

## Physicochemical Characterization of Novel Chitosan-Soy Protein/TEOS Porous Hybrids for Tissue Engineering Applications

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**Keywords:** Tissue Engineering of cartilage, Biodegradable, Sol-gel, Soy protein, Chitosan, Scaffolds, TEOS

**Abstract.** In this paper we report a new type of cross-linked porous structure based on a chitosan-soy protein blend system developed by means of combining a sol-gel process with the freeze-drying technique. The final structure was investigated by Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR), contact angle measurements and the morphology by scanning electron microscopy (SEM). The water uptake capability and the weight loss were measured up to 14 days and their mechanical properties were assessed with compression tests. Results showed that the addition of tetraethyl orthosilicate (TEOS) to the chitosan-soy protein blend system provide specific interactions at the interface between the two polymers allowing to tailor the size and distribution as well as the degradation rate of the hybrids. Finally, TEOS incorporation induces an increase of the surface energy that influences the final physicochemical properties of the materials.

### Introduction

Damaged or diseased cartilage is a major clinical problem since this tissue shows limited ability of self-repairing [1] and consequently leads to progressive debilitation, and decrease of patients quality of life [2]. Despite the appealing advantages in use autologous transplantation due to use of native hyaline cartilage containing mature chondrocytes [3], concerns still exist regarding the lack of available donor tissue [4], and morbidity that happens at the donor cartilage harvest sites [3]. Moreover, the difficulty to restore the chondral functions emerged the need to conduct experimental research with the goal to obtain an adequate healing of such defects. Such type of strategies comprise the Tissue Engineering approach as a mean to replace and restore, maintain or even improve the tissue functions by combining cells and scaffolds into which can be incorporated bioactive agents aimed at control cellular activities. For this purpose a highly porous scaffold with an adequate pore size, distribution and interconnectivity is required to allow cell ingrowth proliferation and differentiation in order to facilitate tissue regeneration [5]. Because of the above shortcomings, Tissue Engineering rapidly became to play an important role in tissue regeneration. Our group has been proposing for such type of application, the blending of natural polymers, such as chitosan and soy-protein [6, 7], in order to develop hybrid materials with tailorable properties. However, due to the immiscibility of the two phases some problems may arise. An approach to solve this problem comprises the increasing of the interfacial interactions by creating an intimate mixing between the two phases, through the sol-gel process. The chemical linkage of soy protein to chitosan arises from the availability of several reactive groups in the soy, such as  $-NH_2$ ,  $-OH$ , and  $-SH$  [8], that can enable the linkage to chitosan through a silica network. In addition to the possibility to control the degradation rate of the biomaterial it has also been demonstrated that the presence of silanol groups (Si-OH), arising from the hydrolysis of silane-coupling agents, can

induce the formation of bone-like apatite layer [9]. We believe that the herein proposed cross-linked sol is an attractive candidate for certain tissue engineering applications such as the regeneration of cartilage because of the ability to fill irregularly shaped tissue defects and easy of incorporation of cells and bioactive agents [5]. Interestingly, it is also possible to fabricate scaffolds and control its architecture using a freeze-gelation and freeze-drying methodologies [10].

In this work, cross-linked porous structures based on chitosan and soy protein blend were developed by means of combining a sol-gel process with the freeze-drying technique. A strong emphasis was placed on understanding the effects of chemical composition, *i.e.* concentration of silane-coupling agent, catalyst and time of reaction, on the overall physicochemical properties of the porous structures.

### Materials and Methods

Reagent grade chitosan (with a deacetylation degree of  $\sim 91\%$ , Vanson, USA), soy protein isolate (SI) (Loders Crocklaan, The Netherlands) and tetraethyl orthosilicate (TEOS, 99.999%, Aldrich) were selected for this study. Chitosan was dissolved in 0.2 M acetic acid solution at concentration of 4 wt% to obtain a homogeneous solution. Soy suspension (1 wt%) was prepared by slowly suspending the soy protein powders, under constant stirring, in distilled water with glycerol. After adjusting the pH to  $8.0 \pm 0.3$  with 1 M sodium hydroxide solution, the dispersion was heated in a water bath at  $50^\circ\text{C}$  for 30 min. Later on the two solutions were mixture together (75/25 wt% chitosan-soy protein) and homogenized, under constant agitation during 1 hour. Different amounts of TEOS and 0.5 M chloridric acid (HCl) solution were added to the blend, under constant stirring for 24 and 72 hours. Then, the sol-gel was poured into a Petri dish and allowed to dry at room temperature for several days. By its turn, the porous hybrids were obtained by transferring the sol-gel into a mould, freezing at  $-80^\circ\text{C}$  overnight, followed by freeze-drying for the period of 4 days to completely remove the solvent. The neutralization of both membranes and porous hybrids was performed by soaking in a 0.1 M sodium hydroxide solution for 10 min and washing abundantly with distilled water until reaching pH 7. The preparation conditions shown in Table 1 (blend formulations, TEOS concentration, and time of reaction) were varied to study its effect on the physicochemical properties of the porous hybrids. The characterization of the final porous structures or scaffolds was analyzed by Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) (Perkin-Elmer 1600 series equipment). The microstructure of all samples was examined by scanning electron microscopy with an attached energy dispersive electron probe X-ray analyzer (SEM-EDS) (Leica Cambridge S-360, UK). Water uptake and degradation tests were performed by soaking the scaffolds into a phosphate buffer saline solution (PBS) with pH of 7.4 at  $37^\circ\text{C}$  for times up to 14 days. The surface properties of the hybrids were assessed on membranes by means of static contact angle ( $\theta$ ) measurements using the sessile drop method with ultra-pure distilled water (polar) and diiodomethane (non-polar) (OCA equipment, Germany and SCA-20 software). The surface energy ( $\gamma$ ) was calculated by Owens and Wendt method. The mechanical properties were assessed using compression tests in dry state (Instron 4505).

Table 1: Experimental parameters used in the preparation of the porous chitosan-soy protein/TEOS hybrids.

| Sample | Blend chitosan-soy protein (g) | TEOS:HCl (wt%) | Reaction time (hours) |
|--------|--------------------------------|----------------|-----------------------|
| CS     | 79.2                           | -              | -                     |
| CST1   | 79.2                           | 1:1            | 24                    |
| CST2   | 79.2                           | 1:0.1          | 24                    |
| CST3   | 79.2                           | 1:1            | 72                    |
| CST4   | 79.2                           | 1:0.1          | 72                    |

### Results and Discussion

The analysis of most of the formulations under SEM revealed a homogenous dispersion of the soy in the chitosan. The SEM micrographs showed that all porous structures possess a well-defined

orientation and anisotropic porosity, with pore sizes ranging between 50 and 350  $\mu\text{m}$  (Fig. 1). The chitosan-soy protein/TEOS hybrids presented a different structure when compared with the CS, probably due to the presence of TEOS which provided specific interactions at the interface between chitosan and soy protein. These interactions allow a better miscibility of the blend system traduced in the increase on the size and uniformity of the pores as well as an increase of the interconnectivity. Thus, by changing the TEOS/HCl concentration it became possible to tailor both pore size and distribution.

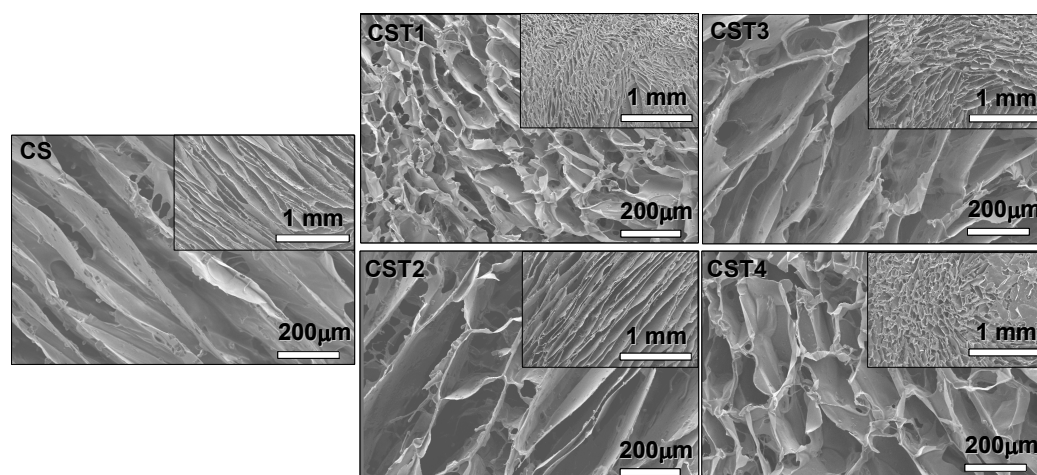


Fig. 1: SEM images of the porous chitosan-soy protein (CS) and chitosan-soy protein/TEOS (CST) hybrids listed on Table 1, prepared by means of a sol-gel with a freeze-drying method.

Fig. 2 shows the FTIR spectra of CS and CST materials, possessing the typical bands of chitosan and soy-protein at  $1700\text{-}1630\text{ cm}^{-1}$  and  $1580\text{-}1490\text{ cm}^{-1}$ , respectively assigned to amine I and II. Although, the FTIR analysis of CST revealed the appearing of the Si-O-Si asymmetric stretching occurring as a doublet at  $1140$  and  $1080\text{ cm}^{-1}$  (\*) which indicated that TEOS was present in the blend system.

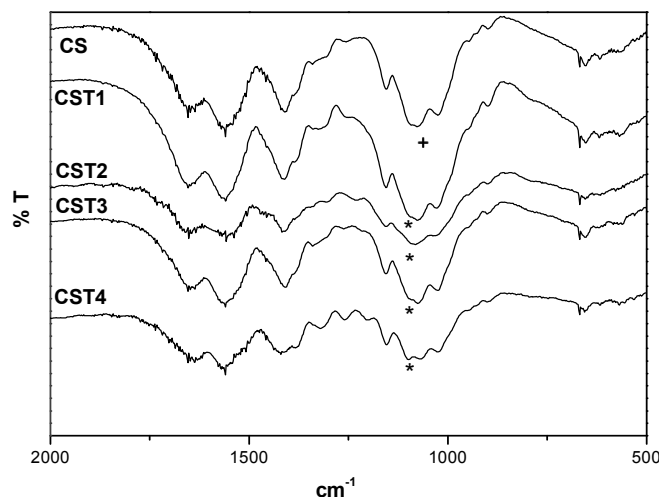


Fig. 2: FTIR spectra of the porous chitosan-soy protein (CS) and chitosan-soy protein/TEOS hybrids (CST) with the several formulations listed on Table 1.

The intensity of the Si-O-Si vibrational modes is weakly detected for CST hybrids due to the mixed stretching of Si-O-Si bonds that overlaps with the -C-O-C- (+) of the glycosidic linkage of chitosan. Due to small amount of TEOS used, the Si-OH groups which are known to promote the formation

of an apatite layer were not detected. Future  $^{29}\text{Si}$  NMR or XPS studies will be conducted to clarify the presence of these groups.

By soaking the materials in phosphate buffered saline (PBS) solution (pH 7.4) after 14 days, it was possible to assess the degradation profile of materials. Fig. 3A shows that the water uptake of CST hybrids increased when compared with CS due to the higher materials porosity. The weight loss data (Fig. 3B) shows that some of CST hybrids exhibit a lower degradation rate when compared with CS. This can be explained by two possible factors; (i) the formation of a siloxane network which prevented the material to degrade faster and (ii) due to different surface area of the porous structures. Moreover, when increasing the reaction time one can see that the materials degraded faster which may be explained by the increase of the surface area of the porous structures. These results suggest that, by increasing the reaction time, it was possible to tailor the degradation rate.

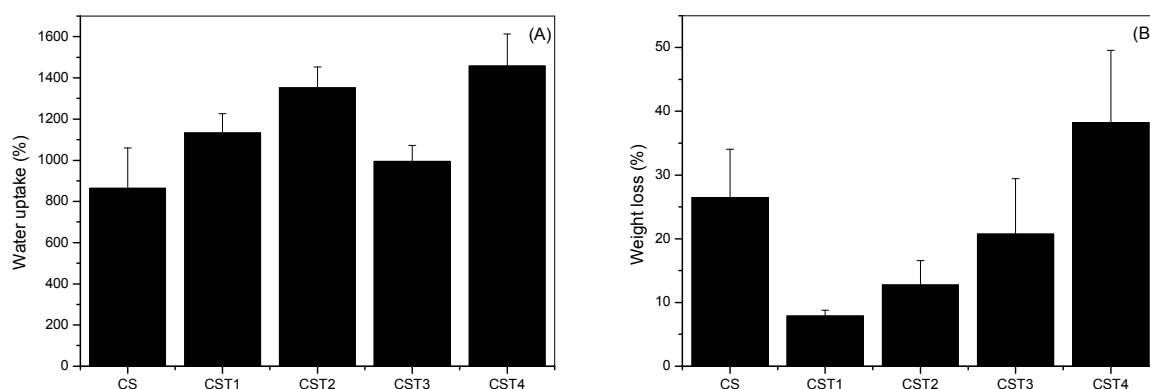


Fig. 3: Water-uptake (A) and weight loss (B) of the porous chitosan-soy protein (CS) and chitosan-soy protein/TEOS hybrids (CST) after soaking into a PBS solution after 14 days.

The measured contact angles and the calculated surface energy of the prepared membranes are summarized on Table 2. The contact angles of CST decrease when compared with those of CS. It has been reported that a high density of cross-linking considerably decreases the materials hydrophilic character [11]. Therefore, this fact may be related with the formation of a less cross-linked structure of the materials. Although, it is possible to observe that the expected trend was not detected for CST3. This can be related with the formation of a non-homogeneous structure. Despite, the surface energy of CST increases in comparison to CS which may be the result of the formation of a cross-linked structure.

Table 2: Contact angle ( $\theta$ ) and surface energy ( $\gamma$ ) of chitosan-soy protein (CS) and chitosan-soy protein/TEOS (CST) hybrid membranes.

| Membrane | $\theta_{\text{Water}} (^{\circ})$ | $\theta_{\text{Diodomethane}} (^{\circ})$ | $\gamma \text{ (mN.m}^{-1}\text{)}$ |
|----------|------------------------------------|---|-------------------------------------|
| CS       | $121.7 \pm 0.1$                    | $85.9 \pm 0.1$                            | $17.08 \pm 0.1$                     |
| CST1     | $98.1 \pm 0.1$                     | $72.5 \pm 0.1$                            | $21.6 \pm 0.1$                      |
| CST2     | $112.7 \pm 0.2$                    | $61.7 \pm 0.1$                            | $28.1 \pm 0.1$                      |
| CST3     | $119.1 \pm 0.3$                    | $79.3 \pm 0.1$                            | $21.2 \pm 0.9$                      |
| CST4     | $107.4 \pm 0.1$                    | $66.7 \pm 0.1$                            | $25.4 \pm 0.1$                      |

Compression tests (dry state) showed an increasing of the elastic modulus (secant at 1% strain) of CST1 and CST4 when compared to CS porous structures. Fig. 4A shows that chitosan-soy protein/TEOS based porous structures which reacted for the period of 24h (CST1) presented an increasing trend of E-modulus when the TEOS/HCl ratio increased from 1:0.1 to 1:1. This observation is mainly due the formation of a cross-linked structure due to the sol-gel reaction (hydrolysis and condensation of silane-coupling agent). When HCl is present at higher concentrations the hydrolysis and condensation of silane-coupling agent is more effectively achieved, thus promoting the formation of a stable siloxane network. However, when the reaction time increase from 24h to 72h, the formulation with the lowest TEOS/HCl ratio (CST4), presented an increased mechanical stability. These results may be explained by the change on the size and

orientation of the pores (see Fig. 1) which affected the final mechanical properties of the porous materials. In general, the scaffolds containing the siloxane network revealed a lower deformation behaviour (< strain %) when compared to the control CS (Fig. 4B). Thus, the siloxane network formed by sol-gel process resulted in the formation of a more rigid structure, which promoted a slight decrease of the strain.

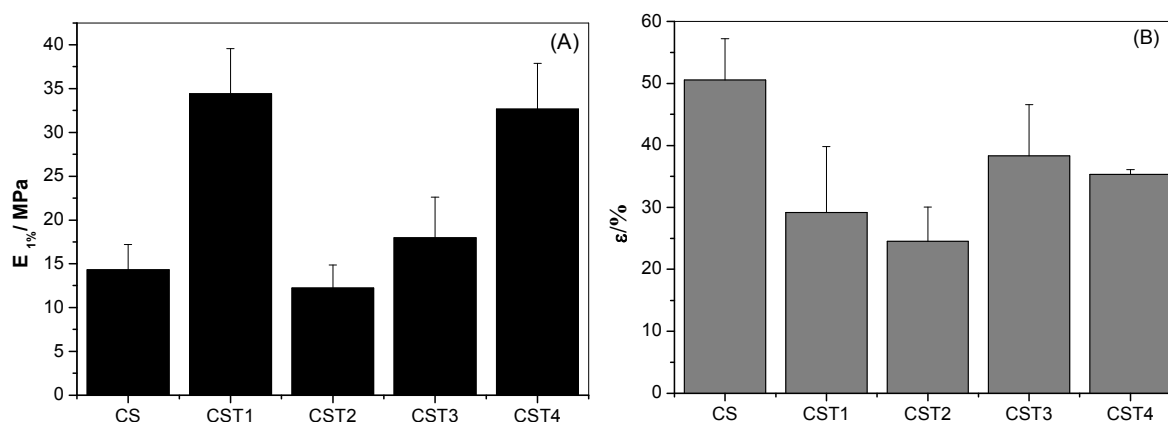


Fig. 4: Mechanical properties of chitosan-soy protein (CS) and chitosan-soy protein/TEOS (CST) hybrids obtained by compressive tests: modulus (A) and percentage of maximum strain (B).

## Conclusions

This work demonstrated that porous structures with a suitable porosity and adequate interconnectivity can be successfully prepared by means of combining a sol-gel with a freeze-drying method. We conclude that TEOS can be used to introduce specific interactions in the interfaces between chitosan and soy protein, and improve its mechanical stability and degradability.

These novel 3D porous structures exhibit physicochemical properties that appear to have potential to be used as a scaffold for tissue engineering of cartilage.

## Acknowledgements

S.S. Silva thanks the Portuguese Foundation for Science and Technology (FCT) for providing PhD scholarships (SFRH/BD/8658/2002) under POCTI program. This work was also partially supported by the European Union funded STREP Project HIPPOCRATES (NMP3-CT-2003-505758).

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