Silk-Based Bioinks for 3D Bioprinting

Shikha Chawla, Swati Midha, Aarushi Sharma, and Sourabh Ghosh*

3D bioprinting field is making remarkable progress; however, the development of critical sized engineered tissue construct is still a farfetched goal. Silk fibroin offers a promising choice for bioink material. Nature has imparted several unique structural features in silk protein to ensure spinnability by silkworms or spider. Researchers have modified the structure–property relationship by reverse engineering to further improve shear thinning behavior, high printability, cytocompatible gelation, and high structural fidelity. In this review, it is attempted to summarize the recent advancements made in the field of 3D bioprinting in context of two major sources of silk fibroin: silkworm silk and spider silk (native and recombinant). The challenges faced by current approaches in processing silk bioinks, cellular signaling pathways modulated by silk chemistry and secondary conformations, gaps in knowledge, and future directions acquired for pushing the field further toward clinic are further elaborated.

1. Introduction

Functionality of each tissue of human body is governed by a defined architecture, biochemical composition, and mechanical properties. Tissue engineers attempt to develop human tissue-like constructs by culturing cells on various 3D porous scaffolds, in order to replicate the structural and functional dynamics of native tissues. However, such engineered constructs, based on porous scaffolds, such as lyophilized, porogen-leached, hydrogels, textile-based materials (for example, nonwoven, knitted, woven, braid, electrospun nanofibers)\(^1\) are critically devoid of (i) 3D architecture with anatomical relevance to native tissues and organs, (ii) orientation/alignment of cells for biological functionality, branching morphogenesis, tortuosity, curvature, porosity, and (iii) reproducibility to scale up tissue models for clinical applications for transplantation. Hence, current tissue engineering strategies fail to coherently recapitulate the complexity of 3D anatomy and functionality of human tissues. Also, lack of microcapillary fluid mechanics such as fluid shear stress and inability to create precise hypoxic gradients within engineered constructs inhibit vasculogenic sprouting.\(^2\) As a result, till date very few engineered constructs could reach human clinical trial level.

Therefore, tissue regeneration strategies need a paradigm shift toward more defined biological systems offering structural and mechanical cues for rendering spatio-temporal microenvironment to guide cellular orientation, tissue differentiation and morphogenesis.\(^3\) There are two different approaches using 3D printing technology in tissue engineering. The first strategy is to develop acellular 3D porous scaffolds, and top-seed cells post-printing;\(^4\) while the second approach is used to create tissue equivalents by directly depositing cells (in the form of dispersed or aggregates) within the bioink, a process known as bioprinting.\(^5\)–\(^7\) The intent of 3D bioprinting strategy is to create a tissue-specific construct with 3D stacks/filaments of a hydrogel-based matrix with explicit control over deposition of cell seeding modalities\(^6\) under strictly regulated microenvironment with gradients of growth factors, cytokines/chemokines and other biological moieties in optimal concentrations.\(^8\) Hence 3D bioprinting offers untapped potential to capture the complex architectural and functional hierarchy of native tissues and develop biomimetic equivalents offering patient-specific transplant for clinical setup. Moreover, beyond their potential use as clinical transplants, such bioprinted tissues could be used as in vitro model systems to screen efficacy of drug candidates, in order to advance our understanding of the human developmental mechanisms and disease progression, considering both intra- and inter-cellular signaling pathways operational within the 3D tissue microenvironment.\(^9\),\(^10\)

Bioink design is a crucial aspect in the process of 3D bioprinting as it not only provides the stable 3D architecture for tissue development but acts as the first point of contact for cells, which subsequently provides necessary cues for synthesizing regulatory proteins and cytokines to form an appropriate 3D niche. From a physico-chemical standpoint, to do so successfully, the bioink needs to be printable taking care of several parameters including rheology, swelling ratio, surface tension, and gelation kinetics.\(^11\) From a biological standpoint, factors like spatio-temporal cell positioning for replicating the homotypic–heterotypic cell interactions in 3D, degradability and extracellular matrix (ECM) regulation within the bioink should mimic the tissue niche in situ, and most importantly the process of cell differentiation should closely simulate the developmental pathways of organogenesis. However, progress is currently impeded due to limited choice of bioinks that comply with the requirements of spinnability, cell encapsulation and tailor-made degradability (Figure 1).

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Silk, being a strong and robust material with inherent spinability and adequate cytocompatible properties, has become a popular choice for bioink preparation. The reason as to why silk is being exploited is that simply by controlling the shear force (for example, by applying pneumatic pressure), it is possible to induce sol-to-gel transition by changing the secondary conformations (random coil to \( \beta \)-sheet crystallization) of the silk fibroin polymers; hence eluding the requirement of high temperature or toxic organic solvent into the material (Figure 2). This feature of silk fibroin has encouraged several scientists worldwide to explore the applications of silk in 3D bioprinting. Through this review we will discuss and summarize the progress toward 3D bioprinting made by silk fibroin bioinks; silkworm silk, and spider silk (native as well as recombinant silk proteins) and investigate how chemistry and rheological features of silk bioink may be manipulated to achieve high resolution printing, and identify the current challenges that need attention for achieving clinical success. In order to elucidate the progress of the field it is imperative to critically address cell-free 3D printing of silk protein solution, followed by use of silk bioinks for 3D bioprinting. Historical progress of the biofabrication field or different printing techniques will not be covered in this manuscript, as many good reviews in recent literature elucidated those aspects. Due to the advantages, versatility, and popularity, the scope of this manuscript is limited to the extrusion-based bioprinting of silk-based bioinks, applicability, and unsolved challenges.

2. Material Design and Printing

Most inks currently being developed for 3D printing are made of thermoplastics, synthetic polymers, silicones, pluronics or for bioprinting natural polymers such as collagen, gelatin, or alginate or their modified versions such as nano-cellulose, gelatin methacryloyl. But the need to operate in an aqueous or hydrogel environment to fabricate cell-laden bioprinted constructs limits the choice of materials to ensure smooth translation of this technology to the clinic. The materials must offer controlled degradation overtime and sustain proper functionality throughout the lifetime of the implant. Bioink composition should be such that the cells embedded within the bioink should be able to erode and remodel their surrounding matrix, and progenitor cells should be able to differentiate along a specified lineage. The bioink should promote wound healing, support ECM synthesis and deposition and ensure integration with the native tissue, while it should not elicit innate or adaptive immune response after implantation. Additionally, pre-and post-processing parameters must be able to preserve biocompatibility of material in order to incorporate cells and biological molecules into bioprinted structures. To facilitate the creation of such a construct, there is the need for a multidisciplinary approach demanding simultaneous progress in biomaterial chemistry, rheology and fluid dynamics, cell biology, and instrumentation engineering. In addition, sophisticated programs that allow for precise placement and incorporation of multiple cell types and matrix components within the 3D hydrogel pattern need to be developed. It is still a major challenge to develop advanced softwares and algorithms for “off the shelf” product development for individual patient-specific manner (Figure 1).

In most cases, the so called “ideal” bioink features for printability differ drastically with the biological demands of the cellular components. For example, maintaining highest extent of cell viability during and after printing is critically important. So far, reported literature on 3D bioprinting has demonstrated the cell viability range from as low as 45% to as high as 98% post 14 d in culture. Increasing polymer content and viscosity promote printability and finer resolution of printed structures, but cell viability decreases with increasing polymer concentration in the bioink or printing pressure. The temperature, pH changes and crosslinking methods that may be required to induce sol-to-gel transition in order to induce solidification can rupture cell membrane.
inflict stress to induce apoptosis and/or necrosis,\cite{23,24} or denature other biological components (such as growth factors, ECM proteins, cytokines) that are added to the ink for biomimetic tissue development. Therefore, carefully standardizing the physico-chemical parameters of hydrogel precursors are important factors for printability of a cell-laden functional construct.\cite{13,25} Taken together, some key requirements of 3D bioprinting include:

1. Optimizing the bioink in terms of its rheological behavior is of utmost importance for bioprinting. For instance, high viscosity bioinks exhibit gel-like properties that aid in extrusion of self-supporting filamentous structures. The viscosity before printing must allow easy mixing and homogeneous 3D distribution of cells throughout the printing process without affecting the viability.

2. Bioinks for extrusion-based bioprinting should exhibit shear-thinning behavior to allow ready flow of fluids through confined nozzle diameters of the bioprinter. At the same time, immediately after extrusion, the fluids must demonstrate instant shape stability to assemble a 3D structural assembly without collapse.

Figure 1. Challenges in silk-bioink development. Successful 3D bioprinting demands careful optimization of bioink composition, rheology, 3D printer design, printing parameters. But most critical aspect is to understand how the bioink would affect cell behavior and can ignite signaling pathways for organogenesis. Though the schematic illustrates general challenges in the field of 3D bioprinting, we have attempted to define the same in context of silk-based bioinks.

Figure 2. Sol to gel transition (due to random coil to $\beta$-sheet crystal conformation) of the spider silk spidroin and silkworm silk fibroin polymers in critically important for printing silk protein. Magnified image of direct-write silk fiber\cite{12} in alcohol coagulation bath shows rough fiber surface. But exposure to alcohol would induce cell death, so this strategy cannot be used for 3D bioprinting, but might be useful as scaffold for tissue engineering.\cite{4}
(3) Optimal gelation strategy should be adopted to deposit self-supportive structures. This in turn will help in avoiding cell sedimentation that might lead to nozzle clogging and/or uncontrolled non-homogeneous cell distribution through the printed construct.
(4) The bioink should possess optimum surface tension to avoid getting attached on the nozzle tip, and exhibit physical forces to overcome the droplet formation enabling smooth extrusion of filaments.[26]
(5) The hydrogel matrix should possess a hydrated network to sustain cell viability via continuous exchange of gases, nutrients and metabolite wastes.
(6) In extrusion-based bioprinting, the diameter of the extrudate/filament is determined by nozzle diameter, ink rheology, flow rate and extrusion pressure.[14]

Thus, extrusion-based fabrication of bioprinted constructs with precisely deposited cells and matrix proteins within a hydrogel matrix, pertains to well-defined architecture, porosity, biomechanics, and composition, wherein each parameter can be independently controlled for creating high precision structures ranging from submicron to several millimeters.[12] Thus bioprinting process can render 3D bioprinted constructs with varying cell modalities, i.e., dispersed cells or spheroids, which considerably affect the extent of homotypic adhesion and differentiation.[6] While inferior resolution and frequent nozzle clogging restrict its application, a major concern with extrusion-based bioprinter is the larger filament size (100–300 µm).[37]

In droplet based bioprinter, hydrogels are deposited by print heads using thermal or acoustic process akin to methods adopted to deposit discrete liquid droplets on 2D surface. However, one major limitation with inkjet printing is that it uses low viscosity fluids (2–20 mPa s) which demand high shear rates (10^2–10^6 s^-1) for extrusion in the form of droplets measuring ≈50 µm in diameter for smooth printing.[28,29] For instance, while Tao et al. successfully prepared functional silk inks (significantly diluted to ≈3 wt%) doped with a variety of additives such as enzymes, nanoparticles, growth factors and antibiotics. This helped in achieving a printable ink with 0.046 ± 2 N m^-1 surface tension and 3 mPa s of dynamic viscosity.[30] Major points of concern here include: (i) lower resultant β-sheet crystal content due to low protein concentration (3%) which may have resulted in poor mechanical properties and faster degradation of material in vivo,[31] (ii) compromised cell functions such as attachment and differentiation due to relatively lower percentage of amino acid segments,[32] and (iii) absence of continuous structures. Overall, 3D extrusion-based printing using a broad spectrum of ink enables the fabrication of controlled feature sizes with continuous filamentous form and complex architectures.

2.1. Why Silk

A major problem with most of the natural polymeric bioinks is that they generate relatively fragile, easily deformable structures, leading to faster degradation in vivo, thus necessitating the requirement of immediate crosslinking methods to maintain structural fidelity of printed constructs.[15] Unfortunately, these additives are often harsh and liberate cytotoxic residues upon crosslinking which impede cell growth.[33]

Silk biomaterials, on the other hand, are food and drug administration (FDA) approved and commonly accepted in medical practice as sutures and surgical meshes.[34] Composed entirely of amino acids, silk-based bioinks are amphiphilic in nature and therefore can be exploited to generate protein drops in the pico- to nano-liter range (for droplet printing), or extruded filaments (for extrusion-based printing) by optimizing ink rheology (e.g., viscosity, surface tension) with variable values of pH and ionic strength. Moreover, the extent of secondary conformations (precisely β-sheet content) in silk fibroin could be modulated resulting in controlled mechanical properties, hence rendering controlled degradation in vivo.[35] Here we have attempted to develop a deeper mechanistic insight about silkworm silk fibroin or spider silk-based 3D bioprinting by discussing the individual properties of each in context of 3D bioprinting applications. This will help in generating insights about cell–material interactions and aid in developing novel silk-based regenerative strategies as 3D bioprinted tissue equivalents.[36] Table 1 provides an extensive overview of the various polymeric bioinks used and their comparison with silk bioink for 3D bioprinting applications.

2.2. Sources of Silk

Various types of silk protein exist in nature, although the most commonly investigated so far are derived from silkworm silk and spider silk.[48,49] Of the two sources, silkworm silk Bombyx mori is undoubtedly the most frequently studied due to easy availability (Table 2). Also, since the entire sequence of B. mori is known,[50] this makes it very convenient to manipulate the material for bioengineering purposes specific to the target application. In contrast, availability of spider silk is rather limited, a problem that has been solved by recombinant protein production technology.

2.2.1. Silkworm Silk

Silk fibroin of B. mori origin comprising a heavy (~350 kDa) and light (~26 kDa) chain with a glycoprotein, P25 (30 kDa) in the ratio of 6:6:1. The heavy and light chains are linked by a disulfide bond, while non-covalent interactions predominates their association with P25.[51] The fibroin chain consists of amino- (N-) (130 amino acids) and carboxyl- (C-) terminal (100 amino acids) peptide domains, while the C-terminus is conserved among species of silkworms and spiders, N-terminus is conserved only among spiders and the sequence varies considerably in silkworms.[52] In order to process silkworm silk for 3D bioprinting applications, the reverse engineering technique of converting silk fibers back into solution form needs to be applied to prepare a bioink. This is generally achieved by facilitating the disruption of hydrogen bonding and hydrophobic interactions that regulate the intra- and inter-chain interactions within the silk fibroin chain facilitating structural deformation.[52] Once the silk is
## Table 1. Comparative analysis of silk versus other polymeric bioinks.

<table>
<thead>
<tr>
<th>Bioink</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Silkworm silk</strong></td>
<td>i. Ease of structural modification</td>
<td>i. Need to mix with another polymer for optimal printability</td>
</tr>
<tr>
<td></td>
<td>ii. Possibility to introduce physical crosslinks (β-sheets) to induce sol-to-gel transition avoiding solvents or heat or UV light exposure</td>
<td>ii. Need to optimize rheology of the bioink</td>
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<td></td>
<td>iii. Biomineralization mechanism similar to Col[32]</td>
<td>iii. Batch-to-batch variation as the amino acid sequences of fibroins show extensive diversification among the different species, either due to environmental effects, or due to evolution.</td>
</tr>
<tr>
<td></td>
<td>iv. Controlled degradation</td>
<td></td>
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<tr>
<td></td>
<td>v. Flexible structural modification</td>
<td></td>
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<tr>
<td></td>
<td>vi. Cleavable by MMP’s facilitating cell migration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vii. High cellular viability for at least 1 month</td>
<td></td>
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<td></td>
<td>viii. FDA approved for many medical applications</td>
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<tr>
<td><strong>Agarose</strong></td>
<td>i. Stiffness in the range of native soft tissues</td>
<td>i. Non-degradable</td>
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<td></td>
<td>ii. Non-toxic</td>
<td>ii. Hybrids with another polymer for rendering biological properties[33]</td>
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<tr>
<td></td>
<td>iii. Maintains shape fidelity across broad range of temperatures</td>
<td>iii. Needs high temperature for dispensing (70 °C) and gels at low temperatures, not suitable for bioprinting</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td>i. Main ECM component of the connective tissue</td>
<td>i. Slow gelation rate for bioprinting leads to non-homogenous cell suspensions due to gravity pull down</td>
</tr>
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<td></td>
<td>ii. Provide abundant integrin binding domains for cell attachment and growth</td>
<td>ii. Batch-to-batch variation</td>
</tr>
<tr>
<td></td>
<td>iii. Easy degradation</td>
<td>iii. Deformation and inconsistent constructs due to shrinkage limit its application for high resolution 3D printing</td>
</tr>
<tr>
<td></td>
<td>iv. Minimal immunogenicity</td>
<td>iv. Mechanically weak (elastic moduli ≈1 kPa), so cannot produce self-supporting scaffolds</td>
</tr>
<tr>
<td></td>
<td>v. Easy to process and modify</td>
<td>v. High viscosity not suitable for inkjet printing[24]</td>
</tr>
<tr>
<td></td>
<td>vi. High printability</td>
<td></td>
</tr>
<tr>
<td><strong>Gelatin</strong></td>
<td>i. Contains cell-adhesive Arg-Gly-Asp motifs</td>
<td>v. Sterilization leads to structural modifications</td>
</tr>
<tr>
<td></td>
<td>ii. Facilitates cellular proliferation, ECM synthesis</td>
<td></td>
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<tr>
<td></td>
<td>iii. Thermoresponsive phase transition behavior[36]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>iv. Decreased immunogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>v. Cleaved by MMP’s facilitating cell migration</td>
<td></td>
</tr>
<tr>
<td><strong>Matrigel</strong></td>
<td>i. Supports osteogenic and angiogenic phenotype in vitro</td>
<td>i. Mechanically weak at physiological temperature</td>
</tr>
<tr>
<td></td>
<td>ii. Mechanically strong 3D bioprinted structures</td>
<td>ii. Requires additional polymeric dopants or crosslinking for stability of printed constructs[39]</td>
</tr>
<tr>
<td></td>
<td>iii. High cellular viability</td>
<td></td>
</tr>
<tr>
<td><strong>Pluronics</strong></td>
<td>i. Cytocompatible</td>
<td>i. Provides cellular viability only up to 5 d and decreases thereafter</td>
</tr>
<tr>
<td></td>
<td>ii. High printability</td>
<td>ii. Erodes quickly in medium, lack of structural integrity.</td>
</tr>
<tr>
<td><strong>PEG and PEO</strong></td>
<td>i. Biocompatible, facilitates cell entrapment</td>
<td>iii. Cured by UV light to enable crosslinking, the intensity, type and duration can hinder cell viability</td>
</tr>
<tr>
<td></td>
<td>ii. FDA approved for many medical applications</td>
<td>iv. Temperature dependency for printing depending upon conc. used</td>
</tr>
<tr>
<td></td>
<td>iii. Easy to control transition temperature</td>
<td>v. Often hybridized with PEG/PEO rendering hydrophilic surface and hence poor cell attachment</td>
</tr>
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<td></td>
<td>iv. Easily modified, anneals smaller fragmented sequences</td>
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<tr>
<td><strong>Alginate</strong></td>
<td>i. Biocompatible, low toxicity, and low cost[40]</td>
<td>i. Resistant to protein adsorption</td>
</tr>
<tr>
<td></td>
<td>ii. Can be used for bioprinting across wide range of conc. (2–4%)</td>
<td>ii. Lack of cell adhesion motifs</td>
</tr>
<tr>
<td></td>
<td>iii. Ease of crosslinking</td>
<td>iii. High hydrophilicity leading to very low cell adherence</td>
</tr>
<tr>
<td></td>
<td>iv. Stability of constructs</td>
<td>iv. Poor mechanical strength</td>
</tr>
<tr>
<td><strong>Photopolymerizable</strong></td>
<td>i. High mechanical strength</td>
<td>i. Cellular responses vary with source of origin</td>
</tr>
<tr>
<td>hydrogels (gelatin methacrylate)</td>
<td>ii. Low swelling ratio</td>
<td>ii. Low cell attachment and protein adsorption due to highly hydrophilic nature[45]</td>
</tr>
<tr>
<td></td>
<td>iii. Low viscosity at room temp. so easy to extrude</td>
<td>iii. Lack of cell-binding motifs</td>
</tr>
<tr>
<td><strong>Hyaluronan gel</strong></td>
<td>i. Offers cell adhesion, migration, proliferation</td>
<td>i. Cured by UV, release of free radical residues impact cell viability.</td>
</tr>
<tr>
<td></td>
<td>ii. Controllable mechanics, architecture, and degradation</td>
<td>ii. Low proliferation rate of cells</td>
</tr>
<tr>
<td></td>
<td>iii. Fast gelation</td>
<td>iii. Only suitable for bioprinting with low conc. of photo-initiator (~0.1%) which yields mechanically weak structures[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv. Photopolymerized free radicals generated in hydrogel may cause poor cell sustainability</td>
</tr>
</tbody>
</table>
available in solution form, subsequent steps proceed toward the disruption of water of hydration leading to β-crystallization of silk solution into 3D forms (Figure 2). There are several ways to achieve this, for instance, exposure to shear force,[53] immersion in concentrated alcohol solution,[4] exposure to high temperatures[54] or low pH,[55] shear-induced stress,[12] application of electric fields, sonication-induced mechanical vibrations,[56] etc. Overall, each of these protocols has a distinct mechanism of action to modulate the dynamics of the silk protein chains in order to induce β-sheet crystallization to varying extents. Exposure to alcohol, for example, induces instant gelation,[12] whereas other protocols such as water vapor annealing[54] cause slow and controllable crystallization. Most of these strategies work in the case of 3D printing (where cells are not encapsulated in the bioink), but the situation becomes complicated with encapsulated cells in the case of 3D bioprinting. An in-depth investigation of such gelation strategies in the context of silk-based bioinks is not only crucial for successful development of 3D bioprinted constructs, but it is important to generate insight about the impact of these strategies on cell behavior and subsequent tissue differentiation, which will form an integral part of the upcoming sections. Table 3 provides a brief summary of the properties of silkworm fibroin and how they can be exploited in context of 3D Bioprinting.

Table 2. Comparison of silkworm silk and spider silk properties.

<table>
<thead>
<tr>
<th></th>
<th>Silkworm silk</th>
<th>Spider silk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Silk glands</strong></td>
<td>Salivary</td>
<td>Major ampullate</td>
</tr>
<tr>
<td><strong>Quantity of silk produced</strong></td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td><strong>Divisions of silk glands</strong></td>
<td>Posterior, middle, anterior</td>
<td>Tail, sac, duct</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td>Fibroin</td>
<td>Spidroin</td>
</tr>
<tr>
<td><strong>Components of silk</strong></td>
<td>Light chain, heavy chain, fibroin p23, sericin</td>
<td>Major ampullate spidroins (MaSp1 and MaSp2)</td>
</tr>
<tr>
<td><strong>Repeat sequences</strong></td>
<td>(CAGACAGACAGAG), motifs</td>
<td>Alternating poly-A and glycine rich repeats</td>
</tr>
<tr>
<td><strong>pH gradient during fiber formation</strong></td>
<td>7.2–6.9 in posterior silk gland, 5 in middle silk gland, 4.8 in anterior silk gland[48]</td>
<td>≈7 in sac, 6–6.5 in duct</td>
</tr>
<tr>
<td><strong>Ionic concentration</strong></td>
<td></td>
<td>1. Na, K, Mg, Zn increase from posterior to anterior gland 2. Ca decreases from posterior to anterior gland</td>
</tr>
<tr>
<td><strong>Silk fiber diameter</strong></td>
<td>10–16 µm</td>
<td>3–6 µm</td>
</tr>
</tbody>
</table>

2.2.2. Natural Spider Silk

Spider silks are known to possess superior mechanical properties over their silkworm silk counterpart. Synthesized in the major ampullate gland of genera Nephila, Argiope, Latrodictus, and Araneus, the dragline silk forms the toughest fiber.[55] Two major polypeptides identified in dragline silk, Major ampullate spidroin 1 and 2 (MaSp1 and MaSp2), comprise a core sequence of highly repetitive amino acids, conjugated by non-repetitive C- and N-terminal domains on both ends, hence forming an amphipathic protein structure.[58] This property results in encapsulation of the hydrophilic domains inside the micelles, while the terminal hydrophobic domains form the edges, rendering stability to the proteins, a property that should be exploited for generating precise protein drops for 3D printing purposes (Table 3).[59,60]

These high molecular weight spidroins (200 to 350 kDa) primarily differ in terms of proline content and hydrophobicity. Distinct amino acid motifs comprising Gly-rich repeats and poly-Ala blocks predominantly exist in the core domain enabling the formation of secondary conformations (random coil or α-helical and β-sheet), contribute toward superior mechanical properties[48] and slow degradation of bioprinted constructs due to high crystallinity (Figure 2). However, other factors such as the implantation site, mechanical environment, and fabrication strategy play critical roles in determining their in vivo degradation time. In addition, the C-terminal domain plays a role in the alignment of secondary conformational changes by enabling hydrophobic interactions for driving self-assembly into extruded filaments[59,60] Further, extensive research has been performed to improve the...
Table 3. Summary of the properties of silkworm fibroin in context of 3D bioprinting.

<table>
<thead>
<tr>
<th>Silk property</th>
<th>Structure</th>
<th>Structural features</th>
<th>Relevance to 3D bioprinting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change of secondary conformation (random coil to α-helix or β-sheet) under shear force&lt;sup&gt;[61–63]&lt;/sup&gt;</td>
<td>Repeating hexapeptides sequences render the protein its hydrophobic character. The hydrophobic amino acids (e.g., Ala) lead to self-assembly of silkie fibroin via β-sheet conversion, while glycine residues facilitate cross-linking between β-sheets predominantly by hydrogen bonding.</td>
<td>(i) This property of self-assembly of silkie fibroin is utilized as post-printing gelation strategy for construct stability, hence eradicating the need for harsh cross-linking agents. (ii) The predominance of β-sheet domains gives high mechanical strength and toughness to silkie fibroin, hence regulating cellular mecano-transduction in 3D bioprinted constructs for guiding orientation/differentiation. (iii) Once crystallized, degradation of silkie is slow which would prolong the biodegradation of such constructs in vivo, esp. targeted for hard tissue regeneration.</td>
<td></td>
</tr>
<tr>
<td>Processed into water-soluble solution up to 30 wt% silk protein&lt;sup&gt;[12]&lt;/sup&gt;</td>
<td>Prevalent intra- and inter-molecular hydrophobic interactions inhibits self-assembly between the hydrophobic domains due to strong static repulsion. The hydrophilic domains direct the formation of disorganized and extensible amorphous structures in contrast to their hydrophobic counterparts.</td>
<td>(i) A water-based hydrogel system ensures sufficient swelling of the construct necessary for hydration, hence facilitating cell survival for extended intervals for 3D bioprinting activity. (ii) Hydrated hydrogel-based matrix aids in entrapment of matrix biomolecules and proteins required for cell functioning. (iii) Flexibility of improving the silkie fibroin protein concentration to 30 wt% increases the probability of more exposed cell adhesive amino acids on the surface of construct for biomolecules and consequently cells.</td>
<td></td>
</tr>
<tr>
<td>Amphiphilic nature of silk protein chains&lt;sup&gt;[65]&lt;/sup&gt;</td>
<td>Hydrophilicity is imparted by Asp and Glu at the N- and C-terminus resp. and –COO as side chains.</td>
<td>Amphilic domains facilitate self-assembly of silkie fibroin into micelles. Stimuli-responsive C- and N-terminal domains play a role in fiber formation under shear force. This helps in generation of precise volume of fibroin protein drops in pico- to nanoliter orders (for inkjet printing) or continuous filaments (for extrusion-based printing) when shear force is applied. The silk protein structure could be tethered with various biomolecules targeted for triggering specific cellular signaling (viability, proliferation, differentiation) with respect to different cell types and tissues.</td>
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</tr>
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<td>Tailoring of chemistry of silkie fibroin (For eg., diazonium coupling&lt;sup&gt;[56]&lt;/sup&gt;, EDC–NHS chemistry&lt;sup&gt;[57]&lt;/sup&gt;, Carbodimide coupling&lt;sup&gt;[58]&lt;/sup&gt;)</td>
<td>Functionalization of specific amino acid side chains (∼10%). For eg., Trp and Tyr</td>
<td>The sequence yields viscoelastic behavior with elastic modulus of hydrogel in the order of 0.02 kPa in similar order to that of soft human tissues. A major advantage being that physically crosslinked recombinant silkie fibroin are dominated by crystalline domains exhibiting hydrophobic interactions and physical entanglements; all of which contribute towards reversibility of volume, hence shear-thinning&lt;sup&gt;[64]&lt;/sup&gt;. Once extruded, the crosslinks can be disrupted, and the bioink instantly regains its original stiffness. Several recombinant silkie fibroin sequences have now been synthesized with precisely modulated material properties, while their application to 3D bioprinting is still in infancy; the sequences have certified potential to act as cytocompatible bioinks for biomedical applications.</td>
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Mechanical properties, cellular attachment, and mineralization of the native silkie silk through the synthesis of recombinant silkie silk proteins<sup>[61–64]</sup>.

2.2.3. Recombinant Spider Silk

By using recombinant DNA technology, synthetic spidroin genes that encode parts of dragline silkie proteins have been designed for silkie protein production in heterologous hosts (yeast, bacteria, plant, or mammalian cells). Further, important sequence repeats for controlled structural assembly can be genetically designed into an expression vector, followed by expression in a suitable host. The unique printability of recombinant silkie silks originates from this controlled self-assembly of their unique amino acid sequences. While the C-terminal domain houses the hydrophobic regions responsible for β-sheet assembly, the N-terminal domain interconnects the spidroins into endless protein chains by predominance of helical conformation. This unique arrangement of smaller hydrophilic links annealed between repetitive crystalline β-sheet regions help reinforce fiber alignment upon shear thinning<sup>[64]</sup>.

Several spider silkie-like polypeptides were designed focusing on this controlled interplay among the sequence-secondary structure–properties (Figure 3). To achieve controlled secondary conformational behavior, researchers systematically vary the ratio of hydrophilic to hydrophobic blocks<sup>[63]</sup> to regulate its transition into β-sheet conformation and hence render control over ink (viscosity, rheology) and material (degradation, mechanical stiffness) properties. One such example is the recombinant silkie silkie eADF4 hydrogels consisting of very small amino acid chains showing shear-thinning (non-Newtonian) behavior<sup>[49]</sup>. This synthetic sequence is identical to the core of dragline silkie fibroin consisting of a repetitive domain of 44ADF4 from European garden silkie Araneus diadematus. The sequence yields viscoelastic behavior with elastic modulus of hydrogel in the order of 0.02 kPa in similar order to that of soft human tissues. A major advantage being that physically crosslinked recombinant silkie silkie are dominated by crystalline domains exhibiting hydrophobic interactions and physical entanglements; all of which contribute towards reversibility of volume, hence shear-thinning<sup>[64]</sup>. Once extruded, the crosslinks can be disrupted, and the bioink instantly regains its original stiffness. Several recombinant silkie silkie sequences have now been synthesized with precisely modulated material properties, while their application to 3D bioprinting is still in infancy; the sequences have certified potential to act as cytocompatible bioinks for biomedical applications.
3. Challenges in Silk-Bioink Development

3.1. Challenges of Bioprinting Silkworm Silk

Like many other biopolymers (alginate, collagen), regenerated silk fibroin protein solution does not fulfil all requirements of printability due to several reasons. First, silk fibroin inks display shear thinning behavior at low concentrations (i.e., <20 wt%) upon application of physical stress or applied pressure; i.e., the concentration range is not optimal for printing. However, at concentrations >20 wt%, it shows typical Newtonian fluid behavior,[12] where viscosity remains constant even after shear rate increases (Figure 4). Primary reason being that at higher concentrations of polymers, the mobility of constituent macro-molecular chains is reduced, hence impeding their capacity to self-assemble and align.

Second, 3D printing of regenerated silk fibroin solution was achieved by direct-writing of aqueous silk fibroin solution into a coagulating alcohol bath (86% methanol), such that the deposited fibers suffered instant solidification by induced

![Diagram](image)

Figure 3. a) Illustration of Native spider dragline silk and its molecular structure. b) The development of the recombinant spider silk to achieve higher mechanical strength. c) The attachment of the silica with the recombinant spider silk to achieve control over architecture of the matrix using R5 protein.[61] d) Modification of the recombinant spider silk with RGD peptide sequence for improved cell adhesion.[62] e) The addition of the metallic salts, such as Ca$^{2+}$, K$, Mg^{2+}$, to improve the mineralization in recombinant spider silk scaffolds.[63]
β-sheet crystallization, to fabricate complex 3D microperiodic architectures.[12] 3D microperiodic filaments as low as ≈5 µm in diameter were produced, which is not possible to attain by using other rapid prototyping methods, which produce fiber diameters in the range of 150–400 µm.[12] However, during the printing process, the fibroin chains underwent shear force (<100 s⁻¹) resulting in conformational changes from random coil to β-sheet formation, resulting from thermodynamic and kinetic processes.[4] This resulted in clogging of micro-nozzle due to β-sheet crystallization induced by shear stress, obstructing smooth flow of the printed filaments. In addition to the problem of frequent nozzle choking and disruption in

![Article Image](image-url)
printing, alcohol based coagulating baths cannot be used while depositing cells. Therefore, it is very challenging to print silk fibroin solution alone without additives.[13]

Gelatin was added to develop a physical blend of silk fibroin-gelatin (SF-G) ink, to enhance flowability of silk fibroin by imparting shear thinning behavior[6,66] due to the difference in isoelectric points of silk and gelatin. Moreover, addition of gelatin imparted biofunctionalization (Arg-Gly-Asp (RGD) motifs) to the bioink.[4] Physically blended SF-G bioink formulations might be advantageous as it avoids any chemical or photo crosslinking, and develops a gel on account of combined effect of hydrogen bonding and/or hydrophobic interactions, electrostatic interaction, or hydrogen bridges. However, these interactions are reversible[11] due to thermoresponsive behavior of gelatin, exhibiting aqueous phase at physiological temperature (37 °C) while gel transformation occurs at temperatures <30 °C. Therefore, these interactions are weak and often result in uncontrolled degradation requiring further crosslinking of these cell-laden SF-G constructs to render stability at physiological temperature.

Third, dissolution process of silk cocoon to reconstitute silk fibroin solution results in random degradation of the fibroin protein chains subjective to the processing parameters including the temperature applied and the duration and extent of degumming.[67] This significant degradation of silk fibroin self-assembly, leads to degradation of fibroin chains in the broad range of 40–50 kDa, which further impacts the viscosity of inks for 3D printing.[11,32,65] Consequently, to facilitate bioprinting, such low viscosity bioinks require faster crosslinking mechanisms (by using alcohol coagulating bath) to facilitate the layering of the 3D printed constructs, which would adversely affect the encapsulated cells.[11] To circumvent these issues, use of native silk directly from glands may provide a good source of silk but in that case the availability will be limited.[11] Separation of sericin from fibroin will be another obstacle deterring from the use of this silk type. Therefore, considering the available choices, use of regenerated silk is the most viable option for 3D bioprinting.

Another major challenge with 3D bioprinting of silk fibroin is optimization of intrinsic physical properties (surface tension, viscosity, rheology) to facilitate printability of the ink and material properties (swelling ratio) properties for accuracy of tailor-made printed geometry. While surface tension property (0.04 and 0.07 N m⁻¹) aids in generating solution drops of silk, which in association with the amphiphilic nature of silk, regulates flowability of ink under shear force for 3D bioprinting applications.[30] In terms of rheology, different hydrogels can possess different behaviors. For instance, collagen, primarily used as a hydrogel for soft tissue regeneration such as skin, has the tendency to drastically contract during in vitro incubation period. This can invariably affect resultant physical properties (pores, interconnects, geometry) of the hydrogel scaffold having consequential effects on its suitability for the target-tissue used (Table 1). Silk fibroin, on the other hand, depicts a tendency for swelling behavior[6] in the range of 15–25% (Figure 5a,b) by absorbing water or media (in case of in vitro cultures). This property may invariably affect the resultant geometry and porosity of the construct, and hence this aspect of silk-based bioink must be considered for high resolution 3D bioprinting.

3.2. Challenges of Bioprinting Spider Silk Ink

As opposed to silkworm silk, bioink derived from spider silk can smoothly extrude from the micronozzle without choking.[65] However, a major disadvantage associated with spider silks is that it does not degrade at physiological temperatures due to the lack of enzyme degradation sites[49,65] which may not be ideal for in vivo applications. This property is majorly overcome using recombinant spider inks. Since the ink is generated from shorter, known sequences constituting of parts of silk chains, they are adhered together using synthetically derived polymers such as polyethylene glycol (PEG), polyethylene oxide (PEO), or polyacrylic acid. In the process, 3D constructs acquire...
extreme hydrophilicity which renders surface unsuitable for cell anchorage and subsequently affects biological functions (Table 1). \[49\]

A similar response was reported by Schacht et al. \[49\] when they prepared a 3D construct using a cysteine variant of the dragline spider silk protein ADF4 of A. diadematus ADF4(C16) (sequence: 16 repeats of module C: GSSAAAAAAAS-GPGGYG PENQGSPGGYGPJGP) and cultured mouse fibroblast cells for 24 h. The 3D printed cell-laden constructs demonstrated weak cell adhesion as indicated by round and aggregated cellular morphology due to lack of cell adhesion motifs. Thus, hydrophobic interactions and surface topography of the constructs mostly influenced the cellular adhesion in this case. However, when cell-adhesive RGD motifs were attached to the sequence (eADF4(C16)-RGD), fibroblast adhesion increased 15 times compared to the former. These studies paved the way for the synthesis of a number of synthetic fragments possessing cell attachment domains to fabricate cytotocompatible 3D constructs.

Another major challenge associated with the application of larger bioengineered silk mimetic polypeptides is the uncontrollable and irreversible aggregation of the alanine-rich repeats (Figure 2). Tailor-made block copolymers and silk polypeptide fragments often self-assemble rapidly, leading to precipitation of insoluble complexes. \[48\] Lithium bromide is often used in high concentrations to disrupt the existing hydrogen bonding between the macromolecular chains in order to promote solubility of protein. \[43\] However, most spider silks resist this disruption in bonds as compared to silkworm silk, which demands some chemical modification in the sequence chain to maintain the predominant random coil conformation until fabrication of unstable, deformed structures. Therefore, when cell-adhesive RGD motifs were attached to the sequence (eADF4(C16)-RGD), fibroblast adhesion increased 15 times compared to the former. These studies paved the way for the synthesis of a number of synthetic fragments possessing cell attachment domains to fabricate cytotocompatible 3D constructs.

Using a different modification strategy, an engineered recombinant silk protein was strategically conjugated with methionine residues in a study conducted by Valluzzi et al. \[72\] The underlying mechanism was to incorporate a polar sulfide by oxidation of the residues to increase protein polarity and reversibly place a hydrophilic residue. \[68\] Subsequent experiments revealed that as soon as the methionine residue is reduced, it triggers the self-assembly process of silk. Reoxidation of methionine could revert this process; a property not present in native silk. \[73\] But triggering mechanisms can prove to be relatively harsh on the consequent material properties. A relatively milder mode of enzymatic modification was introduced by Winkler et al. by installing serine phosphorylation/dephosphorylation sites to induce silk assembly. \[68\]

This flexibility rendered by silk polymer to control/modulate the self-assembly process will lead to viable methods to simulate the natural process of silk spinning wherein 30% (w/v) protein concentrations remains stable in aqueous medium, hence aiding in the design and development of stable 3D bioprinted tissue constructs.

4. Optimization of Silk Bioink Rheology

Native silkworm fibroin solution undergoes shear-thinning while passing through spinneret, during which β-crystallization is induced through shear force, hydrophobic interactions, and ionic interactions, and β-crystallites are aligned by flow-induced orientation. In addition, increase in elongational viscosity during stretching of extruded filament plays crucial role in stabilizing the fibroin filaments during spinning by spiders or silkworms. \[74\] Targeting similar biomimetic spinning, researchers attempted to use regenerated silk solution to spin filaments by wet spinning or microfluidic spinning. Modulus of a hydrogel bioink depends upon two factors; G′, which refers to the deformation energy stored during physical/shear stress (i.e., solid phase of the hydrogel) and G″, referring to the dissipated energy during physical/shear stress (i.e., liquid phase of the hydrogel). Another critical factor, known as the loss factor (\(\tan \delta\)) is computed by a simple calculation of \(\frac{G″}{G′}\). Therefore, if \(G″ > G′\) (i.e., \(\tan \delta > 1\)), the hydrogel flows like a liquid while, alternatively, when \(G″ > G′\) with tan \(\delta < 1\), the hydrogel would resist flow and thus behave more like a solid. In case of polymeric solutions (Figure 4b,c), higher value of G″ over \(G′\) is initially observed, but with increasing frequency a crossover point is reached (attaining \(G′\) greater than \(G″\)), either by concentration exceeding their entanglement concentration, or by chemical and physical crosslinking. On the contrary, a solid hydrogel demonstrates \(G″ > G′\) at all values of frequency and time. In case of native silk fibroin solution, viscoelasticity is higher, \[73\] with \(G″ > G′\) at higher frequencies, whereas at low frequency, it exhibits crossover to liquid behavior with \(G″ < G′\).

These observations are typical of a concentrated polymer solution exceeding the overlap threshold, where existing inter-chain entanglements determine the rheology of polymer. \[74\]

In extrusion-based bioprinting process, a fluidic bioink is printable under applied deformation if a continuous steady-state hydrogel filament proceeds without a break. Proper optimization of silk bioink’s viscosity, surface tension, and inertial forces (\(G″, G′\), due to entanglement or crystallization), applied pressure, velocity of the linear stream, and the cross-sectional
area of the nozzle ensure break free filament formation. But, there are still several features that differentiate it from the rheology and spinnability of native silk spinning dope. First, elongational viscosity component is absent in 3D bioprinting as there is no provision of stretching after releasing from the micronozzle, which may lead to a localized defect causing disruption in filament. Second, extrude swell is an extremely important factor to be taken into account especially in the case of extrusion-based bioprinter by robotic fluidic dispensing system as high resolution structure formations are involved. When silk fibroin solution is injected through the micronozzle tube, the bioink is compressed by the given confinement, and the polymer chains get stretched within the nozzle. After extrusion, the polymer jet would expand/bulge due to the release of stored elastic energy within the viscoelastic polymer chains. In shorter capillary, a large proportion of the elastic energy would be stored resulting in large swelling. But in larger capillary part of the stored elastic energy will be released, resulting in lower swelling of extrudates. Therefore the main challenge is to optimize the extrude swell of the silk ink to prevent dripping and ensure smooth extrusion process for printing. Third, in native silkworm silk obtained from B. mori, typically shear-rate is low (<0.1 s⁻¹), with viscosity in the range of 10⁻¹⁵⁻¹⁰ Pa s, and the cross-over from solid to viscous state (at G' ≈ G″) occurs at angular frequencies between 1–10 rad s⁻¹. On the other hand, Ochi et al. represented evidence of silk fibroin in solid hydrogel form (G' > G″) at all frequencies tested. Therefore, it may be logical to believe that these inconsistencies or contradictions may reflect inherent variations within biological samples hence impeding standardization of rheological outcomes, even for the same system (silk species), constituting a major obstacle for further development of the silk-based bioinks.

For the first time we demonstrated that 3D microperiodic silk fibroin structures can be printed using extrusion-based direct-write printer using concentrated silk fibroin solution in alcohol coagulating bath (Figure 2). Interestingly, when the shear rate was raised above 100 s⁻¹, silk solution showed a sharp shear thickening behavior, due to transition toward β-sheet crystallization; causing micro nozzle choking during direct ink writing technique. Interestingly, using highly concentrated silk solution, previous studies have reported rheology up to a maximum of 100 s⁻¹ of shear rate; hence, shear thickening behavior noticed in this particular study was not reported earlier (Figure 4d). Ideally, to facilitate smooth extrusion through micronozzle, the yield stress of the ink must be overcome by the exceeding applied shear stress in the print head to fluidize ink, and once extruded, and instantly regain their original yield stress and shear elastic modulus, G'. However, if large shear forces are applied on silk solution (>100 s⁻¹), the thermodynamic and kinetic processes immediately lead to β-sheet conformation within the nozzle even before extrusion, which is a major limitation of silk inks.

To overcome this problem, gelatin was added to silk⁴⁻⁶ to prepare SF-G blend which exhibited shear thinning across a range of concentrations resulting in drastic increase in the elastic modulus of the blend, most likely facilitated by the interchain interactions due to hydrophobic as well as electrostatic interactions between the two protein macromolecules. For instance, G' and G″ values drastically increased by ≈2000 times and 100 times respectively, for 5SF-50G blend over silk fibroin solution only, under consistent oscillation (1% strain) and frequency (1 Hz). In cases where G’ < G″ (>2000 Pa; for higher gelatin concentrations, 5SF-50G and 5SF-40G) the hydrogel behaved more solid-like hindering smooth extrusion through micronozzle (Figure 4c-g). But after addition of low gelatin concentration, where G'' > G' (i.e., elastic modulus is less than 2000 Pa), SF-G ink extruded smoothly through the micronozzle. Therefore using 5SF-30G and 5SF-20G blends, we were able to optimize the parameters for optimal viscosity to obtain smooth flow of polymer ink to produce self-supportive filamentous structures via direct-writing.⁴

Further, interaction of the cell suspension and biopolymers can drastically affect the rheology of the bioink. In that respect Maisonneuve et al. reported that the viscosity of cell suspension in the presence of biopolymers, at different volume fractions, vary at different stages of shear rate as a function of the applied shear stress due to the transient cell–cell and cell–biopolymer dynamics. This perceptive will help to envisage and deduce the non-Newtonian behaviors of cell suspensions under the influence of shear flows exerted by the biopolymer. Similarly, we also noticed an abrupt change in viscosity of the SF-G bioink before and after encapsulation of mesenchymal stem cells (MSCs) during the flow of bioink that altered the time of enzymatic crosslinking in our system (unpublished data). This also highlights the requirement for the extensive rheological analysis using sophisticated micro rheology techniques to measure rheology of the cell encapsulated bioink systems prior to cell encapsulation, in order to develop new and efficient bioink compositions for their use in bioprinting. However, once printed and incubated in media for in vitro cultures, another critical feature that is often ignored in the case of bioprinting is that cytoplasm of cells and cellular cytoskeleton are thixotropic in nature, as they demonstrate time-dependent decrease in viscosity at steady shear rate and subsequent recovery when the flow is stopped, which may considerably affect the resultant rheological properties of the bioink. Hence, it may be interesting in future to investigate the dynamic behavior of cells encapsulated in the bioprinted constructs in context of both silk bioink material as well as cellular-induced rheological modifications.

Similarly, in a recent study, silk-based bioink formulations were optimized to suffice suitability for soft tissue reconstruction bearing optimum physical and mechanical properties. SF-G-glycerol blend was prepared to develop materials that demonstrate optimal properties under physiological printing conditions. The results depicted that gelatin concentration above 10% (w/v) was optimal for printing in physiological conditions (37 °C extrusion and 20–25 °C deposition). This was in accordance to our previous reports where a lower gelatin concentration (<40% (w/v)) smooth ink extrusion by enhanced viscoelasticity, while too low a concentration resulted in extremely low elastic modulus hence depicting issues in solidifying. However, in the given study, authors have shown that gelatin concentrations exceeding 15% (w/v) lead to choking of the micronozzle tip. This was not observed in our study. We selected SF (10%, w/v)-G blend with a sol-to-gel transition temperature of 18 °C. A possible reason for the differential working concentrations of gelatin in both studies could be that the gelatin used had different bloom numbers or molecular weights. The Bloom strength is defined as a
measures at a yield stress of 179 ± 367 cP) and plastic viscosity (1978 to the structure without any additional polymerization steps. Typically, with increasing Bloom number, the solubility of gelatin in water decreases, hence dictating the concentration conditions. As we know, the elastic modulus. But, in this case, the addition of glycerol (as low as 1%) lead to increased gelation of the SF-G blends, hence producing self-supporting structures. Although the structures were mechanically stable, the blended formulations yielded prints in the feature size range of 250 μm. As we know, SF-G constructs have an inherent tendency to swell in aqueous medium, which can significantly change the resultant geometry of the construct, has not been taken into account here.

In the recombinant spider silk eADF4(C16), RGD-motif was induced in the established bioink. Like most concentrated polymer networks, spider silk hydrogels depict viscoelastic behavior, with stress increasing proportionately linear with strain. The elastic modulus of 3% (w/v) of eADF4(C16) hydrogel was ≈ 2 kPa, which is significantly lower than the elastic modulus of the SF-G blends, hence producing self-supporting structures. Although the structures were mechanically stable, the blended formulations yielded prints in the feature size range of 250 μm. As we know, SF-G constructs have an inherent tendency to swell in aqueous medium, which can significantly change the resultant geometry of the construct, has not been taken into account here.

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5. Cytocompatible Gelation Strategies

The bioink properties before, during and after gelation are essential for its printability. For efficiently fabricating stable 3D bioprinted constructs, it requires printing a cell-laden polymer solution with gelation induced rapidly after extrusion. To print structures with high printability and structural fidelity, the bioink must be either viscous or instantly cross-linked post-printing; however, both methods are unsuitable for sustaining cell viability in bioprinted constructs. Therefore, slower gelation strategies are being devised for cell-laden constructs, however, care needs to be taken to minimize the time of gelation as much as possible as it leads to sedimentation of cells in the bioink resulting in clogging of the nozzle and non-homogenous cellular distribution. Hence, gelation of bioinks is extremely tricky, considering its consequential effects on material's mechanics, degradation, diffusion rate and biological responses.

While instant gelation can be induced in microperiodic silk only constructs using 86% methanol immersion (Figure 2), frequent choking of nozzle and exposure to alcohol render the ink unsuitable for bioprinting. For cell encapsulated constructs, silk was mixed with gelatin in a physical blend which was stabilized by hydrogen bonds, hydrophobic interactions and/or ionic interactions and entanglements of high molecular weight polymer chains. However, these networks entail weak bonding, reversible and their structural stability is difficult to control, esp. owing to the thermo-responsive nature of gelatin. Therefore, physical cross-linking was performed using sonication which induced β-sheet crystals in silk fibroin as a result of enhanced molecular vibration, hydration of hydrophobic domains and transient increase in local temperature of bioink. Optimization studies revealed that increase in temperature and sonication time were directly proportional to the extent of gelation of induced. For instance, 8SF-15G blend exposed to 10 s of sonication at 50% amplitude induced gelation within 1 min 30 min at 37 °C, while no gelation was induced at 4 °C (Figure 5c,d). These factors cumulatively led to silk-induced physical crosslinking of the hydrogel with no effect on the conformation of gelatin. Another useful strategy for two-part room temperature curing was reported which utilized the self-organizing properties of aqueous silk fibroin to produce intermediate crystallization, further stabilized into evaporation-induced buckling of deposited silk inks, hence avoiding application of harsh curing methods (high temperatures, UV) used in conventional rapid prototyping techniques. The silk-polyol blends facilitated predictable micro-extrusion volumes, resulting in constructs with improved mechanical properties precisely stacked into complex 3D architectures.

To overcome this limitation, chemical functionalities can be introduced to improve the mechanical strength of the hydrogel by creating covalent crosslinks, thereby resulting in an irreversibly linked network. Coupling with chemical crosslinking using methanol, glutaraldehyde, EDC–NHS varying concentrations of photoinitiator and exposure to UV light (time, duration) which makes them unsuitable for cell printing was a common method. Another method for crosslinking of natural hydrogel derivatives of bioink such as gelatin, hyaluronic acid, dextran was done using methacrylate or methacrylamide. The authors reported the use of fabricated bioelastomer in 10 and 15% (w/v) methacrylated tropoelastin gels containing 31% methacrylation by controlling the extent of methacrylation and protein concentration. However, photopolymerizable groups such as acrylates, require illumination by UV light immediately after plotting which due to liberation of free radicals inside the hydrogel may question the long-term sustainability of encapsulated cells. Therefore, fabricating 3D bioprintable constructs with preserved viability over long-term culture periods, are presently very challenging to obtain.
Alternatively, strategies incorporating other materials which may require polymerization methods, such as chemical crosslinking have also been introduced. One example is enzymatic crosslinking induced by mushroom tyrosinase.⁵ Without disrupting the covalent links, tyrosinase enzyme oxidizes ≈10–11% of residues of silk (275 and 10 tyrosine residues in heavy chain and light chains of B. mori respectively) and ≈20% of the residues in gelatin into reactive o-quinone moieties. These oxidized residues club with available nucleophiles (amine groups of gelatin/silk) and trigger non-enzymatic reactions or have the option of condensing with neighboring quinine moieties. Using enzymatic gelation with tyrosinase, we demonstrated successful crosslinking in 3D bioprinted SF-G constructs with cytocompatible properties as illustrated by survivability of human turbinate mesenchymal stem cells for at least a month. However, the extent of gelation could be altered as a function of temperature and enzyme concentration. For instance, SSF-15G blend started to gel after the addition of 500 U of tyrosinase at 28 and 37°C within 30 and 15 min, respectively. When tyrosinase concentration was reduced to lower than 300 U, gelation did not initiate even after 1 h of incubation at respective temperatures.

Further, using robotic dispensing, hydrogel-based structures made from recombinant silk offered process-compatibility and high structural fidelity. A silk solution, eADF4(C16), was bioprinted with applied shear force of 1.0 to 1.1 bar with human fibroblasts.⁶⁰ However, one major advantage with recombinant silk bioinks is the non-requirement of post-processing steps. For smaller sequences, transition toward β-sheet crystals is usually modulated by precisely controlling the ratio of hydrophobic to hydrophilic chains. However, one major drawback of using large bioengineered recombinant spider silk sequences is instant self-aggregation or assembly. To deal with this, researchers commonly use fusion proteins (thioredoxin)⁷¹ and amino acids (methionine)⁷² and tether them to the engineered sequences to avoid self-assembly until desired. However, one major concern of such sequences being subjected to bioprinting application is that once these proteins are purified or removed, silk chains instantly aggregate. Therefore, controlling the rate of this gelation strategy and the ultimate viscosity of the resultant ink would be a huge challenge.

6. Biological Characterization

6.1. Cell Viability

The two major concerns regarding cell viability in 3D bioprinted constructs include achieving a high percentage of viable cells during printing and sustaining the viability over extended culture periods. There are several steps in bioprinting which can significantly affect cell viability either at printing stage (nozzle diameter, design, shear stress), construct design, and geometry (orientation of cell-laden filaments, pore size and porosity, interconnectivity, tortuosity) or post-printing stabilization methods (physical, chemical or enzymatic cross-linking). Of these, the diameter/length ratio of the extrusion micro-nozzle and shear stress applied are two critical factors which have an immediate effect on the cell viability.

Most studies have reported a dip in cell viability immediately post-printing⁶¹ due to shear-induced stress, but cell number gradually increases with culture time. This phenomenon, while currently ignored at in vitro level, may have dire consequences if the 3D bioprinted constructs are being targeted for clinical application. Sudden cell death occurs via necrosis, which is an uncontrolled mode of cell death typically caused by external factors that involves the early loss of the cell membrane integrity, hence inducing an acute inflammation due to the leaked cellular content in the lack of lysosomal activity.⁴⁴ Therefore, such constructs may be regarded as completely unsuitable for implantation purposes. Again, this argument will only be valid in case of extreme shear stresses as slight changes in stress rate may induce apoptosis, a programmed cell death mechanism, with no adversaries associated with inflammatory responses.⁴⁴ Therefore, we believe that it is extremely important to properly characterize the ink rheology, viscosity as well as composition to sustain a healthy, viable population of resident cells inside the matrix. For the first time, we showed that SF-G bioink had the potential to support high cell viability (82–87% of human turbinate-derived MSCs) for at least a month⁵ with no evidence of decrease in cell viability immediately post-printing (Figure 6a,c), hence warranting their use in preclinical implantation studies to push them closer toward clinic. Another interesting aspect would be to investigate the alterations in the cell nuclear activity as a function of the mechanical compression of cells offered by the increased shear stress to extrude bioink. Biophysical forces acting on the cellular cytoplasm while passing through a nozzle might result in rupturing of the nuclear envelope, subsequently leading to the formation of a bleb and chromatin hernia due to leakage of chromatin contents.⁸⁰ However, the deformation of the nuclear envelope restores overtime. This may be applicable in the case of bioprinting since only small shear stress is applied due to cells present inside the hydrogel, hence inducing reversible changes in the nucleus and cytoskeleton forces. But where larger shear forces are applied due to high viscosity or concentration of bioink, this the damage may be irreparable, consequently affecting cell survival and causing inflammatory responses due to leaked cellular contents.⁴⁴

However, getting closer to the subject, while the initial cell viability is a function of printing parameters, the preservation and sustainability of cell numbers overtime are dependent on several other factors. Most studies focusing on 3D bioprinting have shown viability maximum up to 7–14 d.⁸⁹,⁹⁰ Therefore long-term cell viability is often a challenging proposition in context of bioprinted constructs. For instance, a 3D bioprinted construct created using adipose derived MSCs encapsulated with decellularized adipose tissue’s matrix to closely mimic the native tissue environment in vivo.⁹¹ A significant decline in cell viability was conspicuous from the peripheral zones toward the central core of the construct within 14 d. This may be due to several reasons including restricted matrix diffusivity curtailing adequate nutrient diffusivity, composition of bioink, concentrated cellular distribution, construct geometry, etc. Dimension of complex bioprinted constructs may be in the order of few centimeters. Silk-based bioinks offer great advantage in that respect as being a hydrogel it can be precisely tailor-made to crosslink in order to control oxygen transport and mass transport.⁹⁰
Apart from material characteristics, cellular components such as the cell seeding modality (dispersed cells or aggregates), the cell type being used, target tissue type etc. also play a key role in defining long-term viability in vitro. Precisely, we used SF-G cell-laden constructs wherein two cell populations, either hMSCs or chondrocytes, encountered varied responses toward viability within similar SF-G-laden matrix.[6] While hMSC aggregates experienced a drastic dip (≈3 times) in cell number by day 14 (Figure 6b), owing to the hypoxic microenvironment toward the core of aggregates (confirmed by HIF1A expression) (Figure 6e), chondrocytic aggregates remained largely unaffected. This was attributed to their adaptability to the native hypoxic environment of cartilage tissue. Moreover, a gradual progressive increase in cell number was evident in case of homogenously dispersed cell-laden constructs as compared to clustered cells in aggregated form bestowing wall-to-wall contact and higher cell density within the aggregated region.

Whereas with regards to spider silk, progress in relatively slow with paucity of data in terms of biological characterization of bioprinted constructs. As discussed in the previous section, an ideal scenario for developing a bioink suitable for cell deposition would be the need to avoid additional components or post-processing parameters which may harm the cells. Although it is proven that spider silk constructs support high cell viability, albeit the values are lower than other molecular engineered hydrogels (>90% viability)[92] and established bioinks. A recently conducted study demonstrated successful bioprinting of recombinant hydrogel of eADF4(C16) spider silk solution with human fibroblasts and printed using 3D robotic dispensing system. The constructs demonstrated live cells until 7 d and no decline in cell viability as a function of the printing process (printing speed, shear force) was reported. However, extended culture periods are required to monitor their potential as tissue mimics.

6.2. Motility, Migration, and Remodeling

Cell motility, proliferation, and matrix remodeling are important factors in the development of a functional 3D bioprinted construct. Since the cells are encapsulated in a hydrogel, post-printing they should be able to proliferate within the hydrogel bioink and self-assemble into complex anatomically relevant 3D cellular architectures. Integrins expression and stable focal adhesion formation with the bioink helps cells to anchor with pericellular niche (Figure 7a) and govern cell–cell communication or contraction (Figure 7b). Therefore, the bioink has an undying role in deciding the fate of encapsulated cells. First, the chemical composition of the bioink regulates deposition and entrapment of secreted proteins, such as, signaling proteins, or adhesion molecules. Cells may secrete a series of cytokines, matrix degrading enzymes, and morphogens to develop an environment for migration. Then, 3D bioprinted hydrogel matrix similar to the ECM of native tissue provides architectural and mechanical cues that guide cells for motility and alignment. This is particularly observed in the case of alginate hydrogel bioinks cross-linked by Ca\(^{2+}\), wherein the hydrogels depict faster degradation rate, whereas lack of adhesion motifs rendering cells unable to express focal adhesion points or integrins, leading to poor cell proliferation; eventually cells fail to remodel the surrounding matrix.[5,15]

Characteristic features of silk-based bioinks support proliferation of bioprinted cells within the construct. Das et al. bioprinted SF-G constructs with hTMSCs and induced gelation by different cross-linking methods (tyrosinase crosslinking or sonication) which directly affected the stiffness of the resultant
matrix. While alginate constructs failed to undergo cellular proliferation, maximum increase in cell numbers was observed in tyrosinase crosslinked SF-G matrices containing relatively lesser β-sheet content and therefore lesser stiffness.[5] A probable cause could be that a lesser compact matrix would facilitate easy diffusion of nutrients as well as proteolytic enzymes for cell regulated matrix differentiation and remodeling, which does not hold true for more stiffer sonication-induced structures.

The data was further corroborated with another study from our laboratory where we used MSC-laden SF-G bioink and demonstrated increased in the DNA content over a 21 d culture period.[6] Concomitantly, through gene expression profiling we noticed significant levels of MMP2 (matrix metalloproteinase 2) activity within 14 d of culture. Apart from its known gelatinase behavior,[6] literature suggests that silk films exposed to MMP2 enzyme are known to degrade by decrease in the amount of β-sheet content as early as 5 d of exposure. Therefore, it is only

Figure 7. Morphogenesis in 3D bioprinted constructs. a) Cells formed strong attachment with silk-gelatin bioink, degraded and remodeled pericellular matrix, b) cells expressed lamellipodia, filopodia to contract bioink matrix, c–e) cells migrate from embedded spheroids (arrows in c,d), probably triggered by HIF1α expression (red) and form new aggregates,e) immediately after printing cells were entrapped in randomly oriented manner, but (b) with time cells showed coordinated directional sensing and cellular alignment toward the 3D printed bioink filaments (Figure 3 of ref. [7]).
logical to believe that the presence of vacant pericellular spaces evident in the H&E micrographs of 3D bioprinted SF-G constructs after 14 d was a result of degradation of the matrix due to cell synthesized MMP2 activity (Figure 7a). This pericellular space created by matrix degradation subsequently led to cell motility, proliferation, conspicuously evident in the aggregates (Figure 7c) where hMSCs oriented along the peripheral surface of aggregates forming individual cell clusters were prominent. The creation of new cell clusters by the self-assembly of cells through change of cell polarity, cell–cell and cell–ECM interactions in vacant/empty spaces of the SF-G matrix lead to the development of complex morphogenetic features specific to target tissue. This phenomena was specifically accredited to the presence of degradable SF-G matrix, as cellular aggregates without any surrounding matrix showed significantly decreased hMSC viability, strongly hinting toward restricted movement and consequential cell death within the hypoxic core. Not only limited to cell mobility and migration, but MMP sensitive hydrogels such as SF-G bioink have suggestive roles in promoting active matrix remodeling by facilitating timely release of entrapped ECM proteins (cell synthesized or extracellular) and growth factors. This was in accordance to the extensive glycosaminoglycan (GAG) production and deposition observed specifically within the pericellular regions of the hMSC-laden constructs within 14 d (Figure 7d), in part triggered by the hypoxic central core (Figure 7e). This cell enabled synthesis, accumulation, and release of morphogens is only possible in the presence of an optimal hydrogel matrix which further directs lineage commitment and cellular differentiation.[6]

Another interesting observation from the given study was that hydrogel stiffness (β-sheet content in case of silk-based bioinks) majorly dictates the fate of cellular differentiation.[5] While the stiffer sonication-induced SF-G matrices (~2 times higher β-sheet content) expressed significantly upregulated osteogenic markers (Runx2, alkaline phosphatase, osteopontin), the lesser stiff tyrosinase crosslinked matrices (with lesser β-sheet content) induced chondrogenic differentiation of encapsulated hTMSCs. Future studies may explore in detail about the underlying signaling mechanisms that trigger such cell–material interactions and direct cellular functions.

With evolving concepts on matrix properties used for 3D bioprinting, very few studies have reached the pre-clinical testing phase. In vivo evaluation of one of the 3D printed SF-G compositions constituting 1% (w/v) glycerol revealed that the non-porous printed matrices exhibited significant structural stability. The structures were mechanically more stable than SF-G or silk alone formulations. Cells infiltrated within the constructs by 2 weeks with a significant increase by week 4 concomitantly with material degradation.[33] The gel structure was evidently degraded after 3 months with significant collagen deposition. However, complete absence of multinucleated giant cells throughout the study period investigated suggested minimal inflammatory response. Hence the printed structures were suited for applications where shape and volume retention is needed. Though further experiments are required to determine tissue-specific applications of the SF-G-glycerol bioink material.[33]

Furthermore, as discussed above, cellular mobility can be affected by several microenvironmental conditions, such as geometry of construct, substrate stiffness, hydrophobicity/hydrophilicity of material, all of which require further standardization with respect to recombinant spider silks. One possible common highlighted in most studies associated with larger segments of recombinant spider silks is the reduced viability over other established bioinks which is due to hydrophilicity of PEG/PEO which impedes effective cell attachment ultimately leading to apoptosis. However, 3D bioprinting application of recombinant spider silk inks is still in its infancy and hence detailed biological characterization in terms of proliferation, differentiation, and gene expression analysis has not been clearly undertaken.

6.3. Morphogenesis in 3D Bioprinted Constructs

The ideal strategy for developing tissue engineered 3D bioprinted constructs relies upon structural cues of the surrounding matrix for guiding tissue morphogenesis. In this context, 3D bioprinting holds added advantageous over conventional tissue engineering because it can integrate multiple architectural, biochemical, and mechanical cues by spatially and temporally tuning cell–cell junction stability, cell body shape, and alignment by dictating the cell–cell cohesiveness necessary to build a functional tissue.[93] Although the concept of tissue bioprinting is quite remarkable, current evidences are not suggestive of any concrete involvement of 3D printed construct in guiding self-organization of tissues in vitro. Recently, we used bone marrow stromal cells bioprinted in a SF-G hydrogel niche as dispersed cells and observed cellular behavior overtime in vitro. Qualitative fluorescence microscopic analysis performed on regular time intervals depicted typical rounded cellular morphology with resident cells distanced from one to other inside the hydrogel matrix after 1 d of incubation (Figure 7f). Whereas after 7–14 d, bone-marrow derived mesenchymal stem cell (BMSCs) exhibited typical polarity with extended cellular body due to matrix interactions dictated by structural and mechanical cues from the SF-G construct (Figure 7g). The coordinated directional sensing and defined cellular alignment toward the deposited filaments was evident. Such dynamic cell–cell junctions are critical in mediating the behaviors of cellular clusters/or a group of cells in which individual cells coordinate their directionality and alignment by prominent cell–cell sensing.[94] Corroborating these results, based on another study from our lab,[6] we bioprinted a similar 3D matrix of silk-gelatin MSC aggregates using a 210 μm nozzle diameter. After 14 d, we observed migration of elongated singular cells oriented tangentially parallel to the peripheral zone of the cellular spheroids within the 3D construct in vitro. Cellular differentiation was initiated by 14 d of culture. This promotional effect may be attributed to improved and timely release of degraded construct, a phenomenon previously reported in context of 3D bioprinted sweat gland.[95] The encapsulated MSC synthesized extensive amounts of MMP2, which plays a major role in the degradation of SF-G matrix. As the material erodes around the pericellular zones (Figure 7a), the entrapped biological molecules will release along with the migrating cells, further dictating cell orientation, differentiation and morphogenesis. A validation to this hypothesis comes from the fact that chondrocyte aggregates
cultured in a similar matrix with identical differentiation conditions, showing depleted levels of MMP2 activity, did not exhibit cell migration or upregulated matrix synthesis.

6.4. 3D Bioprinted Cartilage

Development of a functional, clinically conformant cartilage is still a farfetched dream in the field of tissue engineering even after three decades of efforts for the development of tissue engineered cartilage.[1] The currently available tissue engineering based cartilage repair approaches are mostly focused on the development of 3D aggregates, 3D porous, or hydrogel based scaffold[96,97] which show improper integration with the defect size because of the obvious architectural mismatch between the engineered neo-cartilage and the host defect site.[98] 3D bioprinting offers a solution to all such problems by offering the development of site specific and patient specific constructs in any desired shape and size through the deposition of cell-laden bioink. En route to this approach we have endeavored the use of 3D bioprinting for the design and development of cartilaginous tissue constructs using human BMSCs and articular chondrocytes[6] to assess the differentiation potential of the bioprinted cells (both aggregate and dispersed) with respect to development of superior cartilage tissue equivalent (Figure 8a,b). Interestingly, we observed superior cell viability along with significant decline in markers of cartilage hypertrophy in the BMSC-laden SF-G ink based 3D bioprinted constructs as compared to chondrocyte-laden constructs. The reason could be attributed to the upregulated hypoxia as observed by upregulated gene expression of HIF1α and HDAC4 which lead to an upregulated expression of chondrogenic markers like aggregcan (ACAN) and COMP.1. The study drew attention to the probable role of our standardized SF-G bioink in controlling hypoxia and immobilization of growth factors leading to enhanced chondrogenic potential of the BMSCs encapsulated in 3D bioprinted constructs as compared to 3D aggregates. Nevertheless, the study did not shed much light on the inherent regulatory pathways of SF-G bioink that lead to reduction of cartilage hypertrophy in the BMSC-laden 3D bioprinted SF-G constructs. Shi et al. conjugated BMSC-specific-affinity peptide E7 with silk-gelatin which facilitated proliferation, supported chondrogenic differentiation and cartilage specific ECM synthesis by the cultured BMSCs.[99]

A major limitation of cartilage tissue engineering is lack of understanding to distinguish the development of neo-cartilage construct with regard to phenotypically stable articular cartilage or transient cartilage. Hence, instead of using primary cells having varying differentiation potential, we used a stable specialized BMSC cell line that is capable of maintaining a stable phenotype during chondrogenic pellet cultures.[100] Thus, in the follow-up recent study we tried to assess a number of conditions for optimizing the in vitro articular cartilage differentiation combining the useful effect of the 3D bioprinted SF-G bioink and TVA-BMSC cell line. TVA-BMSC is an immortalized cell line engineered to express specifically targeted transcription factors and activate specific signaling pathway with respect to the target tissue to be engineered on demand.[100] The developed 3D bioprinted constructs were assessed for long-term cell viability and expression of articular cartilage markers like Autotaxin and Proteoglycan 4. Our results suggested that TVA-BMSCs cultured in the presence of TGF-β1 show transient cartilage like properties, while when encapsulated in 3D bioprinted SF-G bioink, while in the absence of TGF-β1, TVA-BMSC laden, 3D bioprinted constructs developed into a stable articular cartilage. TVA-BMSCs cultured in 3D bioprinted SF-G matrix showed up to 88% cell viability up till 14 d post printing. Furthermore our study delineated the significant regulatory role of our standardized SF-G bioink in controlling the hypertrophic differentiation of BMSCs by governing Indian Hedgehog and Canonical Wnt signaling pathways. The gene interaction network in our study revealed stark similarities of the signaling pathways active in our 3D bioprinted SF-G constructs with the embryonic cartilage development pathways. Our study thus provided substantiated evidences that the 3D bioprinted articular cartilage tissue equivalent could contribute the necessary developmental cues for fabricating clinically conformant patient specific cartilage constructs.

6.5. 3D Bioprinted Bone

Congenital defects, oncolgical disorders, and trauma may lead to critical size defects in the bone. The currently available bone regeneration approaches mostly use either the autologous or allogeneic bone for transplantation or 3D porous scaffolds, which comes with a number of associated drawbacks.[101] For example, while the scaffold free regeneration approaches are associated with problems like donor site morbidity and lack of sufficient bone resources for transplantation; the 3D porous scaffold based approaches show lack of architectural control and control over pore size. In that context, the use of 3D bioprinting technology would provide efficient solutions to the above mentioned problems by providing patient-specific and donor-site specific engineered bone constructs ready for transplantation. In our lab we are trying to develop such 3D bioprinted bone tissue equivalents using BMSC-laden SF-G bioink where we are further emphasizing the role of targeting the developmental biology inspired differentiation protocols[102,103] that may enhance the osteogenic potential of the developed 3D bioprinted SF-G constructs both in vitro and in vivo (Figure 8c,d). Osteogenic gene expression was carefully monitored over 3–5 weeks in vitro by the expression of Runx2, alkaline phosphatase, osteocalcin, etc. which showed promising upregulation of these markers in 3D bioprinted SF-G constructs compared to cell aggregates cultured without the SF-G matrix. This controlled study highlighted the considerable involvement of the matrix in inducing cell-enabled responses toward tissue differentiation and development.

6.6. 3D Bioprinted Skin

We have recently developed 3D bioprinted skin tissue constructs using the SF-G bioink, by incorporation of human fibroblasts, keratinocytes, and melanocytes. The structures were designed in such a way that the crossing filaments would represent the dermal papillae at the interface of dermis and epidermis layer. We tried to artificially fabricate the intricate geometry of different layers, to replicate undulation in basement membrane of native skin which cannot be achieved by any technique.
other than 3D bioprinting. The construct composed of dual layers was developed by initially printing the fibroblast layers followed by keratinocyte layers after allowing the fibroblasts to proliferate throughout the construct. The bioink and the optimized printing parameters support viability of entrapped cells for more than 21 d as evaluated by live and dead staining. The cellular functionality was evaluated by gene expression, protein production, and immuno-labeling studies by noticeable expression of characteristic markers such as fibronectin and cytokeratin I (Figure 8e,f). Detailed mass spectrometric based proteomics analysis (MALDI-TOF and Orbitrap) revealed the expression of several proteins involved in cell proliferation, cellular motility and ECM remodeling having a similar protein expression pattern as of native human skin.

We have also utilized 3D bioprinting technology to develop in vitro skin scar model, where 3D bioprinting helped to

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**Figure 8.** Protein expression of Cartilage (expression of collagen type II, and negligible expression of collagen type X), bone (expression of osteopontin and osteonectin) and skin (cytokeratin I and fibronectin) specific markers in 3D bioprinted silk-gelatin based cartilage, bone, and skin constructs respectively, as observed by immunofluorescence analysis.\(^{5,7}\)
attain a parallel arrangement of cells and the synthesized ECM in an aligned manner similar to in vivo skin scar.[105] Further, SF-G bioink provided opportunity for non-covalent crosslinking of the inflammatory cytokines thus attaining in vivo like multimeric complex formation where ECM-cytokine-cell and cell surface receptor interact to induce biological functions of the cells for example toward scar specific differentiation.[106] The developed model replicated several key features of hypertrophic skin scar for example: (1) differentiation of fibroblasts to myofibroblasts with promising expression of α-smooth muscle actin stress fibers; (2) enormous production of ECM proteins like collagen and fibronectin; (3) contraction associated with scarring; (4) excessive synthesis and deposition of fibrotic scar specific proteins like SPARC and Tenascin; and (5) reduction in matrix metalloprotease secretion. Therefore, future studies warrant a detailed investigation into role of SF-G in understanding of the structure-function relationship of the developmental skin model that would lead to better mimicry of native tissues with extended applications in therapeutics.

7. Conclusion

Tissue engineering combines material science with the concepts of embryonic tissue development to reproduce cellular events (morphology, alignment, differentiation, and morphogenesis) in a controlled 3D microenvironment with a time-dependent delivery of biological molecules. Traditional tissue engineering approaches developed randomly porous scaffolds and deposited cells on top, which would result in non-homogenous cell distribution, lack of cellular infiltration, inhibited fluid flow inside the scaffold, exogenous administration of morphogens at a constant rate. Resulting engineered constructs were far from being considered as anatomically relevant tissues. Therefore this void for fabricating tailor-made, tissue graft substitutes with anatomy and functional dynamics identical to native tissues paved the way for 3D bioprinting.

3D bioprinting, a robust dispensing-based technique, has a promising potential for generating anatomically relevant sized constructs for tissue grafting in clinic in the order of several centimeters (Figure 9). The technique has made it possible to

![Figure 9](image-url)
mimic the complicated tissue hierarchy and heterogeneity by using multi-hydrogel based materials rendering differential properties such as stiffness, topography, roughness, composition with multiple cell populations especially required in the case of complex tissues such as osteochondral tissue. Of the available hydrogel bioinks, optimized blends of B. mori silk-gelatin bioink has immense potential for 3D bioprinting of functional tissue equivalents possessing high resolution, controlled feature sizes, structural fidelity, cytocompatibility, and ability to support lineage commitment of encapsulated cells in aqueous medium. Apart from the commonly used B. mori silkworm silk, molecular level changes could be induced in recombinant spider silk proteins to incorporate functional features by fusing relevant peptides/proteins to the existing amino acid sequences. Such recombinant hybrids of spider silk possess novel functionalities and also avoid post-translational modifications post-printing.

By combining 3D bioprinting of silk with advanced robotics and DNA programmed assembly, we could further streamline cellular signaling mechanisms by encapsulating programmed/functionalized cells into the bioink in order to replicate tissue heterogeneity and multi-cellular organizations.\[101\]

Though far from clinical application, the developed 3D in vitro model systems using silk-based hybrids may find application in pharmaceutical companies for testing the efficacy of drug molecules etc., hence lowering the burden on animal testing. Silk-based structures incorporated with other polymeric/inorganic materials and anisotropic fillers to improve mechanical strength of the printed constructs are being developed for specific target applications.\[69\] Also, the field of bioprinting may benefit from the inclusion of supramolecular hydrogel materials with biocompatible cross-linking mechanisms that are reversible in nature\[107\] and materials for 4D printing of biomimetic constructs with stimuli responsiveness.\[108\] Apart from the scientific challenges, there are still many regulatory issues that need to be dealt with in time before such 3D bioprinting technology can hit commercialization level.

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Conflict of Interest
The authors declare no conflict of interest.

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