3D printing of silk and applications

J.Lavanya[1], Dr.S.Umamageshwari[2] and Dr.N.Gokarneshan[2]

[1] Department of Fashion Design, SRM Institute of Science and Technology, Kattankalathur, Chennai, Tamil Nadu.

[2] Department of Textile Chemistry, SSM College of Engineering, Komapapalayam, Tamil Nadu.

Abstract

Silk is a natural polymer sourced mainly from spiders and silkworms. Due to its biocompatibility, biodegradability, and mechanical properties, it has been heavily investigated for biomedical applications. It can be processed into a number of formats, such as scaffolds, films, and nanoparticles. Common methods of production create constructs with limited complexity. 3D printing allows silk to be printed into more intricate designs, increasing its potential applications. Extrusion and inkjet printing are the primary ways silk has been 3D printed, though other methods are beginning to be investigated. Silk has been integrated into bioink with other polymers, both natural and synthetic. The addition of silk is primarily done to offer more desirable viscosity characteristics and mechanical properties for printing. Silk-based bioinks have been used to fabricate medical devices and tissues. This article discusses recent research and printing parameters important for 3D printing with silk.

Key words: 3D printing; bioink; bioprinting; printing parameters; silk; tissue engineering.

Introduction

Since its introduction in the 1980s, three-dimensional (3D) printing (also referred to as additive manufacturing, rapid prototyping, or solid free-form fabrication) has revolutionized the manufacturing field, with applications ranging from houses to human organs (1). With over 114,000 people on the organ transplant list, and only 35,000 organs transplanted last year (2), viable methods of printing human organs are essential. Due to this critical need, significant progress has been made on improving methods (printers) and materials (bioinks) to create biomimetic materials for tissue engineering. 3D printing has the ability to properly produce structured scaffolds to control cell behavior and promote tissue development (3). For a material to be considered printable, it must have both rheological properties that allow for printing and solidification properties that result in a mechanically stable structure (4). The final properties of the print determine if the construct will successfully function in certain applications (5). For the material to be used in biological or biomedical applications, it must not cause an immune response and have desired biodegradation characteristics (4). One candidate that is well suited for such biomaterial applications is silk. Silk is a natural polymer that not only has great biodegradability, mechanical properties, and elicits minor immune responses (6,7), but also has favorable shear thinning and viscosity characteristics that make it an ideal candidate for 3D printing (8). Silk has been successfully used as a scaffolding material for tissue engineering applications for years. However, the common methods of fabricating silk scaffolds (phase separation, solvent casting, particulate leaching, molding, foaming, etc.) do not permit customizable, high-precision geometries (9). 3D printing methodologies create more reproducible, complex designs, recapitulating microarchitectures conducive to tissue engineering (10,11). With the added advantage of producing devices and treatments specific to an individual patient's geometry, 3D printing techniques combined with silk-based biomaterials will likely result in a significant improvement to tissue repairs and regeneration (11,12).

Silk structure and characteristics relevant to 3D printing

Silk naturally occurs in several animal species, including spiders, of the class Arachnida, and moths, of the order Lepidoptera in the worm or larvae stage (13). The structures of many natural silks are similar, consisting of a core protein, arranged in fibrils, with an additional protein coating that acts as a glue, holding the fibrils together (14). The core protein is similar across silks, being composed of both heavy and light chain sections in the sub-level structures of the protein (15). Although the general structure is similar, the core protein's amino acid sequences and cohesive material properties vary between silks produced by spiders and those by silkworms. For example, spiders can produce eight types of silk with differing material properties, each originating from unique glands, whereas silkworms can only produce one type of silk (13,14). The material properties of silk are specific to the species of arthropod that produced it. For example, silk from silkworms differs significantly between mulberry (i.e. Bombyx mori) and non-mulberry (i.e. Antheraea mylitta) species in that non-mulberry silk has lower solubility, lower protein yields, and contains specific binding proteins (7). External conditions, such as proper nutrition and environment, can affect the mechanical and structural properties as well (7,14). Thus, it is important to take the silk source into consideration when using it as a biomaterial. Currently, the most investigated silk source is the Bombyx mori (B. mori) silkworm, a source that has been thoroughly domesticated and industrialized through centuries of use in the textile industry (16). It has been engineered into many formats for biomedical applications, including scaffolds, films, microparticles, and hydrogels (13,17,18). Spider silk has not been investigated as thoroughly, mostly due to its high cost, low yield, and the predatory nature of spiders (13). However, through the recent development of recombinant DNA technology, spider silk has emerged as a feasible biomaterial (13,19). The difference between silk from silkworms, specifically B. mori, and spiders, specifically N. clavpies, begins on a molecular level. The core structural proteins, fibroin and spidroin, from silkworms and spiders, respectively, have different structures (13,14,17). Fibroin is a copolymer that consists of two protein chains, one light (26 kDa) and one heavy (390 kDa), connected by a disulfide bond; a glycoprotein (P25) non-covalently links to the copolymer (13–15,17). The heavy chain consists of repeated sequences of glycine, alanine, and serine, which are responsible for the hydrophobic, anti-parallel, βsheet domains (17). Silk fibroin primarily exists in two configurations, silk I and silk II. Silk I is dominated by the α-helix secondary structure, making it amorphous and water-soluble, while silk II is the crystalline, β-sheet dominated state (14). Spidroin is composed of major ampullate spidroin proteins 1 and 2, with the primary structures of the proteins being dominated by repeated sequences composed of glycine, alanine, proline, arginine, and glutamic acid (13,14,19). Sections of spidroin that are rich in alanine can form sections with tight β-sheets, similar to fibroin (14). The relative abundance of the β-sheets in silkworm and spider silk is directly correlated with the crystallinity, toughness, and strength of the silk threads (17,19).

For silk to be used in 3D printing applications, the raw threads need to be converted into an aqueous form. As an aqueous bioink, human biocompatibility must be considered. For silk sourced from silkworm cocoons, specifically Bombyx mori silk, this is addressed by the removal of sericin, the glue-like protein coating, and the isolation of the fibroin protein core, since sericin, in combination with fibroin, has been proven to trigger an immune response (13). The sericin coating of silkworm fibers is removed by submerging the raw cocoons in a boiling alkali solution, most commonly a sodium carbonate solution (13,14,17,23). Next, the solid, β-sheeted, silk threads can be dissolved in ionic solutions (24,25), such as lithium bromide (LiBr), disrupting the hydrophobic interactions and hydrogen bonds creating an aqueous solution that can be purified for printing (19,26).

Aqueous regenerated silk exhibits shear thinning and Newtonian viscoelastic properties (15,27). However, native silk feedstock shows non-Newtonian viscoelastic properties (15). Shear thinning is a decrease in viscosity and anisotropy as the strain rate increases (28). Shear thinning is advantageous because it lowers the pressure requirements during the printing process and increases the resolution of the print (8). However, such characteristics of silk fibroin in aqueous form isolated directly from the silk glands of B. mori silkworms were found to vary among silkworms (15). Additionally, the viscosity of reengineered aqueous silk fibroin solution was found to be variable on the basis of the type of solvent used to dissolve the silk fibers (29). The Vollrath research group determined that shear thinning occurred when the shear rates increased above the critical shear rate. The critical shear rate varies from sample to sample because it depends on the molecular weight and concentration of the polymer solutions. Above the critical shear rate, the influence of intermolecular interactions increases, decreasing the degree of shear thinning (30). It was determined that shear rates higher than 2 s^{-1} caused phase separation, inducing β-sheet formation through the exclusion of water from the fibers (30). The elimination of water allows the silk fibers to form hydrogen bonds with other silk fibers creating the β-sheet structure (30). Temperature and pH play key roles in the rheological properties of silk. Silk solutions experience sol-gel transitions at increased temperatures. A paper published by the Asakura research group found that B. mori silk experienced this sol-gel transition at 38°C (31). Similarly, altering the pH of the silk solution induces gelation. Lowering the pH causes gelation that can be reversed by raising the pH if the exposure to the acid is relatively short (30).

Types of 3D printing

There are many methods of 3D printing, including stereolithography (SLA), digital light processing (DLP), selective laser sintering (SLS), fused deposition modeling (FDM), inkjet printing, and direct extrusion. 3D printing allows for complex, custom geometries to be constructed that are either difficult or impossible to generate with traditional manufacturing techniques. 3D printing is already being used for medical applications such as prosthetics, orthopedic products, and dental implants (32,33). Since these products do not contain biological components, such as cells, enzymes, or growth factors, they can be produced using any method of additive manufacturing (33). Due to the required processing conditions for SLA, SLS, and FDM printing, such as high temperature requirements or the use of harmful lasers, inkjet and direct extrusion printing are most commonly used when printing for biological or biomedical applications (8).

Inkjet 3D printing

Inkjet printing is a common method of printing biological components (34) and is one form of “bioprinting” (35) in the tissue engineering field. This method involves depositing droplets of ink (with or without cells) onto a substrate. The ink is heated or compressed by a piezoelectric or ultrasound waves to increase pressure and cause deposition of a droplet (34) (Figure 1). The droplet size can be controlled by altering printing conditions, such as the temperature or the viscosity of the ink (35,36). Inkjet printing is ideal for printing biological materials due to its ability to print versatile constructs precisely, as well as its biocompatible printing conditions (35,37,38). This is possible because inkjet printing prints layer-by-layer, allowing for greater control over the final design (39). It also has the ability to create patterned structures (40). However, inkjet bioprinting requires printing with liquid ink, limiting the potential printable materials. The ink must also be able to dry and form 3D structures with the proper organization, which is not always feasible with liquid inks (34). Inkjet 3D printing was first used to print silk fibroin in 2006 by the Kaplan research group. They were able to print a 0.6%

w/w silk solution into lines on clear vinyl plastic. Bone marrow stem cells were seeded onto the silk and vinyl plastic, but cell growth and differentiation were specific to the silk lines (41).

Extrusion 3D printing

Extrusion 3D printing is a commonly used method of printing non-biological and biological materials. It is capable of printing materials with a large range of viscosities. Extrusion printing has commonly been used to print a blend of two materials at the same time, one with a higher viscosity, to offer functional support, and the other with a low viscosity, to provide a conducive environment for cell growth and proliferation (34). Materials with shear thinning properties are easily printed with extrusion printing (34). Similar to inkjet printing, extrusion 3D printing requires specific solidification characteristics; materials must solidify quickly and controllably to create mechanically stable prints (4). Depending on the application, microextrusion printing can be controlled pneumatically, with a piston, or through a screw configuration (Figure 1). The Lewis research group was the first to successfully use extrusion printing methods to print with silk fibroin. Silk fibroin bioink was prepared to a concentration of 28–30% and printed into a square lattice and circular web. Researchers were able to seed human mesenchymal stem cells (hMSCs) onto the prints and saw an increase in glucosaminoglycan accumulation, indicating differentiation into chondrocytes (42).

Printing parameters Proper printing technique and ink need to be chosen while considering parameters such as printing speed, pressure, voltage, frequency, resolution, viscosity, and cell density/viability (Table 2). Specific to silk applications, an important consideration for inkjet printing is the nozzle diameter (43). Researchers in the Edirisinghe group investigated the effect the nozzle design played in printing silk using an electrohydrodynamic printer. Electrohydrodynamic printers are similar to conventional inkjet printers, with the addition of an electric field that is applied to increase the resolution of the print from the micrometer to the nanometer scale. It was determined that not only were the width of the lines printed greatly affected, but the level of stress the materials were put under was affected by the nozzle geometry (44). See reference for more specific information on nozzle geometry. Printing techniques need to be chosen to obtain the desired construct. During the printing process, silk will naturally form β-sheets due to the exposure to shear stresses. Studies have been done to enhance the crystallinity of silk fibroin during inkjet printing even further (39). For example, layers of silk can be printed followed by methanol to induce hydrogen bonding between silk fibers creating the β-sheet structure (39,45). Silk droplets that are not treated with methanol remain water soluble and quickly degrade (39). By controlling the ratio of silk fibroin and methanol, the degree of crystallinity (and thus the mechanical properties and degradation rate of the material) can be controlled (39).

Bioink

Bioink is the structural material used for 3D printing that provides support and the proper environment for cells. Bioinks can be composed of biomaterials, biological components, cells, or any combination of the three (3). For bioinks to be printable, the rheology, swelling, surface tension, and gelation kinetic characteristics must be considered (19). To function biologically, the ink must have a high enough cell density, be degradable, and offer extracellular matrix (ECM) like support (19,57). Furthermore, bioinks must be biocompatible, have a high printing resolution, and hold their shape after printing (8). The most important considerations for bioink printability are the ink's viscosity, density, and surface tension (43).

Bioink parameters

Choosing or developing ink with desired characteristics both during and after printing is important. One of the major challenges with developing bioinks is dealing with the temperature range the bioink will be exposed to. In most 3D printing applications, the temperature is elevated during the printing process, which is detrimental to biological components in the ink. One strategy to overcome this issue is simultaneously printing the polymer and the biological components at different temperatures (12). Incorporating other natural polymers into the bioink can help to lower the temperature requirements during printing, while providing support comparable to the extracellular matrix (ECM) for the cells. Natural polymers such as chitosan, gelatin, and alginate have been shown to have sol-gel transformations at physiological temperatures (58). However, with these materials alone, additional biologically-harmful processing is sometimes required to induce crosslinking to increase the mechanical integrity of the prints (12).

Another major challenge with developing bioinks is making a solution that has a viscosity that protects the cells during printing, while also being printable. These viscosity requirements often differ; higher viscosities are needed to protect cells from the stresses imposed by the printing process while lower viscosities are better for printing (8). Silk has been added to bioinks to increase the viscosity, providing better cellular support (8,59,60). Silk's shear thinning characteristics make it ideal for bioprinting (61–63), which allow it to be printed and then returned to a more organized structure. Printing with silk bioinks is difficult, as the silk will naturally form β-sheets prematurely due to the shear forces the solutions experience, causing the nozzle to clog (8,64). Both inkjet and extrusion printers can be used to print with bioink containing cells. Achieving cell density concentrations conducive to tissue engineering is difficult with inkjet printing as cells must be protected from the shear stresses that are present during the printing process (34). Increasing the cell density concentrations can also lead to issues with nozzle clogging (34,65). Compared to inkjet printing, extrusion printing has a much higher cell density limit (34). However, cell viability is lower than with inkjet printing, due to higher shear stress conditions during extrusion printing (66). Another challenge that must be addressed is the ink's interaction with cells. Ideally, the biomaterials that make up the bioink would contain cell binding domains, a characteristic that some silks, such as B. mori, lack. This reduces the silk's effectiveness as a bioink. However, silk can be modified with RGD binding domains to increase cell adherence (67–69). A study comparing recombinant spider silk proteins with and without the RGD binding domain found that the cell-material interaction could be controlled. The cell adhesion to the spider silk with RGD binding domains was significantly higher than the spider silk missing the binding motif (8,60).

Natural polymer composite bioinks

Many natural polymers have been used in 3D printing applications including alginate (70,71), gelatin (70), and collagen (72,73). However, the mechanical properties of these polymers alone are weak, limiting the structural integrity and applicability of printed substrates (74). To address this challenge, structurally sound silk composites can be printed (Table 3) (4,75). However, failure to choose a suitable ratio of the composite material leads to difficulty in printing, as well as poor performance of the final construct. In particular, Rodriguez and colleagues assessed the printability of various percentages of gelatin in a gelatin/silk bioink through qualitative assessments. They found that limiting gelatin in the composites prevented retention of a solid structure upon deposition, whereas high levels of gelatin resulted in a clogged nozzle (4). The Huang research group overcame this issue by incorporating "fast-gelling" alginate as the sacrificial material in the bioink to maintain the 3D construct's structural integrity (76). The bioink was printed using inkjet printing techniques and had two steps of gelation. The ink was printed into a calcium chloride bath that caused the sodium alginate to gel on contact. The silk was then gelled through enzymatic crosslinking, encapsulating the sodium alginate. The alginate can act as a sacrificial material and be degraded quickly from the construct, leaving just the silk fibroin (76). Similarly, the Reis group used horseradish peroxidase (HRP) to cross-link silk fibroin, creating a fast setting bioink that could be used to make shape-memory implants (11). Researchers also ensured the stability of the 3D-printed scaffolds through the use of rapid freezing technology (75,77). Placing the structure in low temperatures, such as −20°C, has two benefits: effective molding of the top layer and preservation of the bottom layer, which would have otherwise melted (75,78,79). The Kaplan research group combatted temperature requirement issues by combining silk fibroin and a variety of polyols to investigate their compatibility with inkjet printing. They were able to produce complex, mechanically stable, and insoluble 3D structures that cured at room temperature (12). Similarly, silk has been added to bioink formulations to create insoluble, flexible, and strong prints (80). Researchers in the Ghosh group investigated how silk fibroin gelatin-based bioinks, cross-linked by the tyrosinase enzyme, affected cell growth and gene expression. Specifically, they investigated how different methods of dispersing cells throughout the ink affected chondrogenic differentiation for cartilage regeneration (81). The same research group investigated how these silk fibroin-gelatin bioinks supported multi-lineage differentiation of encapsulated stem cells for specific tissue formation (59). Specific to bone repair, extrusion printing methods have been used in multiple studies to produce high strength scaffolds. Researchers in the Kaplan group produced scaffolds using extrusion printing composed of silk and hydroxyapatite to co-culture human bone marrow derived mesenchymal stem cells (hMSCs) and human mammary microvascular endothelial cells (hMMECs) for bone formation (82). Due to the differing mechanical properties of silk and hydroxyapatite, the scaffolds were capable of supporting stem cell and endothelial cell growth, which are both essential for bone repair (82). The Kim research group used extrusion printing to print a composite of collagen, decellularized extracellular matrix, and 3% silk fibroin to create scaffolds for hard tissue regeneration. They saw an increase in cellular activities and physical strength compared to scaffolds containing no silk fibroin (83). Researchers in the Park research group investigating the production of irregular-shaped tissues for tissue regeneration used 3D extrusion printing to print a composition of silk fibroin and a cartilage acellular matrix (CAM)-based bioink to increase mechanical stability. The silk fibroin allowed the viscosity of the bioink to be better controlled and the cross-linking behavior of the silk increased the stability of the prints. These CAMsilk scaffolds performed better in tissue regeneration than scaffolds made of polycaprolactone, a synthetic polymer with ideal mechanical properties (84).

Synthetic polymer composite bioinks Synthetic polymers are also used in 3D printing applications; examples of these materials include polyethylene glycol (PEG) (85,86), polyacrilamide (PAAm) (87), and polylactoglycolides (PLGA) (88). Unlike their natural counterparts, synthetic polymers are easily tunable, allowing for controlled manipulation of properties to improve structure and reduce immune response (89). Despite these advantages, the applicability of synthetic polymers is greatly limited by the necessity of toxic organic solvents during processing and a lack of cellular attachment sites, as these constructs do not mimic the environment of the extracellular matrix (87,90). Moreover, as these constructs are implanted in the human body, synthetic-based composites may yield degradation products that provoke an immune response and cause inflammation (89). Silk and synthetic polymer combinations are combined to create constructs to improve the construct's mechanical strength and integrity as a result of cross-linking, which allows for applications with long-term cultures (4,72,76,90). With regard to printability, researchers in the Kaplan group faced a similar challenge to those who used natural polymers – determining the optimal silkto-synthetic-polymer ratio, such that the construct was not too soft and did not clog the nozzle (90).

Emerging 3D printing methods

Although inkjet and extrusion 3D printing are the most studied methods for printing with silk, other methods are beginning to be studied. A recent publication indicates that digital light processing (DLP)

3D bioprinting could be used to produce scaffolds for tissue and organ engineering applications (65). They used silk fibroin that was methacrylated by glycidyl methacrylate to formulate the bioink. This step allowed the bioink to be polymerized through light exposure (65). Previously, silk fibroin bioinks were not compatible with DLP printing because they could not be polymerized by light. The cytocompatibility of the bioink was investigated and it was determined that the materials were biocompatible. The cells were printed without damage and could be stacked with different cell type layers (65). The Hyun research printed gelatin methacrylate/silk fibroin bioink containing fibroblasts using DPL 3D printing. Silk fibroin particles were added to the gelatin methacrylate to increase the viscosity, preventing the fibroblasts from settling to the bottom of the print plate. When compared to gelatin methacrylate bioink, gelatin methacrylate/silk fibroin bioink produced prints with increased cell dispersion and viability (91). Unlike SLA or masked SLA (MSLA), other printing methods that cause photo-initiated crosslinking, DLP has a shorter production time which is advantageous when printing with bioink containing cells or other biological components (92–94).

Indirect additive manufacturing has been used to produce silk fibroin scaffolds for cartilage tissue engineering. This method of production used an inkjet printed thermoplastic mold. Aqueous silk was placed in the mold, frozen, and ethanol annealed. Although this process was more complex, scaffolds were produced without having to combat the issues associated with 3D printing silk, such as nozzle clogging (10). Another article similarly used a sacrificial mold made through inkjet printing to cast silk fibroin scaffolds. Researchers in the Ng group were able to produce macro- and micro-architectural features on silk fibroin scaffolds that provided suitable environments for cell migration, attachment, proliferation, and nutrient transportation. These scaffolds were highly reproducible (57). 3D freeform printing is a one-step gelation process in which silk fibroin can be suspended in synthetic nanoclay and polyethylene glycol (95). This method of 3D printing is similar to methods of printing into thixotropic gel medias used for other materials, such as PDMS or polysaccharide hydrogels (96–99). 3D freeform printing eliminates processing after printing and does not require crosslinking agents. The silk fibroin is first allowed to gel, then removed from the nanoclay/ PEG bath, and finally sterilized. By printing into the sacrificial medium, silk can be processed into complex structures, previously unattainable (95).

Beyond engineering organs

While tissue engineering has been the focus of much of the current literature on 3D printing using silk (4,10,34,40,59,83–86), exciting research printing silk for other applications can inspire future printing technologies. Silk inks can be customized for specific uses by the addition of components such as nanoparticles, enzymes, antibiotics, growth factors, or antibodies (43). For example, researchers in the Ebbens group were able to use

reactive inkjet printing technology consisting of a mixture of silk fibroin and the enzyme catalase to produce silk fibroin micro-rockets that moved by bubblepropulsion (45). Layers of catalytically active and inactive parts facilitated motion in specific directions (45). Inkjet printing has also been used to print silk doped with additives for bacterial contamination detection (43) and hosting applications (37). Researchers in the Omenetto group printed a polydiacetylene-fibroin-IgG antibody ink onto laboratory gloves that changed from blue to red after bacterial contamination (due to bond stretching) (43). The Tsukruk research group printed silk modified with amino acid polymer side chains, creating environments ideal for cells. They were able to immobilize E. coli cells while providing them an environment conducive to their survival by printing alternate layers of silk-polylysine and silkpolyglutamic acid with cells between them (37).

Reactive inkjet printing (RIJ) has been used to print silk fibroin successfully. Researchers in the Miller research group used this technique to fabricate a dental barrier membrane composed of fibroin. Unlike commercially available membranes, membranes made of fibroin degrade slowly, even in the acidic mouth environment, but also do not require a secondary surgery for removal (100). Microextrusion printing was used by the Huesing group to print aerogels composed of silk fibroin and silica. Silk fibroin was added to silica to increase the mechanical stability and strength. This silica-fibroin aerogel has low density, high surface area, and flexibility in compression and proved to exhibit fire retardancy (101). As a final example, 3D printed silk has been used to produce biophotonic components (102). Silk was printed using a direct write printing technique (similar to extrusion printing), which allowed the production of optical wave guides. Previous production processes required high temperature, harsh chemicals, salts, UV light, or high pressure. Producing optical wave guides with silk eliminated these harmful methods and produced high-quality biocompatible optical materials.

Conclusion

While silk has been used for centuries in the textile industry, its use in 3D printing applications is just at the beginning stages. Silk has desirable characteristics for a biomaterial; it elicits minor inflammatory responses, is biodegradable, and has controllable mechanical properties. Additionally, aqueous silk is viscoelastic and experiences shear thinning, making it ideal for 3D printing applications. Currently, inkjet and extrusion printing are the most studied methods of 3D printing with silk. Though other methods, such as DLP (65) or freeform printing (95), appear to be viable options. Much of the research that has looked into the feasibility of using silk in 3D printing is done using ink that is a combination of silk and another polymer. The addition of silk to bioinks provides tunability of mechanical properties. It is often added to increase the printability and the stability of the prints. Currently, the addition of natural polymers to silk bioink is more investigated, with the majority of these bioinks being used for biomedical applications. However, synthetic polymers offer more reproducible results, which would be advantageous for tissue engineering applications (103). These 3D printed constructs have been used in a variety of applications from medical devices, such as dental barriers (100) and biophotonic components (102), to more industrial applications, such as fire retardant gels (101) and ink that can detect bacterial contamination (43). Tissue engineering with the goal of complete organ regeneration has been a prominent focus in a number of silk 3D printing applications. Scaffolds have been printed to regenerate cartilage (10,77,84,85,104) and bone (72,82,83), and research into silk-based bioinks containing cells continues to be studied.

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