposed and studied systems revealed highly suitable and constitute valid alternatives to them. Even so, the data on those is extensive when compared to those proposed. More research must be performed by other research units to strengthen and validate this data. From the initial screening of materials, we have delivered one that was fully characterised and was studied through *in vivo* functional tests where its therapeutic ability was verified. Gellan gum was originally proposed in this thesis for cartilage TE and the research work conducted has put it close to the clinical trials stage. Our filling of a patent on the applications of this material, also under the scope of this thesis, indicates that this should be the consequent step to take. In parallel, a thorough characterization of materials properties, reaction from the organism, and other related events should be taken in order to assure the safety of this material to patients and cope with the industrial requirements in which it may be included. The main objective of delivering therapeutic tools to solve cartilage regeneration problems in patients can then be finally reached. This path of discovery, experimental study, and generation of a potential solution was the main accomplishment of the work performed in this thesis.