Conference paper

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Biofabrication of 3D constructs: fabrication technologies and spider silk proteins as bioinks

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Abstract: Despite significant investment in tissue engineering over the past 20 years, few tissue engineered products have made it to market. One of the reasons is the poor control over the 3D arrangement of the scaffold's components. Biofabrication is a new field of research that exploits 3D printing technologies with high spatial resolution for the simultaneous processing of cells and biomaterials into 3D constructs suitable for tissue engineering. Cell-encapsulating biomaterials used in 3D bioprinting are referred to as bioinks. This review consists of: (1) an introduction of biofabrication, (2) an introduction of 3D bioprinting, (3) the requirements of bioinks, (4) existing bioinks, and (5) a specific example of a recombinant spider silk bioink. The recombinant spider silk bioink will be used as an example because its unmodified hydrogel format fits the basic requirements of bioinks: to be printable and at the same time cytocompatible. The bioink exhibited both cytocompatible (self-assembly, high cell viability) and printable (injectable, shear-thinning, high shape fidelity) qualities. Although improvements can be made, it is clear from this system that, with the appropriate bioink, many of the existing faults in tissue-like structures produced by 3D bioprinting can be minimized.

Keywords: biofabrication; bioink; biomaterials; biomedical applications; 3D bioprinting; biotechnology; NICE-2014; spider silk.

Biofabrication

In 1907, a protocol was first described for maintaining the viability of isolated tissue outside of an organism [1]. This technique, called *in vitro* tissue culture, catalyzed a boom of biologically-based technology and debate over the possibilities and implications of this development. One of the most exciting technologies which emerged is tissue engineering. Traditionally, tissue engineering is the modular assembly of biomateri-

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als, cells and biochemical factors into tissue-like constructs [2]. Most accept the premise that in order to do this successfully one must, to some degree, mimic the properties of the target tissue. These constructs are immediately implanted or incubated *in vitro* prior to implantation. Relevant applications of tissue engineering include, but are not limited to: implants for regenerative medicine [3], *in vitro* models [4], biobots [5], and alternative food-sources [6]. Although tissue engineering has shown promise towards these applications, few have been approved for consumer use.

The high attrition rates of tissue engineered products are often hypothesized to be due to the modularity of the approach. It results in high variability in the spatial arrangement of the different components (biomaterials, cells, soluble and insoluble biochemical entities). This is problematic for presentation of factors to cells, which direct their behavior, as well as the architecture-dependent mechanical properties of these materials [7]. Due to the intimate relationship between structure and function in biological systems, which is observed across size scales, the success of tissue engineering is thereby limited by this poor control over hierarchical structures and their assembly [8, 9]. To overcome these limits, novel technologies have been established: cell-sheet technology, embedding or molding, centrifuge casting, dielectrophoresis, magnetic-force driven cell-motion, micro-fluidics, biospraying and 3D bioprinting (Table 1). Of these, perhaps the most interesting is the process of 3D bioprinting (3DBP). In this context, biofabrication can be defined as the automated,

Table 1: Published techniques in biofabrication and their basic, generalized process.

Technique	Basic process	References
Embedding or molding	1. Suspension of cells in polymer solution 2. Addition of crosslinker or induction of crosslinking conditions 3. Encapsulation of cells in crosslinked polymer solution, typically within a vessel which results in a defined 3D shape 4. Removal of construct from mold if necessary	[10, 11]
Centrifuge casting	1. Suspension of cells in polymer solution 2. Addition of crosslinker or induction of crosslinking conditions 3. Cell-polymer solution transferred to vessel with defined 3D shape 4. Centrifugation during polymerization of construct 5. Removal of construct from mold if necessary	[12]
Dielectrophoresis	1. Suspension of cells in a viscous polymer solution 2. Application of spatially non-uniform electric field 3. Movement of cells, depending on the set-up, towards low or high field intensities 4. Rapid polymerization of the solution, and encapsulation of cells	[13]
Magnetic-force driven cell-motion	1. Labeling of cells with magnetic nanoparticles 2. Cells cultured under magnetic field until monolayer formation 3. Repositioning of cell monolayer onto a magnetized, positive mold 4. Removal of cell-based constructs from mold	[14]
Micro-fluidics	1. Pre-fabrication of cell-laden constructs as 'building blocks' 2. Flowing of constructs through microfluidic channels to a collection site 3. Fusion of the constructs at the collection site	[15, 16]
Cell sheet	1. Culture cells on a 'smart polymer' surface until monolayer formation; many cultures are done in parallel 2. Release of an undisrupted monolayer from the polymer's surface upon external stimulus (e.g. UV) 3. Layering of monolayers to create 3D constructs	[17]
Biospraying	1. Suspension of cells in polymer solution 2. Placement of polymer solution into a chamber with a nozzle 3. Application of pressure resulting in a controlled spray of the material	[18, 19]
3D bioprinting	1. Generation of 3D image 2. Dissection of image into 2D layers 3. Translation of data to 3D printer 4. Layer-by-layer printing until construct completion 5. Post-processing if necessary	[20–22]

additive assembly of a biological construct by 3D patterning of cells and biomaterials in one processing step [23, 24]. Although each of the named methods has unique advantages, 3DBP is often considered the most valuable technique for tissue engineering/biofabrication due to it having the best spatial control over specific components of the system.

The purpose of this review is to give the theoretical framework of 3DBP, and based on this framework to critically evaluate the recent success of the technology with a particular focus on its use in printing silk-based bioinks; bioinks are materials which are compatible with the 3DBP process.

3D bioprinting (3DBP)

3D printing, first patented by Charles W. Hull in 1986, is rapid fabrication of physical, three-dimensional morphologies [25]. The process can be divided into five steps: (1) generation of a 3D image, (2) re-definition of the 3D image into a stack of 2D layers by an user-demarcated thickness, (3) interfacing this data with the printer, (4) printing a layer of the previously defined thickness one-by-one until the construct is complete, and (5) any necessary post-processing of the material [26–29]. The last step, post-processing, will be discussed in greater detail in later sections, as this is dependent on the material which is used. Although this process applies to most of the existing 3D printers, it should be said that this is a general description: there are many types of 3D printing. As such, the nomenclature for this field is broad, and there is great variety depending on the subfield. For example, some are based on the use of solid or liquid materials in the printing process, while others are based on how the 3D object is created, for example, by adding material a layer at a time (additive manufacturing) [30].

3D printing fitting the definition of biofabrication is referred to as 3D bioprinting (3DBP). Its anatomical elements include: the print head, the material cartridge, the actuator, the nozzle, the working area, and the print stage. The print head is the part which connects precise, motor-controlled movement with actuation of the material. The material cartridge holds the biomaterial and the cells to be printed under user-specified conditions. The actuator is some element which applies pressure to cause material deposition. The nozzle is the orifice, frequently a blunt needle, from which material is ejected. The print stage is the surface which the 3D scaffold is printed onto, and in many set-ups also provides further motor-control. The working area is the volume of space available for the construct. 3D bioprinters are most commonly classified based on their mechanism of material deposition: extrusion, inkjet, or laser-assisted bioprinting (LAB).

Extrusion 3DBP, sometimes referred to as direct-write printing, is a set-up where the mechanical or pneumatic pressure is applied to a cartridge of material to extrude a continuous solution [31]. In the case of inkjet 3DBP, heat or acoustic energy is used to propel droplets of solution; the pressure in the cartridge is kept constant with compressed gas [32]. In LAB, a high energy density beam is directed through a glass slide onto an energy absorbing layer, typically gold or titanium, and the focused energy causes the formation of a concave pocket in the material layer, and subsequently droplets or a jet being propelled towards a collector [33, 34]. Generally, the final printed volume is composed of single droplets; therefore they are correspondingly depicted in Fig. 1. Each of these actuation mechanisms has direct and indirect effects. The direct, downstream effects are on the materials which can be printed and on the shape of the volume which is printed (Fig. 1).

In order to be suitable for biofabrication, the most critical characteristics of a 3DBP process are that it is (1) cell-friendly, (2) reproducible and practical, (3) it allows for printing complex physical and chemical gradients, and (4) geometric structures. The performance of printers is typically evaluated by cell density and viability (fulfills requirement 1), process speed, resolution and accuracy (fulfills requirement 2), and the range of printable materials (fulfills requirement 3 and 4). How well these different printer set-ups generally perform will now be discussed based on these requirements.

Extrusion printing

From this basic set-up there are many interesting variations, for example coaxial needle design [37] or complex robotic joints to increase the degree of geometric freedom [38]. In biofabrication the resolution of

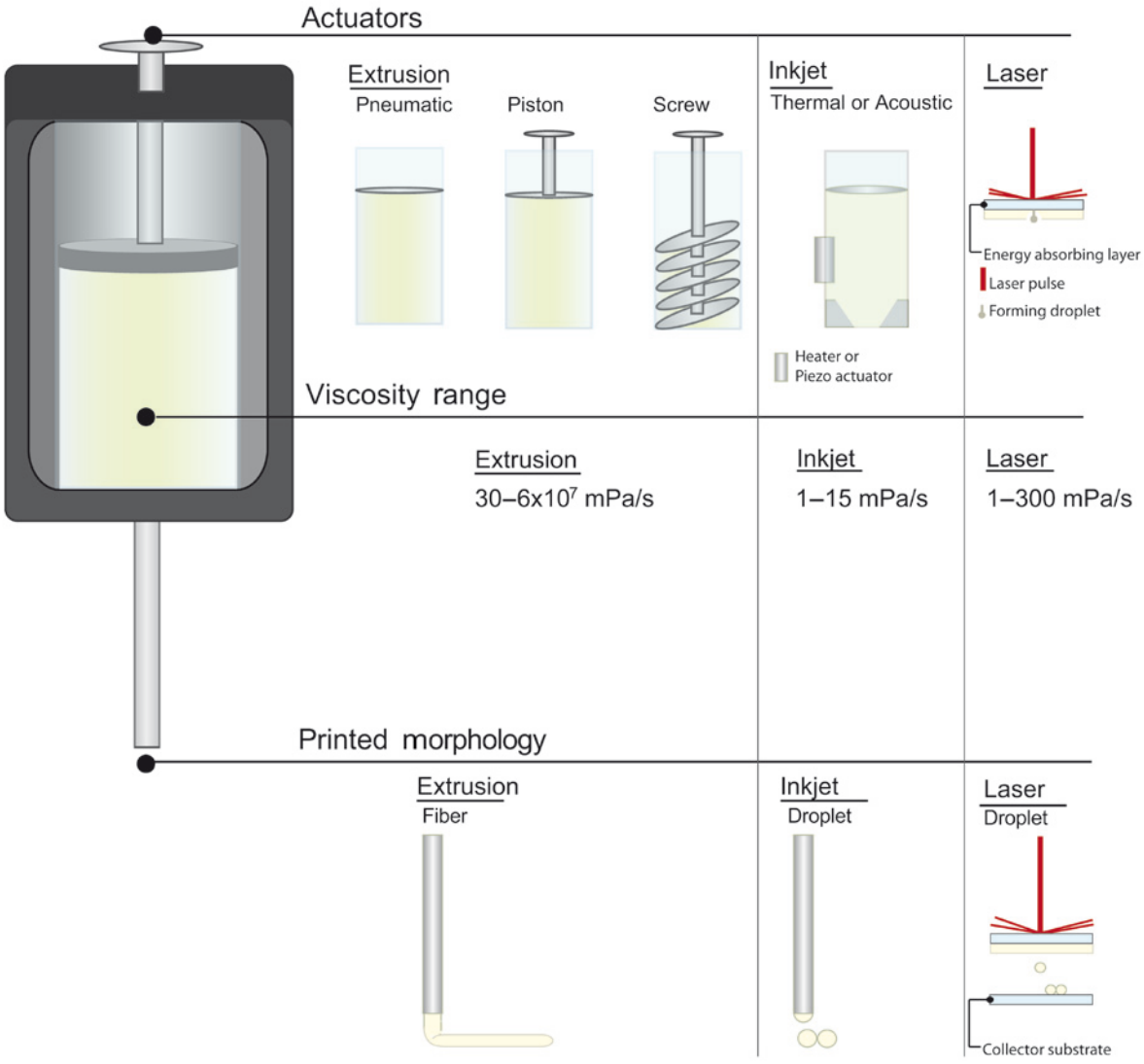

Fig. 1: The different types of 3D bioprinting set-ups. They are defined based on their mechanism of material deposition, the viscosity range of printable materials, and the morphology of the printed volume (i.e. fiber or droplet). These definitions are given in relation to a representation of a print head which shows the actuator, the housing, the material cartridge, and the nozzle (needle) [26, 32, 35, 36].

this technique is mainly limited by requirement 1 mentioned in the chapter above. Cell-free inks enable fiber diameters, and thus resolutions of this method, to be as small as 10 µm [39]. Using cell-loaded bioinks limits the nozzle diameter and leads to a decrease in resolution to dimensions in the range of 200 µm. This limitation in resolution is accompanied by an increase in fabrication speed, as such extrusion printing enables generating 3D structures of clinically-relevant sizes in a reasonable period of time [29]. In terms of the effects on cells, cells can be printed at densities of several million/mL, and there is a wide potential for cell viability post-printing; the cell viability ranges from as low as 40 % to as high as 97 % post-printing [26, 40]. Based on this broad range, which is also compared across similar processing conditions (temperature and shear stress), it is reasonable to conclude that cell viability is significantly affected by the bioink which is used. Further, the attractiveness of this type of system is the wide-range of printable materials. In general, providing printable, biocompatible materials is a greater challenge in the field than the printing technology itself, as will be discussed in the later section, Bioinks.

Inkjet printing

Inkjet systems are the next most commonly used technique for 3DBP. The variables considered for the printed volume (size, shape, speed) are pressure in the material cartridge, rate of nozzle opening and nozzle size. Its performance allows cell viability of ~85 % and a resolution of 50 μm [26]. Compared to the other methods discussed in this review inkjet printing based on commercially available inkjet printers suffers from the lowest cell density (typically <1 million cells/mL) which can be printed [26]. Inkjet printing is also limited to a narrow range of low material viscosities to avoid nozzle clogging or application of cell-damaging forces. There have been, however, some adaptations used to prevent these problems, for example, nozzle-free ejection [26].

Laser-assisted bioprinting (LAB)

LAB is the least commonly used technique due to the complexity of the set-up, and the fabrication systems not being commercially available. However, this should not indicate that it is not a valuable technique. A distinct advantage of LAB is the absence of nozzle clogging, allowing a wide-range of rheological material properties, although the non-dynamic viscosity range is limited compared to extrusion printing [36]. LAB has exceptional resolution in the 10-micron range without affecting the cell viability as compared to the other techniques. The process reliably has cell viabilities above 95 %, and can be used with cell densities of up to 10^8 cells/mL [26]. Unfortunately, in spite of these attractive features from the technical point of view, LAB alone is unable to reach clinically-relevant construct volumes in a reasonable timeframe. This is because of the low volume of printing material in the donor layer as well as of printed droplets. Therefore, LAB might be limited in its practical applications in tissue engineering in the future.

Bioinks

In 3D bioprinting (3DBP) the term “bioinks” is used to describe cell-encapsulating material-matrices which combine printability with cytocompatibility. These demands are quite high and often result in contradicting requirements, making bioinks one great challenge in biofabrication. An ideal bioink can be printed, has high shape-fidelity upon printing, is cytocompatible, and is tailored to its target tissue. Amongst studied bioinks, hydrogels have had the greatest tendency towards success [29].

The major physicochemical parameters determining the printability of a hydrogel are their viscosity and their rheological properties. During 3DBP, the bioink should extrude smoothly and undergo a rapid gelation after printing. If the bioink is already pre-gelled, then the printing process should not result in irreversible damage of the polymer network. Adequate mechanical properties, which can be tailored by polymer concentration or crosslinking of the hydrogel, are necessary to retain the designed and fabricated shape up to clinically-relevant sizes [41]. As previously stated, the requirements imposed by the technique for the bioinks tend to conflict with the biological requirements imposed by the cellular components. The final constructs should allow migration, proliferation and support targeted differentiation of encapsulated cells, which typically calls for a soft substrate. Additionally, the gelation process should be mild and cell friendly [20]. Finally, once the hydrogel precursors have been printed and the cells have survived, the scaffold must degrade at a pre-determined rate when exposed to physiological conditions found in the target tissue [42]. Refer to Fig. 2 for representation of these requirements.

Established bioinks

Existing bioinks include natural (e.g. alginate, fibrin, collagen and gelatin) and synthetic [e.g. poly(ethylenglycol) (PEG), polylactic acid (PLA)], polymers as well as modified versions of these polymers. The most commonly

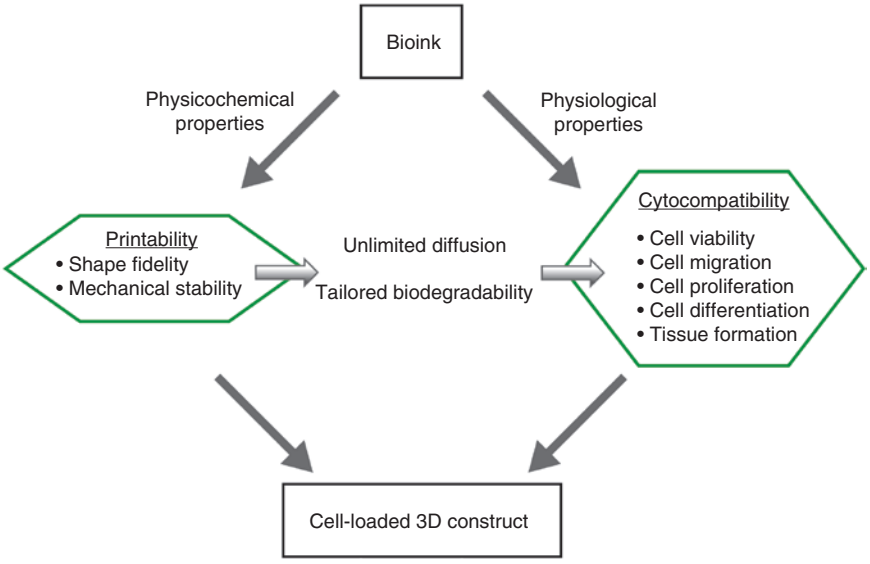

Fig. 2: Physicochemical and physiological requirements of the bioink. Physicochemical properties are related to the printability by the viscosity and macromolecular structure of the material. The printed construct should also allow for diffusion, relating the printed architecture to the cytocompatibility. The physiological activity is related to cytocompatibility by the degradation products, the behavior under physiological conditions, and the biological activity (e.g. cell binding motifs). The final product, the cell-loaded construct, should seamlessly combine these qualities.

used bioinks are the unmodified, natural polymers processed as hydrogels, and will therefore be the focus of this discussion; natural polymers are the only biomaterials whose fabrication process can be directly used for 3DBP [43, 44]. For more detailed information, refer to Table 2 and to Malda et al. [29].

Alginate is one of the most commonly used materials for 3DBP. As a biomaterial in general, alginate has been confirmed to be beneficial for cell viability and differentiation [42], as well as drug delivery [43, 45]. However, alginate-based bioinks also degrade rapidly, translate poorly when used with human-derived cells, and have a limited amount of bioactive binding sites [42, 43, 46]. The next most commonly used bioink is fibrin which has been used due to its success when cultured with neurons [22, 47] and the ability for autologous sourcing [48]. However, fibrin hydrogels possess poor mechanical properties for most applications and degrade before construct maturation [49, 50]. The last most commonly used hydrogel is collagen and its derivative, gelatin. Collagen possesses a major advantage in being biodegradable, biocompatible, easily available and highly versatile [32]. However, collagen-based bioinks show batch-to-batch variations, contraction of constructs, poor mechanical properties, are difficult to sterilize, and have poor water solubility [32, 51, 52]. In an attempt to maintain some of the positive biological activities while reducing these disadvantages, gelatin has also been developed as a bioink [53–55]. Although gelatin shows improvement of the water solubility and viscosity, the gel formation is solely based on physical intermolecular interaction of the gelatin molecules, and the resulting gels are not stable under physiological temperature. Additionally, these gels are also highly variable from batch-to-batch.

In order to expand the range of usable bioinks, there have been many modifications made to these polymers. The most common modifications are chemical ones or polymer blending [35]. Some examples of chemical functionalization include: methacrylation and acetylation of gelatin (modifies degradation) [54, 56], oxidation of alginate (modifies degradation) [42, 64], and synthesis of a block co-polymer comprised of poly(N-(2-hydroxypropyl)methacrylamide lactate) [p(HPMAm-lactate)] and PEG (improves biodegradability) [62]. Some examples of blends include fibrin and alginate (improves biological activity) [22, 54, 61, 65, 66], alginate and gelatin, alginate and gelatin in modified and unmodified forms [55], alginate, gelatin and hydroxyapatite (optimized for bone tissue engineering) [58], thermoresponsive poly(*N*-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAM) blended with methacrylated hyaluronan (HAMA) (to improve printability)

Table 2: Overview of existing bioinks, their gelation method, and their advantages and disadvantages.

Bioink	Gelation method	Advantages	Disadvantages	References
Alginate	Ionic	Biocompatible; supports cellular function and differentiation	Rapid degradation; lack of cell binding motifs	[42, 43, 45, 46]
Fibrin	Enzymatic	Biodegradable; rapid gelation; easy purification process	Poor mechanical properties; fast disintegration	[22, 47–50]
Collagen	Thermal	Biodegradable; biocompatible; availability; versatile	Limited sterilization techniques; batch-to-batch variations; poor mechanical properties	[32, 51, 52]
Gelatin	Thermal	Biodegradable; biocompatible; water soluble	Unstable at body temperature	[53–55]
Gelatin methacrylamide	Thermal/photo	Mechanically stable; biodegradable; biocompatible; water soluble	–	[54, 56]
Fibroin	Self-assembly	Biocompatible; biodegradable; robust mechanical properties	Lack of cell binding motifs or enzyme degradation sites; does not degrade under physiological conditions	[20, 57]
Recombinant spider silk	Self-assembly	Biocompatible; biodegradable; robust mechanical properties	Lack of enzyme degradation sites; does not degrade under physiological conditions	[40]
Bioinks optimized for specific applications by blending				
Alginate/gelatin/hydroxyapatite	Thermal/ionic/chemical/	Biocompatible; biodegradable; robust mechanical properties	–	[58]
Gelatin methacrylamide/hyaluronic acid	Thermal/photo	Mechanically stable; biodegradable; biocompatible; water soluble	–	[55]
Gelatin methacrylamide/gellan gum	Ionic/thermal/photo	Mechanically stable; biodegradable; biocompatible; water soluble	–	[59, 60]
Fibroin/gelatin	Enzymatic/thermal	Biocompatible; biodegradable; robust mechanical properties	–	[20, 57]
Fully synthetic bioinks				
Poly(ethylene glycol) dimethacrylate	Photo	Mechanically stable; cartilage applications	Low cytocompatibility	[61]
p(HPMAm-lactate)-PEG	Thermal/photo	Biodegradable; mechanically stable	Low cytocompatibility	[62]
Glycosaminoglycan-based	Thermal	Chondrogenic	Low viscosity; slow gelation; poor printing properties	[63]
HA-pNIPAAM-HAMA	Thermal/photo	Cytocompatible; fast, reversible gelation; structural fidelity	–	[63]

[63], gelatin with hyaluronic acid or gellan gum (to improve cell behavior towards bone tissue engineering, mechanical properties and printability) [55, 59, 60, 67]. However, even with these modifications, there is an urgent need for further development of bioinks to improve the mechanical properties, gelation process, cytocompatibility, degradation rate, tissue specificity, and adaptability to clinical set-ups.

Silk materials are particularly interesting for technical and biomedical use since they show absence of toxicity, slow degradation, low or absence of immunogenicity, and extraordinary mechanical properties [40, 68–70]. Silk-based biomaterials have been used for medical sutures and breast implant coatings [70–72], biosensing applications [73], and enzyme immobilization [74–76]. Recently, a silk-gelatin blend was used as a bioink [20, 57]. This composite was cytocompatible, crosslinked, showed improved mechanical properties (to gelatin alone), improved cell viability and differentiation (to gelatin, alginate and silk alone), and improved degradation rates (to alginate and gelatin) [20, 43]. However, it was impossible to print silk fibroin without additives; deposition of plain silk fibroin solutions leads to frequent clogging due to shear-induced β-sheet crystallization [57]. In contrast, compared to silk fibroin scaffolds, spider silk bioink can flow through the nozzle without clogging facilitating scaffold manufacturing [40]. This is due to the fact that hydrogels made of recombinant spider silk proteins are physically crosslinked by β-sheet structures and hydrophobic interactions and entanglements, which allows for reversible gelation upon shear-thinning [40, 77]. Further, due to the biotechnological production of recombinant spider silk proteins they can be genetically modified, e.g. with the cell binding motif RGD improving cell attachment [40, 78]. The combination of these mechanical and biological properties raises the number of applications of recombinant spider silk as a novel bioink.

Post-processing and crosslinking

Without delving into complex macromolecular chemistry, it is important to briefly discuss some of the options for solidifying materials in 3DBP when materials do not self-assemble. The basic requirements for a crosslinking process are that it must be rapid for shape-fidelity as well as non-toxic to cells. There are two basic types of crosslinking which can be used: physical or chemical. In the case of physical crosslinking, the most common approach is to maintain the conditions which stabilize the liquid phase in the material cartridge and the conditions which push it towards gelation in the working volume or a tandem print head. An example of this principle is printing a temperature-sensitive hydrogel onto a heated print stage [55]. The advantage of physical crosslinking is that it is often cell-friendly; the disadvantage is that the networks formed are typically weak and their degradation difficult to control. Due to these disadvantages most physically crosslinked hydrogels must be post-processed by chemical crosslinking, and this results in newly formed covalent bonds [29]. This is particularly true for inkjet printing, where the necessity of a low viscosity material mandates some type of post-processing. Some interesting examples of chemical crosslinking techniques include the use of enzymes or UV light [31, 47]. An example of a versatile method for generating UV-crosslinkable hydrogels is by functionalizing 2-hydroxyethyl methacrylate (HEMA) with a photoinitiator. HEMA is a polymeric monomer which can be coupled at hydroxyl groups, making it compatible with many other polymers [79]. However, these types of crosslinking techniques often require synthetic chemistry, making them impractical. Wet-chemical crosslinking allows for predictable, stable network formation, however, the used crosslinking agents may be harmful to cells, and it requires a precise control of crosslinking kinetics to avoid nozzle clogging [29].

3D bioprinting with recombinant spider silk proteins

Recently, the recombinant spider silk protein eADF4(C16) and a variant containing an RGD-motif were established as bioinks. eADF4(C16) consists of 16 repeats of a module C mimicking the repetitive core sequence of dragline silk *Araneus diadematus* fibroin 4 (ADF4) of the European garden spider (Fig. 3a) [81, 82]. The

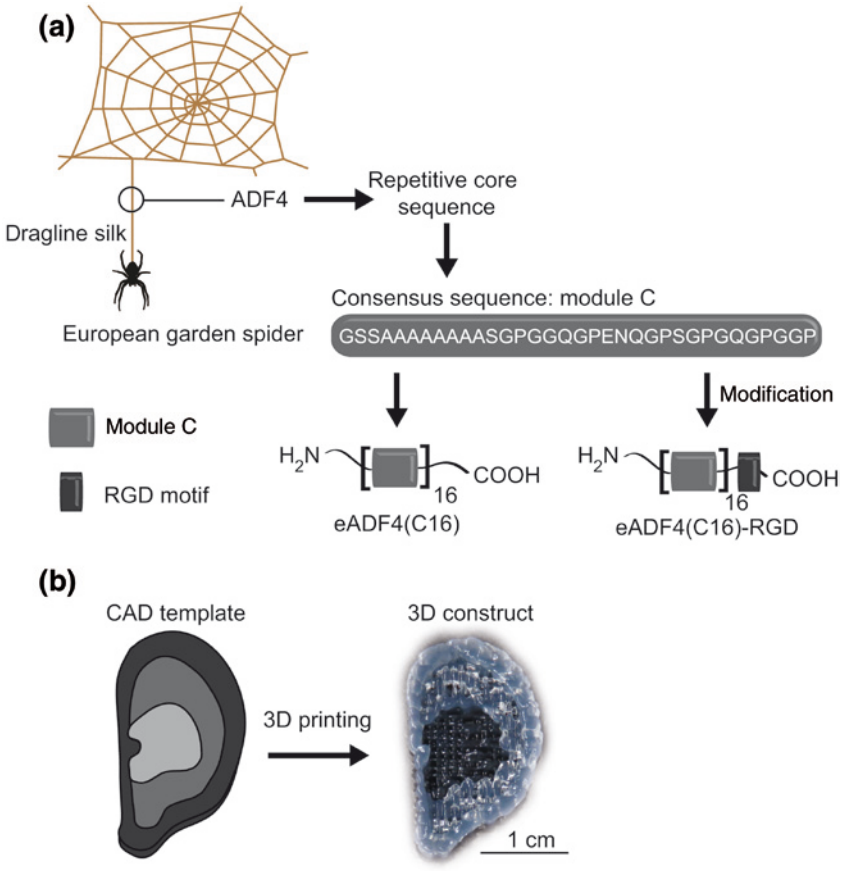

Fig. 3: (a) eADF4(C16) and eADF4(C16)-RGD are made of 16 C modules. The C-module reflects the consensus sequence of the repetitive core sequence of *Araneus diadematus* fibroin 4 (ADF4), one of the main components of the dragline silk of the European garden spider (*A. diadematus*). Dragline silk is the best characterized spider silk, constituting the outer frame of orb webs and serving as a lifeline for the spider [80]. (b) Going from a CAD template (left) to a 3DBP recombinant spider silk construct (right). Recombinant eADF4(C16) was printed by robotic dispensing. In the CAD template, the different shades of gray represent thickness with darker shades representing multiple layers. In the image of the construct it can be qualitatively observed that the construct has the same shape as the CAD file, and the printed strands made of spider silk also have high shape fidelity without the use of post-processing, crosslinking or thickeners.

recombinant spider silk proteins were assessed regarding their printability [40], and spider silk constructs could be printed by robotic dispensing using a print head with an electromagnetic valve. The hydrogels were process-compatible and had high shape fidelity (Fig. 3b). The printability is based on the β-sheet transformation of the proteins during gelation and shear thinning behavior of the hydrogels (Fig. 4).

It was shown that recombinant spider silk proteins can be used as bioink for 3D printing without the need of additional components or post-processing [40]. In contrast, alginate and fibroin need post-processing with crosslinkers or thickeners added to the solution to increase the printing fidelity [20, 58]. For more detailed information of other bioinks, refer to [29] and Table 2.

To produce cell-loaded 3D hydrogel constructs, cells were encapsulated within a highly concentrated silk solution before gelation. The addition of cells to the bioink did not influence the self-assembly into a hydrogel or the printability of the material [40]. The cells survived the printing process and were viable at least 7 days *in situ*. The viability within the spider silk hydrogel could be quantified with 70.1 ± 7.6 %. Although the cell viability in the spider silk constructs is lower when compared to established bioinks such as alginate (~90 %) and gelatin (~98 %), it could be shown that the printing procedure did not significantly affect viability, since after printing 97 % of the cells survived [40, 42, 83].

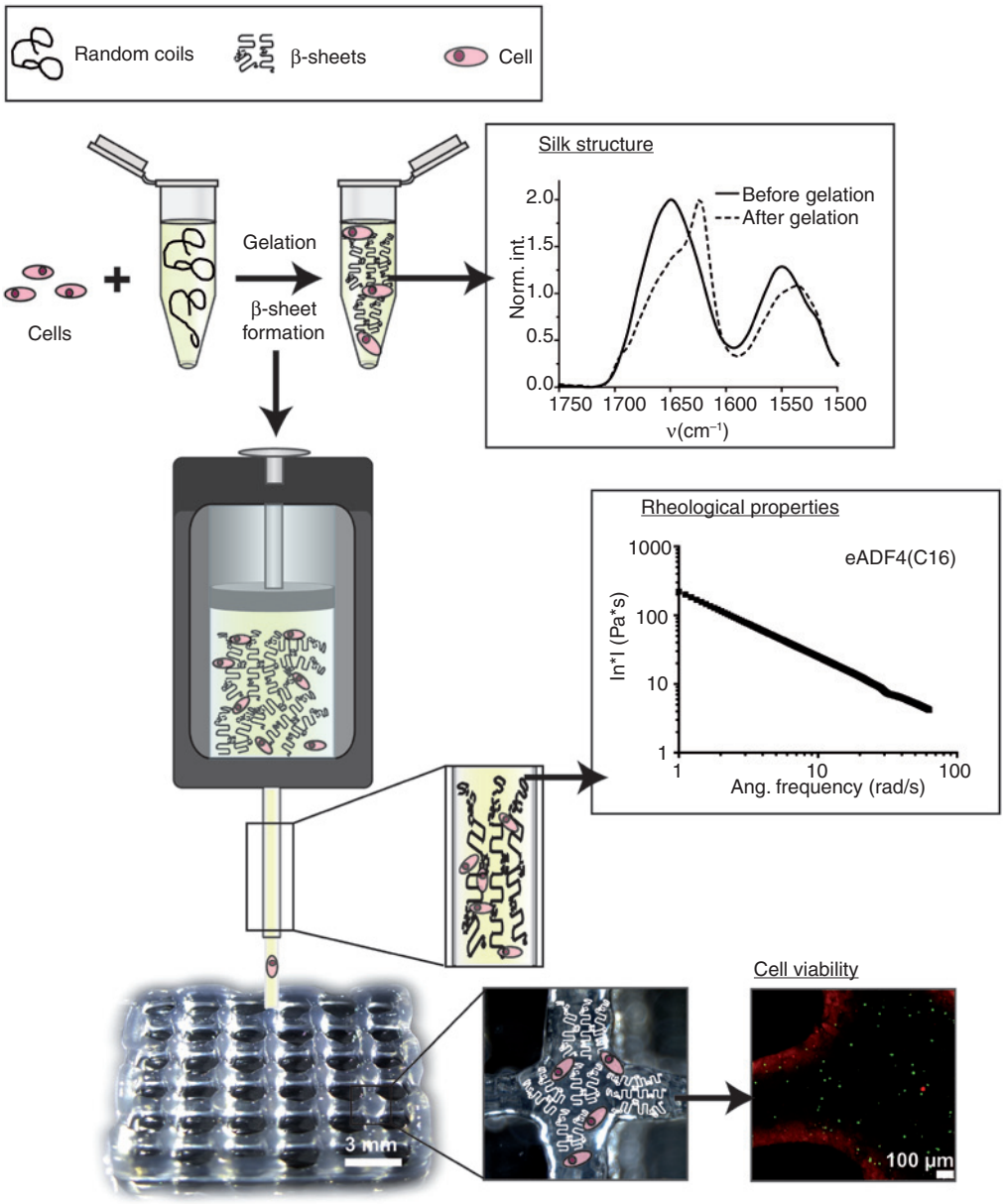

Fig. 4: Printing process for a physically crosslinked recombinant spider silk bioink. Cell-loaded spider silk constructs were printed by robotic dispensing, as mentioned in Fig. 3. The process begins with preparation of the hydrogel from a cell-loaded solution. The corresponding Fourier-transformed infrared spectroscopy (FTIR) structure data shows a peak shift corresponding to β-sheet formation which occurred during self-assembly of the hydrogel. The next step in the process represents the printing of the hydrogel accompanied by alignment of β-sheets under shear-stress, and this corresponds to the given rheological behavior with increasing angular frequency leading to a decrease in complex viscosity, which is called shear-thinning. The final construct is represented by a stereoscope image of the layered structure. The right-hand image represents the presence of viable cells (redlines reflect auto-fluorescence of spider silk; red stained cells are dead and green stained ones viable).

Conclusion and future perspectives

In conclusion, 3D bioprinting (3DBP) techniques hold potential to overcome the current, process-based challenges faced in tissue engineering: high variability and low control over the placement of different scaffold components. Of the different types of 3DBP, it seems as though extrusion printing will be one excellent option for the future of biofabrication, despite some of its drawbacks (nozzle clogging, resolution). Extrusion

printing allows for fabrication of clinically-relevant constructs (size, cell density) and greater ease in bioink development. Additionally, these advantages outweigh the disadvantages. For example, bioinks are critical in cell viability after printing (physicochemical properties) and cell behavior throughout construct maturation (physiological properties). Current bioinks tend to be better in either the "cell friendliness" (e.g. fibrin, gelatin) or printability (recombinant spider silk protein). Future work will most likely focus on polymer blends such that advantages are conserved or enhanced, and the disadvantages minimized or eliminated.

In terms of cell viability after printing, it is reasonable to hypothesize that cell viability is directly correlated with the mechanical stress that the cells are exposed to. In the optimal viscosity range for extrusion printing, there seems to be some protection to shear stress which is absent in inkjet printing; in LAB there are virtually no shear forces on the bioink, due to the nozzle-free set-up. However, LAB is incompatible with higher viscosity ranges, due to the incompability of cells with certain wavelengths and energy densities. Thereby, due to the greater flexibility in bioink development, it seems as though extrusion bioprinting will be the technology that shows the greatest potential in the future. However, it is also possible to imagine future developments will also focus on combining the different types of 3D bioprinting in order to further optimize the process.

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References

[1] R. G. Harrison, M. J. Greenman, F. P. Mall, C. M. Jackson. *Anat. Rec.* **1**, 116 (1907).
[2] R. Langer, J. P. Vacanti. *Science* **260**, 920 (1993).
[3] T. Aberle, K. Franke, E. Rist, K. Benz, B. Schlosshauer. *PLoS One* **9**, e86740 (2014).
[4] M. A. Lancaster, J. A. Knoblich. *Nat. Protoc.* **9**, 2329 (2014).
[5] A. Bozkurt, F. Gilmour R Jr., A. Lal. *IEEE Trans. Biomed. Eng.* **56**, 2304 (2009).
[6] M. J. Post. *Meat Sci.* **92**, 297 (2012).
[7] J. H. Shim, S. E. Kim, J. Y. Park, J. Kundu, S. W. Kim, S. S. Kang, D. W. Cho. *Tissue Eng. Part A* **20**, 1980 (2014).
[8] J. J. Mancuso, J. Cheng, Z. Yin, J. C. Gilliam, X. Xia, X. Li, S. T. Wong. *Front. Neuroanat.* **8**, 130 (2014).
[9] A. Wittinghofer, I. R. Vetter. *Annu. Rev. Biochem.* **80**, 943 (2011).
[10] D. D. Allison, K. R. Braun, T. N. Wight, K. J. Grande-Allen. *Acta Biomater.* **5**, 1019 (2009).
[11] M. D. Tang-Schomer, J. D. White, L. W. Tien, L. I. Schmitt, T. M. Valentin, D. J. Graziano, A. M. Hopkins, F. G. Omenetto, P. G. Haydon, D. L. Kaplan. *Proc. Natl. Acad. Sci. USA* **111**, 13811 (2014).
[12] V. A. Kasyanov, J. Hodde, M. C. Hiles, C. Eisenberg, L. Eisenberg, L. E. De Castro, I. Ozolanta, M. Murovska, R. A. Draughn, G. D. Prestwich, R. R. Markwald, V. Mironov. *J. Mater. Sci. Mater. Med.* **20**, 329 (2009).
[13] D. R. Albrecht, R. L. Sah, S. N. Bhatia. *Biophys. J.* **87**, 2131 (2004).
[14] A. Ito, K. Ino, M. Hayashida, T. Kobayashi, H. Matsunuma, H. Kagami, M. Ueda, H. Honda. *Tissue Eng.* **11**, 1553 (2005).
[15] G. Y. Huang, L. H. Zhou, Q. C. Zhang, Y. M. Chen, W. Sun, F. Xu, T. J. Lu. *Biofabrication* **3**, 012001 (2011).
[16] Y. Ling, J. Rubin, Y. Deng, C. Huang, U. Demirci, J. M. Karp, A. Khademhosseini. *Lab. Chip.* **7**, 756 (2007).
[17] I. Elloumi-Hannachi, M. Yamato, T. Okano. *J. Intern. Med.* **267**, 54 (2010).
[18] A. Abeyewickreme, A. Kwok, J. R. McEwan, S. N. Jayasinghe. *Integr. Biol.* **1**, 260 (2009).
[19] N. K. Pakes, S. N. Jayasinghe, R. S. Williams. *J. R. Soc., Interface* **8**, 1185 (2011).
[20] S. Das, F. Pati, Y.-J. Choi, G. Rijal, J.-H. Shim, S. W. Kim, A. R. Ray, D.-W. Cho, S. Ghosh. *Acta Biomater.* **11**, 233 (2014).
[21] L. Koch, M. Gruene, C. Unger, B. Chichkov. *Curr. Pharm. Biotechnol.* **14**, 91 (2013).
[22] T. Xu, C. A. Gregory, P. Molnar, X. Cui, S. Jalota, S. B. Bhaduri, T. Boland. *Biomaterials* **27**, 3580 (2006).
[23] P. Bajaj, R. M. Schweller, A. Khademhosseini, J. L. West, R. Bashir. *Annu. Rev. Biomed. Eng.* **16**, 247 (2014).
[24] V. Mironov, T. Trusk, V. Kasyanov, S. Little, R. Swaja, R. Markwald. *Biofabrication* **1**, 022001 (2009).
[25] C. J. Luo, S. D. Stoyanov, E. Stride, E. Pelan, M. Edirisinghe. *Chem. Soc. Rev.* **41**, 4708 (2012).
[26] S. V. Murphy, A. Atala. *Nat. Biotechnol.* **32**, 773 (2014).
[27] J. S. Miller. *PLoS Biol.* **12**, e1001882 (2014).
[28] A. Pfister, R. Landers, A. Laib, U. Hubner, R. Schmelzeisen, R. Mulhaupt. *J. Polym. Sci., Part A: Polym. Chem.* **42**, 624 (2004).

[29] J. Malda, J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W. J. A. Dhert, J. Groll, D. W. Hutmacher. *Adv. Mater.* **25**, 5011 (2013).
[30] R. Nayaran, Ed. *Rapid Prototyping of Biomaterials: Principles and Applications*. Woodhead Publishing, Philadelphia (2014).
[31] L. A. Hockaday, K. H. Kang, N. W. Colangelo, P. Y. Cheung, B. Duan, E. Malone, J. Wu, L. N. Girardi, L. J. Bonassar, H. Lipson, C. C. Chu, J. T. Butcher. *Biofabrication* **4**, 035005 (2012).
[32] S. Moon, S. K. Hasan, Y. S. Song, F. Xu, H. O. Keles, F. Manzur, S. Mikkilineni, J. W. Hong, J. Nagatomi, E. Haeggstrom, A. Khademhosseini, U. Demirci. *Tissue Eng., Part C* **16**, 157 (2010).
[33] M. Duocastella, J. M. Fernandez-Pradas, P. Serra, J. L. Morenza. *Appl. Phys. A: Mater. Sci. Process.* **93**, 453 (2008).
[34] C. Mézel, L. Hallo, A. Souquet, J. Breil, D. Hebert, F. Guillemot. *Phys. Plasmas* **16**, 123112 (2009).
[35] C. J. Ferris, K. G. Gilmore, G. G. Wallace, M. I. H. Panhuis. *Appl. Microbiol. Biotechnol.* **97**, 4243 (2013).
[36] S. Catros, B. Guillotin, M. Bacakova, J. C. Fricain, F. Guillemot. *Appl. Surf. Sci.* **257**, 5142 (2011).
[37] R. Cornock, S. Beirne, B. Thompson, G. G. Wallace. *Biofabrication* **6**, 025002 (2014).
[38] I. T. Ozbolat, H. Chen, Y. Yu. *Robotics and Computer-Integrated Manufacturing* **30**, 295 (2014).
[39] J. N. H. Shepherd, S. T. Parker, R. F. Shepherd, M. U. Gillette, J. A. Lewis, R. G. Nuzzo. *Adv. Funct. Mater.* **21**, 47 (2011).
[40] K. Schacht, T. Jüngst, M. Schweinlin, A. Ewald, J. Groll, T. Scheibel. *Angew. Chem., Int. Ed.* **54**, 2816 (2015).
[41] Q. L. Loh, C. Choong. *Tissue Eng. Part B, Rev.* **19**, 485 (2013).
[42] J. Jia, D. J. Richards, S. Pollard, Y. Tan, J. Rodriguez, R. P. Visconti, T. C. Trusk, M. J. Yost, H. Yao, R. R. Markwald, Y. Mei. *Acta Biomater.* **10**, 4323 (2014).
[43] N. E. Fedorovich, J. Alblas, J. R. de Wijn, W. E. Hennink, A. J. Verbout, W. J. A. Dhert. *Tissue Eng.* **13**, 1905 (2007).
[44] B. Derby. *Science* **338**, 921 (2012).
[45] B. S. Kim, D. J. Mooney. *Trends Biotechnol.* **16**, 224 (1998).
[46] N. E. Fedorovich, W. Schuurman, H. M. Wijnberg, H. J. Prins, P. R. van Weeren, J. Malda, J. Alblas, W. J. A. Dhert. *Tissue Eng., Part C* **18**, 33 (2012).
[47] J. C. Schense, J. A. Hubbell. *Bioconjug. Chem.* **10**, 75 (1999).
[48] C. L. Cummings, D. Gawlitta, R. M. Nerem, J. P. Stegemann. *Biomaterials* **25**, 3699 (2004).
[49] W. Xu, X. Wang, Y. Yan, W. Zheng, Z. Xiong, F. Lin, R. Wu, R. Zhang. *J. Bioact. Compat. Polym.* **22**, 363 (2007).
[50] X. Wang, Y. Yan, R. Zhang. *Tissue Eng. Part B, Rev.* **16**, 189 (2010).
[51] C. M. Smith, A. L. Stone, R. L. Parkhill, R. L. Stewart, M. W. Simpkins, A. M. Kachurin, W. L. Warren, S. K. Williams. *Tissue Eng.* **10**, 1566 (2004).
[52] R. Parenteau-Bareil, R. Gauvin, F. Berthod. *Materials* **3**, 1863 (2010).
[53] U. Hersel, C. Dahmen, H. Kessler. *Biomaterials* **24**, 4385 (2003).
[54] E. Hoch, T. Hirth, G. E. M. Tovar, K. Borchers. *J. Mater. Chem. B* **1**, 5675 (2013).
[55] W. Schuurman, P. A. Levett, M. W. Pot, P. R. van Weeren, W. J. A. Dhert, D. W. Hutmacher, F. P. W. Melchels, T. J. Klein, J. Malda. *Macromol. Biosci.* **13**, 551 (2013).
[56] E. Hoch, C. Schuh, T. Hirth, G. E. M. Tovar, K. Borchers. *J. Mater. Sci. Mater. Med.* **23**, 2607 (2012).
[57] S. Das, F. Pati, S. Chameettachal, S. Pahwa, A. R. Ray, S. Dhara, S. Ghosh. *Biomacromolecules* **14**, 311 (2013).
[58] S. Wust, M. E. Godla, R. Muller, S. Hofmann. *Acta Biomater.* **10**, 630 (2014).
[59] F. P. W. Melchels, W. J. A. Dhert, D. W. Hutmacher, J. Malda. *J. Mater. Chem. B* **2**, 2282 (2014).
[60] R. Levato, J. Visser, J. A. Planell, E. Engel, J. Malda, M. A. Mateos-Timoneda. *Biofabrication* **6**, 035020 (2014).
[61] X. F. Cui, K. Breitenkamp, M. G. Finn, M. Lotz, D. D. D'Lima. *Tissue Eng. Part A* **18**, 1304 (2012).
[62] R. Censi, W. Schuurman, J. Malda, G. di Dato, P. E. Burgisser, W. J. A. Dhert, C. F. van Nostrum, P. di Martino, T. Vermonden, W. E. Hennink. *Adv. Funct. Mater.* **21**, 1833 (2011).
[63] M. Kesti, M. Muller, J. Becher, M. Schnabelrauch, M. D'Este, D. Eglin, M. Zenobi-Wong. *Acta Biomater.* **11**, 162 (2015).
[64] K. Y. Lee, D. J. Mooney. *Prog. Polym. Sci.* **37**, 106 (2012).
[65] P. Kesari, T. Xu, T. Boland. *Mater. Res. Soc. Symp. Proc.* **845**, 111 (2005).
[66] X. F. Cui, T. Boland. *Biomaterials* **30**, 6221 (2009).
[67] J. Visser, B. Peters, T. J. Burger, J. Boomstra, W. J. A. Dhert, F. P. W. Melchels, J. Malda. *Biofabrication* **5**, 035007 (2013).
[68] G. H. Altman, F. Diaz, C. Jakuba, T. Calabro, R. L. Horan, J. S. Chen, H. Lu, J. Richmond, D. L. Kaplan. *Biomaterials* **24**, 401 (2003).
[69] A. Leal-Egaña, T. Scheibel. *Biotechnol. Appl. Biochem.* **55**, 155 (2010).
[70] K. Schacht, T. Scheibel. *Curr. Opin. Biotechnol.* **29**, 62 (2014).
[71] P. H. Zeplin, N. C. Maksimovikj, M. C. Jordan, J. Nickel, G. Lang, A. H. Leimer, L. Roemer, T. Scheibel. *Adv. Funct. Mater.* **24**, 2658 (2014).
[72] P. H. Zeplin, A.-K. Berninger, N. C. Maksimovikj, P. van Gelder, T. Scheibel, H. Walles. *Handchir. Mikrochir. Plast. Chir.* **46**, 336 (2014).
[73] H. Mori, M. Tsukada. *J. Biotechnol.* **74**, 95 (2000).
[74] Y. Q. Zhang, W. D. Shen, R. A. Gu, J. Zhu, R. Y. Xue. *Anal. Chim. Acta* **369**, 123 (1998).
[75] I. Drachuk, O. Shchepelina, S. Harbaugh, N. Kelley-Loughnane, M. Stone, V. V. Tsukruk. *Small* **9**, 3128 (2013).
[76] C. Blum, A. Nichtl, T. Scheibel. *Adv. Funct. Mater.* **24**, 763 (2014).
[77] K. Schacht, T. Scheibel. *Biomacromolecules* **12**, 2488 (2011).

[78] S. Wohlrab, S. Muller, A. Schmidt, S. Neubauer, H. Kessler, A. Leal-Egana, T. Scheibel. *Biomaterials* **33**, 6650 (2012).
[79] L. Pescosolido, W. Schuurman, J. Malda, P. Matricardi, F. Alhaique, T. Coviello, P. R. Weeren, W. J. Dhert, W. E. Hennink, T. D. Vermonden. *Biomacromolecules* **12**, 1831 (2011).
[80] A. Aprhisiart, F. Vollrath. *Behav. Ecol.* **5**, 280 (1994).
[81] D. Huemmerich, C. W. Helsen, S. Quedzuweit, J. Oschmann, R. Rudolph, T. Scheibel. *Biochemistry-Us* **43**, 13604 (2004).
[82] C. Vendrely, T. Scheibel. *Macromol. Biosci.* **7**, 401 (2007).
[83] T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, P. Dubruel. *Biomaterials* **35**, 49 (2014).