Editorial

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Journal of
3D Printing in Medicine

Current and future trends of silk fibroin-based bioinks in 3D printing

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"Enzymatic cross-linked SF-based bioinks are now a reality and are potentially one of the most promising and important tool kits in 3D bioprinting and tissue engineering."

First draft submitted: 27 March 2020; Accepted for publication: 2 July 2020; Published online: 9 July 2020

Keywords: 3D printing • bioink • enzymatic cross-linking • silk fibroin • tissue engineering

A key challenge facing healthcare in today's modern society is the change in global demographics attributed to the increasing number of age-related diseases, incidence of obesity and level of population activity [1]. There is, therefore, now an augmented pool of (early-stage) diagnosed patients, which is the main reason for two paradigm shifts, one occurring in medicine and another in the research arena [2,3]. There is now a trend in medicine focused on ending universal therapy through its progressive substitution with personalized approaches that can best fit each patient's needs and the anatomical requirements of the increasingly aged and active population. Accompanying that new trend, there is a great need for the development of new drugs and vaccines. A paramount example is the recent worldwide search for a fast-therapeutic solution for addressing pandemics, such as the one caused by COVID-19. The current demand for new treatments is unprecedented and is pushing the pharmaceutical industry toward decreasing both drug development time and costs. Moreover, such demographic changes are greatly impacting both the 3D printing and tissue engineering research fields [4]. In research, 2D approaches poorly correlate with the human condition and are actually far from mimicking tissue complexity, as compared with the possibilities involving 3D *in vitro* models or 3D-related technologies (e.g., microfluidics and bioreactors). There is, therefore, much research going in to the development of reproductible 3D cell-culturing solutions [5–7].

Bioengineered 3D microsystem technologies and tissue engineering solutions are relatively new and still require a great deal of effort to validate and characterize their properties and suitability, and present many challenges in respect to reproducibility requirements for practical biomedical applications. The 3D bioprinting technique was inspired by these needs and paradigm shifts, in other words, necessity of producing biofunctional 3D scaffolds with high reproducibility (especially with blends), versatility and adaptability to each patient's needs and disease. Importantly, the tissue-engineered constructs obtained using bottom-up approaches should be able to better resemble the wide range of human tissues we are aiming to repair/regenerate.

Despite the promising achievements in personalized tissue engineering due to recent advances in 3D bioprinting, we still face several challenges in the field. 3D bioprinting methods still possess several limitations related to: nozzle clogs; the fact that heating-based systems can damage both protein-based inks and encapsulated cells; methods are time consuming; and the poor resolution and lack of control over spatial distribution, in particular for achieving a 3D single-cell with surrounding ECM and high-resolution printing.

Thus, finding the right bioink and biofabrication method is a difficult and complex process, as the biomaterial/bioink must: be processable using available biofabrication instruments and techniques; be biocompatible with target tissues; not be immunogenic or toxic, to both host and transplanted cells; allow the combination of *in vivo* imaging and surgical methods; and have a production and printing process that is cost-effective and fairly easy for the user, in order to facilitate translation to the clinical setting.

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Among the myriad of biomaterials, *in situ*-forming injectable hydrogels are the most appealing when aiming to develop novel fast-setting bioinks, as they can be used as matrices loaded with cells or bioactive reagents. In this respect, the selection criteria for using a specific biomaterial and blend is of critical importance. Proteins of natural origin such as silk fibroin (SF) may have interesting applications as advanced bioinks, and the significant developments related to this topic are briefly discussed herein.

SF bioinks

Silk proteins can be obtained from different natural sources and consist of a fibrous core protein and glue proteins, the so-called fibroin and sericin, respectively [8]. SF purified from sericins by boiling silk cocoons in an alkaline solution, for example [9], has been attracting a great deal of interest as a biomaterial due to its biocompatibility, well-controlled degradability and versatile processability, thus being suitable for bioink preparation. Moreover, they can be processed as injectable hydrogels and have tunable mechanical properties, which are ideal for drug/cell encapsulation and delivery. In recent years, several robust SF bioinks and formulations obtained by physical and chemical methods, and ionic, photo- and enzymatic-induced gelation, alone or in combination, have been proposed for 3D printing/3D bioprinting (Table 1).

As summarized in Table 1, interesting outcomes are being achieved using different SF bioinks employing blending of SF with different biomaterials and different gelation methods. These involve either ionic- or photo-cross-linking, and physical/chemical gelation processes, but the enzyme-mediated *in situ* hydrogelation reactions have recently gained a new impetus. This is due to their specificity, efficiency and nontoxic nature. Our studies have shown that biomaterials containing Tyrosine (Tyr) groups can be used to prepare these *in situ*, forming amorphous and core–shell hydrogels mediated by the horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) complex [28,29].

When you consider the distinctive biological, physical and chemical properties of SF, the 5.3 mol% amino acid residues of Tyr present in this protein have been regarded as an asset for the production of enzymatic cross-linked SF (eSF) hydrogels with timely and spatially responsive properties. The eSF hydrogels were proposed as artificial biomimetic 3D matrices for different tissue-engineering applications, including hierarchical scaffolds, showing cell seeding, encapsulation and differentiation potential [30]. Thus, eSF hydrogels possess tunable mechanical and degradation properties and biological performance depending on how they are processed and according to the formulation of SF solution/enzyme/cross-linker or oxidizer concentrations.

At the 3Bs Research Group-University of Minho (Portugal), we demonstrated, for the first time, the possibility of applying the proprietary HRP-cross-linked SF as fast-setting bioinks in 3D printing [23,31]. The SF bioinks have shown unique biocompatible, elastic and adhesion properties capable of being printed in an amorphous state. Although the formed structures have shown excellent mechanical properties and memory-shape features after processing, some stability issues can be found during the printing process. Hence, the possibility of controlling the conformational changes from random coils to β-sheets in the HRP-cross-linked SF hydrogels, and improving their structural stability as bioinks and injectable matrices, will make these systems fundamental for the translational research and biofabrication of personalized and memory-shape implants. Ultimately, this should allow the creation of more realistic living artificial tissues and 3D *in vitro* tissue models [32]. The enzymatic cross-linking reaction allows tuning the viscoelastic and degradation properties of the eSF bioinks. However, the spontaneous random coil-to-β-sheet conformational transition of these hydrogels has been demonstrated to possibly affect cell viability and induce apoptosis [28]. Besides the possibility of playing with the blending of eSF bioinks, hybrid solutions making use of simultaneous printing of different bioinks is a promising research line that we are currently pursuing. Such strategies will allow us to address not only the tissue complexity challenges but also the biomechanical and hierarchical/segmental organization requirements of different tissues. In this sense, the combination of eSF and cell-laden Gellan gum-based hydrogels and bioinks are being developed for tissue engineering applications [23,33], with promising early results having already been obtained in the 3D bioprinting of meniscus tissue Oliveira *et al.* (Unpublished Data).

Conclusion & future perspective

The medical field has been revolutionized in recent years and a greater focus has been placed on addressing the need for personalized treatments, which has impacted the lines of research in the field of advanced manufacturing and tissue engineering. It is now clear that important scientific and technological gaps in biofabrication can be solved by advancing the biomaterials field. Enzymatic cross-linked SF-based bioinks are now a reality and are potentially one of the most promising and important tool kits in 3D bioprinting and tissue engineering. Accompanying

Table 1. Promising bioinks and blending strategies using silk fibroin that have been recently proposed for 3D printing and 3D bioprinting applications.

Type of bioink	Type of gelation	Application	Main features	Ref.
GelMA with SF particles	Photocross-linking	Tissue engineering (several tissues)	• Noncytotoxic • Useful in 3D bioprinting as it directly projects a feature onto the bioink	[10,11]
Sil-MA		Tissue engineering (several tissues)	• Tunable for viscosity and shows suitable properties for DLP printing • Can support cell viability beyond 85–95%	[12]
Photoinitiated polymerization of NVP monomer to PVP and interpenetrating network with SF macromolecular hydrogel		Drug delivery, tissue engineering in ophthalmology applications	• Fast gelation • Transparent • Controllable degradation • Not tested for cell encapsulation ability	[13]
Silk/PEG bioink	Physical/chemical cross-linking	Tissue engineering (several tissues)	• Support the proliferation of human bone marrow mesenchymal stem cells *in vitro* • Support fibroblast cells survival *in vivo*	[14]
Collagen-SF-blended bioink		Spinal cord injury	• 3D-printed collagen-SF scaffolds supported neural stem cells grown *in vitro* and *in vivo* • Scaffolds seeded with NSCs can promote the repair of injured spinal cord	[15]
Cross-linker-free silk-gelatin bioink		Cartilage tissue engineering	• Self-gelling ability • Good printing fidelity for fabrication of anatomical structures • Encapsulated chondrocytes remain viable • Tissue-engineered constructs demonstrate *in vitro* and *in vivo* biocompatibility	[16]
SF-PRP		Cartilage tissue engineering	• 3D-printed SF-PRP scaffolds possessed an adequate architecture and good biomechanical properties • Present proper degradation rate for cartilage tissue regeneration	[17]
Alginate-SF bioink	Ionic cross-linking	Tissue engineering (several tissues)	• Supports osteosarcoma cells viability *in vitro*	[18]
Gelatin-SF blended bioink		Bone tissue engineering	• Fibroin-gelatin-$CaCl_2$ bioink promotes the osteogenesis of bone marrow-derived progenitor cells (hMSCs)	[19]
Tyrosinase cross-linked silk-gelatin bioink	Enzymatic cross-linking	Musculoskeletal tissue engineering	• Supports the differentiation and viability of human nasal inferior turbinate tissue-derived mesenchymal progenitor cells	[20,21]
		Cartilage tissue engineering	• Positively regulates the expression of chondrogenic markers (aggrecan, COMP1) by mesenchymal progenitor cells	[22]
HRP cross-linked SF bioink		Musculoskeletal tissue engineering	• Fast setting • Bioprinted scaffolds possess good mechanical properties and memory-shape feature	[23]
		Intervertebral disc	• SF/elastin scaffolds possess adequate structural and mechanical properties similar to the native AF • HRP cross-linked SF bioinks support cell human adipose-derived stem cell metabolic activity and viability up to 21 days of culturing	[24]
HRP cross-linked by SF-TA or G-TA bioinks		Tissue engineering and cell delivery	• SF-TA and G-TA increase gelation kinetics compared with SF alone • Improved mechanical properties • Tunable enzymatic degradation in a concentration-dependent manner • Cyclic RGD and G-TA can improve morphology and metabolic activity of human mesenchymal stem cells *in vitro*	[25]
Blend of alginate-SF bioink	Two-step gelation	Tissue engineering (several tissues)	• Gelation consisting of enzymatic- and ionic cross-linking • Alginate used as a sacrificial matrix • NIH 3T3 fibroblasts proliferate and spread through the hydrogel after printing	[26]
GelMA and SF bioinks		Cardiac tissue regeneration	• 3D-printed scaffold possesses adequate elasticity and stiffness • 3D-printed constructs are nonimmunogenic *in vitro* and *in vivo* • Silk-based ink used as hydrogel allows the encapsulation of human-induced pluripotent stem cell-derived cardiac spheroids and creation of perfusable-vascularized channels	[27]

AF: Annulus fibrosus; DLP: Digital light processing; GelMA: Gelatin-methacrylate; G-TA: Tyramine-substituted gelatin; hMSC: Human mesenchymal stem cells; HRP: Horseradish peroxidase; NSC: Neural stem cells; NVP: *N*-vinyl-2-pyrrolidone; PEG: Polyethylene glycol; PRP: Platelet-rich plasma; PVP: Polyvinylpyrrolidone; SF: Silk fibroin; SF-TA: Tyramine-substituted SF: Sil-MA: Methacrylated silk-based bioink.

10.2217/3dp-2020-0005

these developments, radical 3D bioprinting methods for processing below cell dimensions have been introduced. These can, on the one hand, involve both biomaterial strategies and combinatory approaches using principles of contactless biology and tissue engineering, and on the other hand, facilitate both design and printability of existing bioinks and open up new and disruptive innovations for regenerative medicine applications. Importantly, further advances in functionalization of existing and novel bioinks toward addressing such vision will also constitute a hot topic of research in the near future.

Author contributions

JM Oliveira was responsible for the preparation of the manuscript.

Financial & competing interests disclosure

The author thank the financial support provided through the project NanOptoNerv (PTDC/NAN-MAT/29936/2017), financed by the Portuguese Foundation for Science and Technology (FCT) and COMPETE 2020. This work has been cofunded through the project 0624_2IQBIONEURO_6, Fundo Europeu de Desenvolvimento Regional (FEDER) through the Program Interreg V-A España-Portugal (POCTEP) 2014–2020. The FCT distinction attributed to JM Oliveira (IF/01285/2015) under the Investigator FCT program is also greatly acknowledged. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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