Trends in Biotechnology

CellPress

Review

In Situ 3D Printing: Opportunities with Silk Inks

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***In situ* 3D printing is an emerging technique designed for patient-specific needs and performed directly in the patient's tissues in the operating room. While this technology has progressed rapidly, several improvements are needed to push it forward for widespread utility, including ink formulations and optimization for *in situ* context. Silk fibroin inks emerge as a viable option due to the diverse range of formulations, aqueous processability, robust and tunable mechanical properties, and self-assembly via biophysical adsorption to avoid exogenous chemical or photochemical sensitizer additives, among other features. In this review, we focus on this new frontier of 3D *in situ* printing for tissue regeneration, where silk is proposed as candidate biomaterial ink due to the unique and useful properties of this protein polymer.**

Highlights

In vitro 3D printing techniques have challenges that limit their clinical translation, including multistep processes, mismatches with patient-specific defects, risk of contamination, and post-processing manipulation requirements.

In situ 3D printing, the next frontier for 3D printing, aims to fabricate new tissues and organs *in vivo*, in the surgical setting, directly in the patient.

Inks remain a challenge for this transition to *in situ* 3D printing, requiring fast gelation, high shape fidelity, minimal if any postprocessing, robust mechanical properties tunable to the target tissue, and biocompatibility.

Versatile and appropriate inks, such as those developed from silk fibroin, offer a foundation for this translation, based on their unique amphiphilic structure, versatility in physical crosslinking, mechanical properties, biocompatibility, and tunable degradation.

3D Printing: Present and Future

Organ transplants save lives worldwide, but the shortage of organs and rejection issues have established the need for alternatives. Tissue engineering and regenerative medicine, through the combination of material science and engineering, biology, chemistry, and physics, aim to generate substitute human tissues and organs for *in vivo* and *in vitro* applications [1]. Among the different approaches, **3D printing** (see Glossary) has emerged as a promising strategy to recreate tissues and organs to address current shortages [2]. Different techniques have been developed toward this goal, to mimic complex tissue and organ architectures to recreate functional and structural cues [3]. However, remaining limitations include challenges with conformal prints to match tissue and organ interfaces, *in vitro* fabrication for *in vivo* translation, long processing times, and **postprocessing manipulation**, such as chemical and photochemical **crosslinking** and associated additives that may not be **biocompatible** *in vivo* [4]. Most 3D printed structures are hydrogels, with mechanical weakness for handling [5]. The challenge is to overcome these limitations and translate this important technology into the surgical operating room. *In situ* (or *in vivo*) 3D printing is the next level of canonical 3D printing that could be used not only to overcome the shortage of tissues and organs for transplant, but also to improve patient-specific needs for new tissues and organs designed in real time, and be performed directly in a surgical setting. Introduced in 2007, this approach as a technological development derives from earlier 3D printing and tissue-engineering approaches [6].

Despite the limitations mentioned earlier, some *in vivo* trials have been reported for the *in situ* printing of bone [7], cartilage [8–10], and skin [11]. Among the challenges, fundamental features of inks are key, including **rheology**, biocompatibility, and gelation kinetics to support the right shape and mechanical properties of the construct after the printing [12].

Natural polymers are good candidates in 3D printing applications and, among them, silk **fibroin** protein ink formulations have emerged, because they already have a unique set of features to meet the needs for *in vitro* 3D printing [13]. Indeed, the amino acid sequence and the protein structure (as a high-molecular-weight amphiphilic polymer) make silk adaptable and tunable to meet the biological and mechanical properties required, and crosslinking can be achieved in

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many ways, also avoiding the need for exogenous chemicals (e.g., chemical and photosensitizers) [14,15]. The silk fibroin chemical composition supports biophysical crosslinking, often in aqueous/physiological environments, making it suitable for efficient crosslinking [16].

In situ 3D printing technologies have been described as a new frontier for highly personalized medicine [12,17–20]. Several steps towards the development of this new technology have already been achieved, but many improvements are still required, such as ink formulations and compositions as a central focus [21]. Silk fibroin, as a natural protein with versatility in terms of material format and remarkable mechanical properties, has been exploited in regenerative medicine applications and in *in vitro* 3D printing applications [22–31]. Thus, translating these features *in vivo* represents a logical pursuit to meet the needs of this new frontier.

Looking to the future, the next step in *in situ* 3D printing is the further development of suitable ink formulations; here silk fibroin is proposed as a candidate for this new emerging frontier due to the properties of this fascinating biopolymer (Figure 1, Key Figure).

3D Printing Techniques and Applications

Overview of 3D Printing Techniques

3D printing techniques represent a promising platform to recreate customized, functional substitutes for damaged tissues and organs that are adaptable to conventional manufacturing

Key Figure

In Situ 3D Printing with Silk

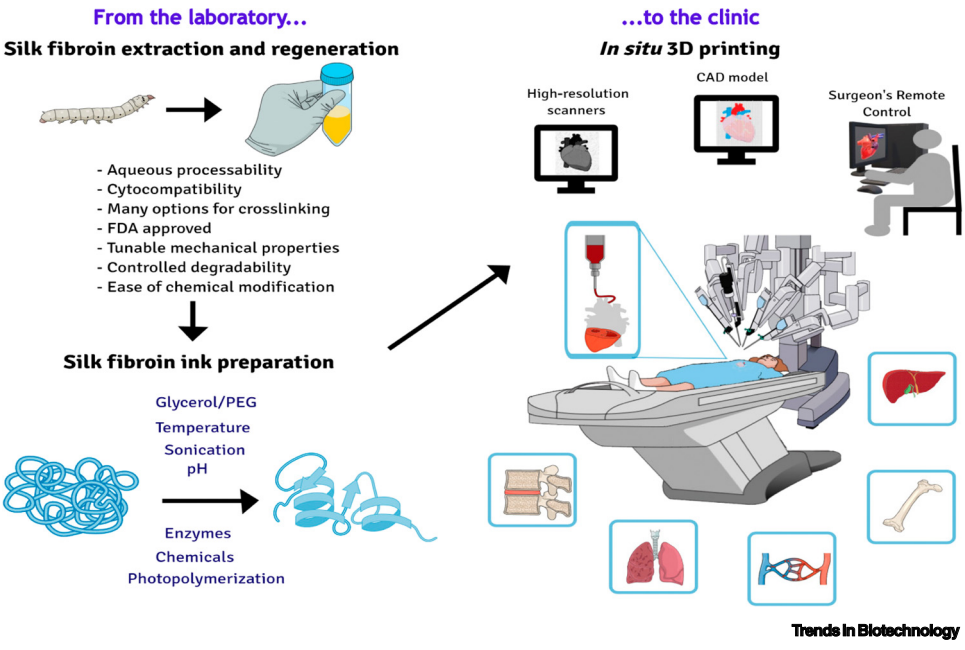

Figure 1. Silk fibroin extracted from *Bombyx mori* exhibits several advantages for *in situ* 3D applications, including many modes of gelation to fabricate inks. In a surgical setting, based on patient-specific data, the ink is directly printed *in vivo* to reproduce 3D reconstructions of the defect site and to monitor the printing process, controlled remotely by the surgeon. Abbreviations: CAD, computer-aided design; PEG, polyethylene glycol.

Glossary

β-sheets: protein secondary structure, characterized by hydrogen bond formation between protein chains. Silk fibroin β-sheet formation leads to crystallization and a thermodynamically stable, insoluble structure.
3D printing: the 3D deposition of biomaterials. Bioprinting is the fabrication of structures comprising combinations of biomaterials, cells, or biomolecules, included in the ink composition, called a bioink.
Arg-Gly-Asp (RGD) sequence: arginine (R), glycine (G), and aspartic acid (D) motifs recognized by cell integrins for adhesion.
Biocompatibility: one of the most important requirements in the design of an implantable structure; the ability of the material to induce a specific host response for the specific application designed for, without cytotoxic effects.
Bioreactor: a device used in *in vitro* experiments that provides media, mass transfer, and sometimes mechanical and/or electrical stimuli to reproduce the physiological dynamic environment in which cells grow and differentiate.
Carbodiimide reaction: chemistry leading to an amide bond formation between carboxylic acid and the primary amine of amino acids, carried out in aqueous and solid phases.
Coumarin: photosensitive crosslinker that, when excited with UV-visible or NIR wavelengths, undergoes cycloaddition reactions.
Crosslinking: specific bond formation to induce biopolymer gelation, thus changing its structural properties. Crosslinking can be created between specific amino acid groups through chemical modification, mediated by enzymes or chemical additives, forming covalent, stable bonds. Physical crosslinking is induced by physical factors such as temperature, solvent removal, pH changes, and sonication, and is less controllable compared with chemical and enzymatic crosslinking, due to the weak nature of physical bond.
Fibroin: structural, fibrous protein extracted from silkworm cocoons produced by insects, providing mechanical support as an insoluble protein matrix. Ease of extraction and processing, along with biocompatibility, and robust mechanical properties, made fibroin a versatile and fascinating natural polymer applied to tissue engineering and regenerative medicine.

techniques [3]. The approach is based on the fabrication of new tissues *in vitro*, later tested *in vivo* and sometimes preclinically [32], with rapid turnaround time, anatomical accuracy, and customized features [33]. In particular, medical imaging data from patients, such as X-ray and computerized tomography (CT), can be readily converted into 3D digital files to enable the printing of complex geometries [34]. A range of 3D printing techniques has been developed; inkjet printing, extrusion-based printing, laser-assisted, and light-based printing, including digital light projection (DLP), and stereolithography (SLA) [35]. Among these, extrusion and light-based techniques are the most widely used, due to the advantages of constructing cell-laden structures by blending cells with the printing ink. Extrusion-based 3D printing uses external forces from compressed air, pistons, or screw rods to extrude ink through a movable nozzle to deposit patterns [3]. A range of biomaterials, including solutions, suspensions, and hydrogels, can be printed by this approach. The printing path of the dispensing nozzle is usually encoded in a list of coordinates along with other parameters, which is generated by open-source software, such as Slic3er. The printing performance of extrusion-based 3D printing is based largely on the viscoelastic properties of the ink, which can be characterized by rheological testing [36]. The **print resolution** is generally in 100s of μm, determined by multiple factors, including ink viscosity, nozzle gauge, and printing speed [3,37].

Light-based printing techniques use projected images or scanning lasers to pattern photocurable inks [38]. A range of photocuring reactions is used, including free radical acrylate, thiol-ene, photo-oxidation, and nitrogen radicals [39,40]. Light-based 3D printers are faster than extrusion-based systems, and provide improved printing resolution below 100 μm [3,41]. The UV light involved in photocuring reactions is usually harmful to encapsulated cells; thus, the use of visible light-based photocuring reactions is increasingly popular [42].

In Vitro 3D Printing Limitations

Tissue and organ regeneration requires mimicry of complex geometries and interfaces to avoid gaps and mismatches, to emulate hierarchical structures, and to generate gradients. However, *in vitro* 3D printing techniques present intrinsic limitations when applied to optimized clinical success. First, the fabrication of the implant *in vitro* may not fit expected and unexpected defects *in vivo,* which may result in longer surgical time, more device handling, and increased risk of contamination. This mismatch is due to the often used flat surface for a base for printing, and the low resolution of imaging acquisition systems, such as X-rays, magnetic resonance imaging (MRI), and CT [4,17]. Second, *in vitro* printed structures often present weak initial mechanical properties due to the fluid-rich nature of the hydrogels used, and this can lead to swelling, shape deformation, and contraction, all of which can affect the overall success of the repair being pursued, along with mismatches to cell and tissue mechanics [5]. Third, biological characterization is performed *in vitro*, usually using **bioreactors**, which cannot fully emulate the complexities of the *in vivo* physiological environment, thus leading to unpredictable outcomes [20].

Clinical Direction: *In Situ* 3D Printing

The *in vitro* 3D printing drawbacks described earlier can be overcome by printing in real time directly in the patient in a surgical setting with high anatomical precision, supported by high-resolution 3D scanners of the defect sizes. This *in situ* 3D printing is based on minimally invasive routes and better control of patient anatomy, where the tissue serves as the substrate for print application and the body serves as the physiological bioreactor or the perfect (natural) environment [21].

There are two main approaches for *in situ* 3D printing. The first is the handheld approach based on portable devices able to print directly. The surgeon can print directly in the defect site and the

Laponite: synthetic nanoclay, a sodium magnesium silicate, used as a rheological modifier in 3D printing, to increase ink viscosity, since it can form viscoelastic gels in the presence of water.
Pluronic F127: commercial name of poloxamer 407, a copolymer comprising two hydrophilic PEG blocks with one hydrophobic PEG block as a non-ionic, hydrophilic surfactant.
Postprocessing manipulation: after printing, further processes may be required, such as washes to remove unreacted reagents and incubations at a fixed temperature to stabilize the structure. In the case of silk fibroin, PEG, alcohol, or freeze-drying are performed to further stabilize the printed object, inducing protein β-sheets (crystals).
Print resolution: the smallest unit of the printed material measured mainly on the x and y axes, less on z axis.
Rheology: study of viscoelasticity properties of soft materials after deformation, allowing the study of the viscosity properties of polymers, providing data helpful in the preparation of inks that need to exhibit viscosity ranges according to the 3D printing technique selected.
Shear stress: in 3D printing, the force applied to the ink during extrusion from the nozzle. Inks should exhibit shear-thinning behavior, the capacity to recover and retain their shape just after printing, minimizing the effect of shear stress.
Silk degumming: silk consists of two main types of protein, fibroin, and sericin. Fibroin is extracted through a process called degumming, which eliminates the sericin in a time-dependent water boiling/extraction step with sodium carbonate. Different extraction times lead to different silk fibroin molecular-weight ranges; a longer degumming time results in lower molecular weight.
Sol–gel transition: inter and intrachains interactions among silk fibroin chains in solution, inducing the formation of β-sheet structures, leading to physical crosslinking and gel formation. β-sheet rearrangements are affected by fibroin concentration, salts, pH, and temperature.
Stiffness: ability of an object (or tissue) to resist deformation applied by force, measured by Young's modulus.
Storage modulus (G′): the elastic behavior of a material, while the loss modulus (G″) represents the viscous

small dimensions of the device allow movement inside and around a wound, as well as ease of sterilization and relatively low cost [43]. The second option is a robotic approach, based on movable systems along a three-axis, surgeon-controlled console. The architecture of the implant to be printed is designed via computer-aided design (CAD) [8,44]. As in the handheld approach, multiple inks can be printed with the same unit by using different ink cartridges. The anatomical location of the defect and the complex structure of some defect sites can be better addressed with the robotic technology, while the combination of the two methods can be useful for mimicking complex architectures [45].

behavior. In rheology, G′ equal to G″ represents the gelation point of the material.

Although many advances in the field are still needed, different trials have been performed, starting from simple systems, and these efforts are already described in recent reviews [21,45].

However, one of the remaining challenges at the core of further development of *in situ* 3D printing is the optimization and crosslinking of the inks used in the process. As mentioned, rapid gelation, shape fidelity, and robust mechanics are fundamental requirements and must be achieved without postprocessing manipulation, the introduction of exogenous chemicals that may not be biocompatible, and damage to other tissues, as well as in a rapid timeframe. Additionally, considering the minimally invasive routes, UV and visible light present depth-of-penetration challenges to curing 3D printed structures or must be conducted on a layer-by-layer continuous exposure to support *in situ* crosslinking; this is different for easily accessible tissues, such as skin [18]. Recently, attempts have been made to overcome these drawbacks applied to *in situ* crosslinking processes, such as the use of extrusion-based printing. Specifically, an extrusion-based portable device, the BioPen, was designed by adding a 405-nm light source close to the nozzle to irradiate the ink just after extrusion and before deposition in the target tissue [46]. Additionally, a coaxial nozzle system allowed rapid crosslinking of the shell, protecting the liquid core, which might contain cells or soft or liquid materials for longer crosslinking times. The ink was based on gelatin methacryloyl (GelMA) mixed with hyaluronic acid and gelatin as additives. The authors studied the parameters to optimize crosslinking efficiency, shape retention, and homogenous reactions. Optimal irradiance was determined at 160 mW/cm^2 and rheological studies revealed that precrosslinking based on physical processes could reduce the exposure time required for the complete gelation after printing, with 1 s of light exposure to induce ink gelation [46]. To overcome the low penetration of UV and visible light, near-infrared (NIR) light (850 nm) was applied for the *in situ* crosslinking of photosensitive polymers. Ink based on branched-polyethylene glycol (PEG) and gelatin backbones was modified with hydrophobic, photosensitive motifs, and crosslinked via biorthogonal two-photon cycloaddition [19]. **Coumarin** derivatives were used as crosslinkers, with NIR two-photon excitation to undergo cycloaddition reactions. The inks were injected into mice via minimally invasive routes, and were tested in brain, skeletal muscle, and dermal tissues. Crosslinking was achieved by irradiating the injected hydrogel *ex vivo*, leading to micrometer-resolution 3D hydrogels. The printing process was supported by 3D image acquisition in real time during the printing and, in all the tissues, no damage to the vasculature or the surrounding tissues was detected, confirming the compatibility of the technology [19]. A bioink formulation for *in vivo* application used methylcellulose and **Laponite** as rheological modifiers to increase GelMa printability. Printing was carried out at 37°C, mimicking physiological conditions, and crosslinking was performed with visible light (455 nm) using Eosin Y as a photoinitiator, exposing the bioink both during and after extrusion. Printing was performed on chicken breast tissue and 2% agarose slices, used as soft tissue model substrates. The printed constructs exhibited mechanical properties in the range of soft tissues, and the rheological modifiers decreased the swelling ratio. The mechanical properties were maintained over 21 days of incubation and NIH-3T3 fibroblast viability was 71–77% after printing, with further improvements needed to enhance cell proliferation [42].

Although all of these studies represent important breakthroughs in the development of *in situ* crosslinking, most of the ink formulations are based on GelMa as the main component, largely applied in *in vitro* 3D printing approaches [47]. The next step is the investigation of other ink compositions, expanding available sources to better mimic the complexity of matrices in the human body. Among the options, natural polymers offer tailorable properties and biocompatibility [48]. Thus, to propel *in situ* technology forward, the development and optimization of inks and crosslinking procedures are major challenges [49].

Silk as Ink

The ink is the building block of 3D printing applications, both *in vitro* and *in situ*, and, thus, must be selected according to specific requirements (Figure 2) [50]. Among the parameters, rheology, nozzle diameter, ink composition and concentration, maintenance of shape fidelity post printing, suitable mechanical properties, and support for cells are some of the variables to be considered [41]. Generally, the ink has to exhibit adequate viscoelastic properties to resist **shear stress** during printing, while elastic recoil serves to assume the shape after printing [40].

During *in situ* 3D printing, rapid and efficient crosslinking is a fundamental requirement to assume the designed shape in the patient's body just after printing, with high fidelity and without postprocessing requirements. Additionally, the **stiffness** range of native tissues is between 3kPa to several GPa; thus, there is the demand for highly tunable inks and hydrogels with respect to mechanical properties [5]. Among natural and synthetic biomaterials (Table 1), silk fibroin derived from *Bombyx mori* silkworm (also called mulberry silk), and compared with other silk sources (Box 1), is a suitable candidate. Silk fibroin is extracted from silkworm cocoons and separated from glue-like proteins called sericins; as a natural fibrous protein, it gained interest in regenerative medicine for its remarkable mechanical and biological properties. The versatility of silk fibroin and ease of processing allow fabrication into various material forms, such as hydrogels, sponges, fibers, and powders [51,52]. This novel protein has garnered interest for *in vitro* 3D printing due to the properties that match the list of requirements detailed earlier, prescribing silk as a versatile material either alone or as a composite biomaterial system [27–31,48,53–61]

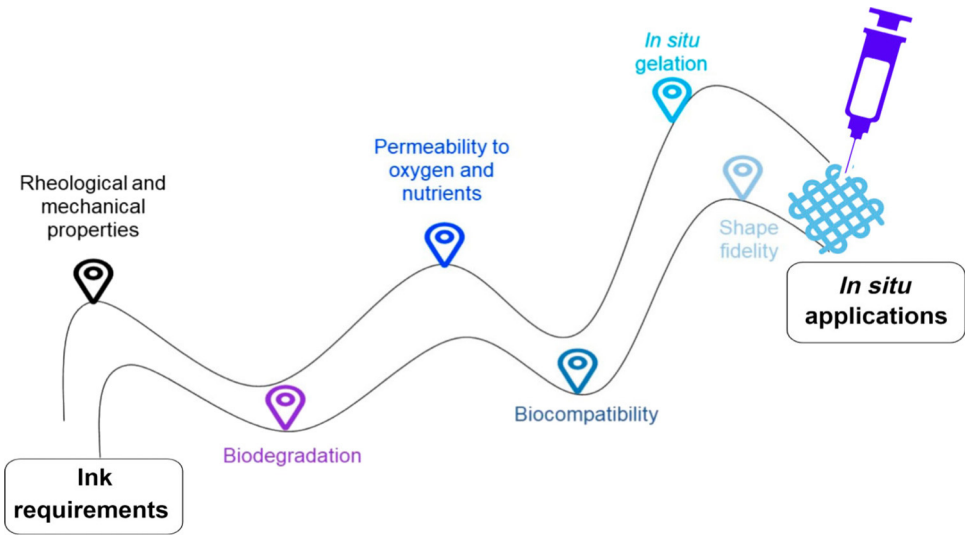

Figure 2. General Requirements for Inks for 3D Printing. Ink formulations require suitable rheological (shear thinning, storage modulus, and viscosity) and mechanical properties. In addition, biodegradability, biocompatibility, and permeability to oxygen and nutrients and, for *in situ* 3D printing, *in situ* rapid gelation and shape integrity after printing are also requirements.

Table 1. Comparison of Natural and Synthetic Biomaterials for 3D Printing

	Crosslinking process	Advantages	Disadvantages	Refs
Silk fibroin (SF)	• Enzymatic • Photocrosslinking • Physical (sonication, solvent removal, heat, pH)	• FDA approved[a] • Low cost • Abundant • Aqueous processability • Controllable degradability • Self-assembly • Several gelation processes • Cell-friendly behavior • Ease of modification • In vivo biocompatibility • Tunable mechanical properties	• Low viscosity if printed individually (high concentrations required) • Lack of RGD sequences	[5,16,66]
Alginate	• Ionotropic gelation (Ca ions) • Photocrosslinking	• Highly hydrophilic • Aqueous processability • Cytocompatible	• Low cell adhesion • Weak mechanical properties • Rapid dissolution	[33,37,50]
Hyaluronic acid (HA)	• Enzymatic • Photocrosslinking • Non-covalent crosslinking	• Important extracellular matrix (ECM) component • Highly hydrophilic • Different inflammatory *in vivo* response according to molecular weight • Ease of modification • Cytocompatible	• Poor cell adhesion • Weak mechanical properties • Rapid degradation *in vivo*	[33,92,93]
Gelatin	• Enzymatic • Photocrosslinking • Chemical reactions • Non-covalent (temperature, pH)	• Good cell adhesion • Tunable mechanical properties with chemical modification • Ease of processability	• High concentration required (from 10 mg/ml) • Poor mechanical properties • Rapid degradation	[58,66,94]
Polyethylene glycol (PEG)	• Photocrosslinking • Chemical reactions	• Synthetic material • Cytocompatible • Highly hydrophilic • FDA approved[b]	• Non-biodegradable • Poor cell adhesion	[50,66,95]
Pluronic F127	• Physical (thermo-reversible) • Photocrosslinking	• Synthetic material • Used as sacrificial material • Water soluble	• Non-biodegradable • Poor cell adhesion • Cytotoxic in long term culture time	[36,96]
Polycaprolactone (PCL)	• Physical (thermo-reversible) • Photocrosslinking	• Synthetic material • Biocompatible • Hydrophobic • Inexpensive • Good mechanical strength • FDA approved	• Very slow degradation • Low water absorption capacity • Requires thermal or solvent deposition	[96,97]

[a]Medical devices: surgical sutures.
[b]Pharmaceutical field.

Box 1. Silk Sources

Silk is produced by species of silkworms, spiders, and mites from the phylum Arthropoda. The most characterized silk sources belonging to this category are [89]:

- Silk fibroin derived from *Bombyx mori* silkworms, also called mulberry silk. This is the most abundant and most studied in the biomedical field. It is produced in huge quantities for the textile industry.
- Silk fibroin derived from non-mulberry silk, characterized by polyalanine repeats in the crystalline structure. Among the different types, silk from the silk moth *Antheraea assama* from India presents RGD epitopes that are absent in mulberry silk.
- Dragline spidroin silk derived from the spiders *Nephila clavipes* and *Araneus diadematus*, among other sources, is limited to genetically engineered options.

Genetically engineered copolymers, such as silk-elastin (SELP), comprising GAGAS from silk fibroin repeats for the stiff domain, and GXGVP as the elastin sequence (for elasticity), where X can be modified to tune the properties of the protein for stimuli-responsive properties, may be possible in the future [90].

The advantages of silk fibroin result from its amino acid composition and unique secondary structure [62], with a heavy and light chain covalently bound by a disulfide bond. The heavy chain comprises a highly repetitive hexapeptide sequence (GAGAGX where X is serine, valine, or glycine; Figure 3A), which folds into hydrophobic, crystalline **β-sheet** domains, representing the component involved in mechanical properties (Figure 3B) [63]. Silk fibroin can self-assemble into these crystalline domains, generating stable hydrogen bonded inter- and intrachain associations between C=O and NH groups, resulting in insoluble, thermally stable, and mechanically durable hydrogels [60,64]. **Storage modulus (G')** and viscosity are fundamental parameters that

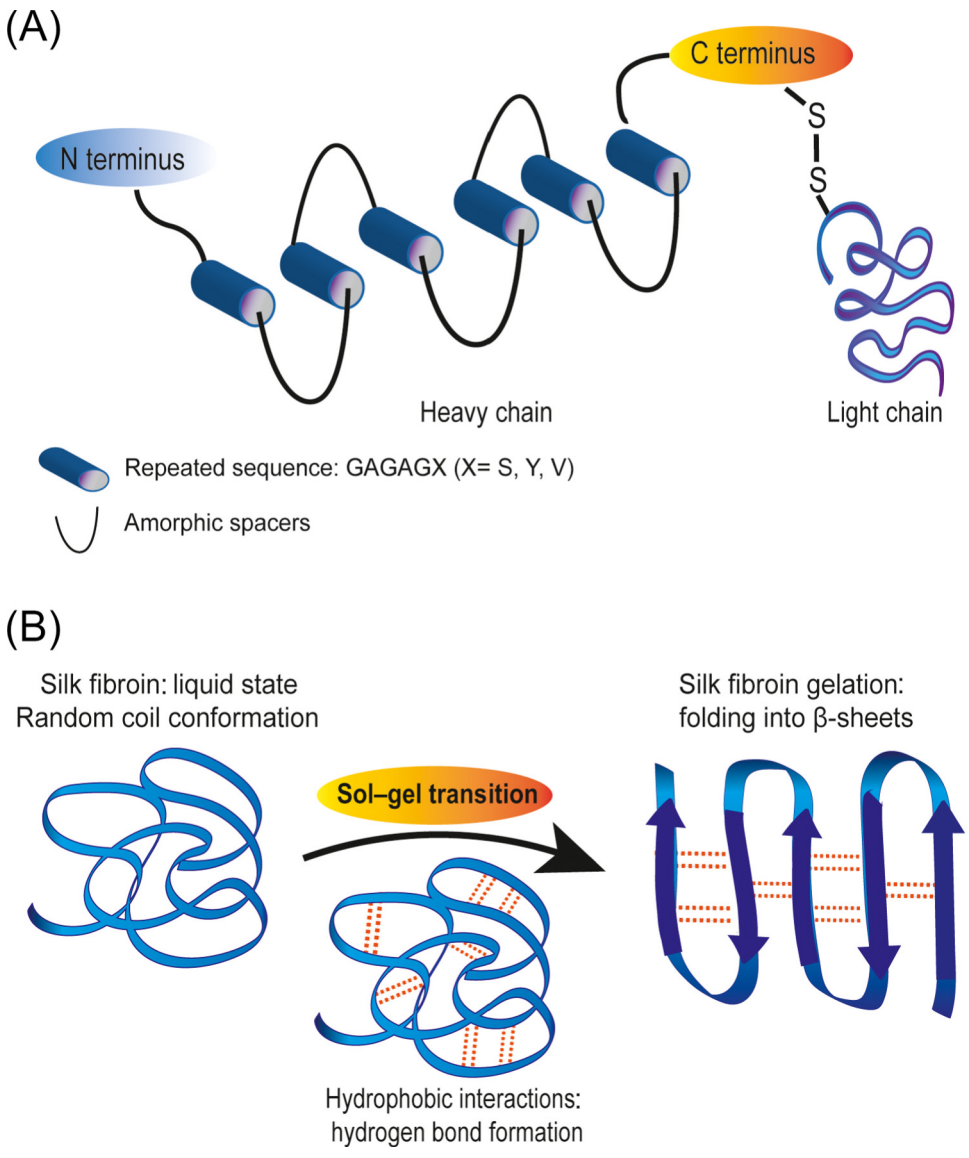

Figure 3. Silk Fibroin Composition and Sol–Gel Transition. Silk fibroin comprises heavy and light chains, covalently bound by a disulfide bond. The heavy chain is composed of hexapeptide repeated sequences of GAGAGX, where X can be valine, serine, or glycine, interspersed into amorphic spacers (A). During the sol–gel transition phase, from a random coil conformation, silk fibroin structure folds into β-sheets with antiparallel domains, through hydrogen bond formation both inter- and intrachain, forming insoluble structures that are thermodynamically stable (B).

determine the **sol–gel transition** and the ability of the material to recover after printing, respectively. Crosslinking and density influence these parameters, leading to the option to tune hydrogel mechanical properties [65]. The gelation of the ink can be achieved by many crosslinking processes, both covalent and physical (Table 2). The latter is characterized by the formation of weak inter- and intrachain interactions and can be achieved via water removal, heating, sonication, pH, and salts, all resulting in polymer self-assembly without chemical reagents or side products [5,66–68]. By contrast, covalent crosslinking can be carried out by enzymatic or chemical reactions, resulting in stronger bonds [39,69].

Enzyme-mediated crosslinking reactions are carried out at mild temperatures, neutral pH, and in aqueous solutions using transglutaminase or horseradish peroxidase (HRP). Transglutaminase forms covalent bonds between free amines and γ-carboxamide groups, or glutamine [5]. HRP-mediated crosslinking requires H_2O_2 and leads to the formation of dityrosine covalent bonds, (tyrosine represents 5% of the total amino acids in heavy-chain silk fibroin) [70]. This crosslinking approach has been utilized for the fabrication of shape-memory implants via 3D extrusion printing, designed based on the patient's specific anatomical data. The implants were fabricated for meniscus regeneration and showed a storage modulus similar to native cartilage, achieved by using postprocessing manipulation via freeze-drying to increase the β-sheet content [71].

Photocurable crosslinking has also been pursued, since the process may provide better control than the enzymatic approach. This gelation process relies on the modification of reactive groups in silk, such as carboxyl and amine groups, usually with acrylate or methacrylate, which in the presence of a photoinitiator and light can polymerize and form covalent bonds to induce gelation [72]. Crosslinking is mainly achieved under near-UV light exposure at 365 nm, with the main advantage of rapid kinetics (seconds) [73,74]. Silk fibroin has been methacrylated with glycidyl methacrylate (GMA) and the ink was applied to digital light processing (DLP) for cartilage regeneration, specifically for the trachea. The printed scaffold supported high cell viability both

Table 2. Silk Fibroin Gelation Processes

Crosslinking process	Examples	Advantages	Disadvantages	Refs
Physical	• Temperature • pH change • Solvent removal • Sonication • Glycerol/PEG • Salts • Electric stimuli	No chemicals Inexpensive	Physical bonding Slow kinetics Poor controllable Stiff gels (β-sheets) Opaque gels	[5,66,67]
Covalent	Enzymatic: • Horseradish peroxidase • Transglutaminase	Mild conditions (temperature, pH) Covalent bonds Transparent gels Elastic gels	Cost Selective Variable kinetics Stiffening with time	[5,70]
	Photopolymerization: • Riboflavin • Acrylate/methacrylate addition • Ruthenium	Controllable Rapid gelation Tunable crosslink density Covalent bonds Clear gels	Postprocessing extraction UV light Toxicity of photoinitiators	[39,66,73,76]
	Chemicals: • Glutaraldehyde • Genipin • Carbodiimide reaction	Genipin, a natural crosslinker Covalent bonds	Toxicity of glutaraldehyde Slow gelation kinetics with genipin	[5,66,77]

in vitro and *in vivo*, with mechanical properties matched to the cartilage tissue [74]. However, *in vivo*, the main challenge is the potential cytotoxicity of chemicals, especially of photoinitiators. Nevertheless, methacrylation is an efficient and useful reaction for silk fibroin, as well as for gelatin (GelMA), collagen, and hyaluronic acid, with different photoinitiators under investigation to avoid cytotoxicity while retaining reaction speed [66] (Box 2). In the photocurable reactions, visible light-driven crosslinking is an alternative to avoid UV-mediated cell damage and utilizes riboflavin (vitamin B2; thus, safe for use in the body) as the photoinitiator to form dityrosine crosslinks [75]. This approach has been utilized for corneal tissue regeneration using photolithography, resulting in a highly elastic and transparent hydrogel, with properties comparable with the silk hydrogels obtained via HRP-mediated crosslinking; crosslinking was achieved within 20 min at 450 nm [76].

Another tool to modulate silk fibroin hydrogels is molecular weight. HRP-mediated crosslinking with different silk molecular weights (derived by different **silk degumming** times) influences the crosslinking density and mechanical properties [15]. Concentration can also be used for a similar outcome; however, viscosity has to be modulated according to the printing technique. The versatility of silk fibroin applied to hydrogel formation was further demonstrated in a recent study performed on HRP-mediated crosslinking. Indeed, gelation kinetics were slower compared with photocrosslinking and mechanical properties may be insufficient for some applications. These features were significantly improved by the addition of phenol groups, conjugating tyramine along silk fibroin sequence via a **carbodiimide reaction** coupled with carboxylic acid residues both on aspartic and glutamic acid, enhancing the gelation kinetics and the stiffness of the hydrogels [77].

Crosslinking time, density, and process are fundamental factors in the selection of inks and in the resultant mechanical properties, while, concurrently, the biomaterial has to exhibit adequate biological properties and degradation to match the tissue regeneration goals. Silk fibroin lacks **Arg-Gly-Asp (RGD) sequence** cell adhesion epitopes, which may be useful in some instances to avoid cell-specific outcomes, although facile functionalization of the protein with this adhesion peptide sequence has been demonstrated by using the HRP reaction: peptide sequences carrying tyrosine groups can be crosslinked to silk fibroin via the enzymatic reaction, while not affecting the gelation of the protein polymer [78].

The *in vivo* degradation of materials printed *in situ* is a key factor to consider in ink formulations. The degradation rate should be balanced with the regenerative processes *in vivo*, to match biological responses and biophysical properties of the printed construct [79]. Silk fibroin degradation kinetics depends on the structure, where the more ordered structures (α-helix and β-sheets) are more resistant to degradation compared with random coils [80]. Although the *in vivo* degradation mechanisms are still under investigation, immune responses are driven by proteases, macrophages, and giant cells [81]. Silk fibroin degradation both *in vitro* and *in vivo* can require days to years depending on the physical and structural properties, including molecular weight,

Box 2. Photoinitiators

Photoinitiators are key to photopolymerization processes with light exposure. The main challenge with photoinitiators is their cytotoxicity. The most common options include [5,66,73,91]:

- Irgacure 2959 [2-hydroxy-4'(2-hydroxyethoxy)-2-methylpopiophenone] (275 nm–UV light): limited water solubility, most commonly used.
- Eosin Y (514 nm–visible light): overlaps with some fluorophores used for cells, works in the presence of co-initiators.
- Riboflavin (330–570nm–UV and visible light): slow gelation kinetics, relative weak gels.
- Lithium phenyl (2,4,6-trimethyl- benzoyl) phosphinate (LAP) (365 nm and 405 nm–UV and visible light): water soluble, works best with UV exposure (365 nm). UV exposure is harmful to cells or neighboring tissues; visible light could be a better option but the efficiency of crosslinking is lower and requires more time.

secondary structure, and concentration. Higher crystalline content and higher molecular weight lead to slower degradation rates. Density, porosity, and surface features also influence the accessibility of enzymes or immune cells to the silk-based material, impacting initial degradation [82]. Consequently, the design of silk fibroin hydrogels with specific structural conformations and content of these features, leads to a tunable degradation rate, a useful feature that can be controlled *in vitro* and *in vivo* for the regeneration of tissues [82].

Concluding Remarks and Future Perspectives

In situ 3D printing is the new frontier of regenerative and personalized medicine. Although early in the technology development, trials were reported toward developing adequate printing technologies and inks, as well as crosslinking processes, compatible with *in vivo* applications. This progress opens possibilities to overcome limitations of current *in vitro* 3D printing approaches, supporting tissue and organ regeneration, while also considering the printing of deformable sensors able to conform to native tissues during printing and during deformation of tissue due to normal activity, moving closer to the clinic and patient-specific needs [4].

Many improvements are still needed to obtain *in situ* systems able to support the mechanical, cellular, vascular, and innervation needs of tissues, while also providing a technology that is user-friendly for surgeons, maintains sterilization, and offers wide acceptability [35,45]. Among these challenges the ink is key, and its design and formulation must be able to recapitulate the complex structure and functions of native tissues and organs (see Outstanding Questions) [3].

Silk fibroin exhibits extraordinary properties and an ability to be printed in complex structures with tunable degradation rates, a range of mechanical properties, biological functionalization as needed, and is free of chemical or photochemical additives [83]. These features are dependent on silk fibroin concentration, molecular weight, crosslink density, and preparation method. Indeed, the variety of silk fibroin gelation mechanisms permits multistep crosslinking reactions to tune the final hydrogel properties, such as required in a surgical setting for *in situ* printing.

However, to make *in situ* 3D printing a reliable technique, many improvements still need to be achieved regarding ink formulation and characterization. For example, the standardization of silk fibroin extraction protocols, the study of the mechanisms underlying *in vivo* degradation, and the setting of sterilization protocols compatible with the clinical environment, are important parameters to be investigated. The ease of modification of the silk fibroin sequence can have an important role in the binding of specific biomolecules, such as growth factors, cytokines, or drugs, which might improve the *in vivo* performance of printed structures, addressing specific biological, and regenerative responses. Additionally, silk viscosity can limit applications in extrusion-based 3D printing techniques, such as shape fidelity after printing [13]. Silk fibroin provides extraordinary functions that can be further enhanced by combinations with other synthetic or natural biopolymers to emulate the complexity of extracellular matrices in the human body. For instance, hyaluronic acid or gelatin can improve the biological performance of hydrogels [24,84], PEG [85], and glycerol [48] as rheological modifiers, improve silk printability, or silk nanofibers can be combined with other biomaterials toward printability and mechanical outcomes [86]. Different formulations of silk fibroin-based inks should be investigated, characterized, and standardized, accessing shape fidelity while avoiding possible *in vivo* cytotoxic effects of photoinitiators when photopolymerization is applied, and scaling up fabrication and, consequently, uses in the clinic [3,87,88].

While tissues and organ complexity likely cannot be reproduced using only one biomaterial, the versatility and tunable features of silk fibroin can provide a foundation for inks for a range of 3D *in situ* printing needs.

Outstanding Questions

For *in situ* printing, how can adhesion to the target and surrounding tissues be achieved?

Ink performance and hydrogel formation depend on printer technology. How can this combination of features be best designed as a technology for versatile use in the surgical setting?

How will sterilization be maintained with the inks, equipment, and process in the surgical room?

How will silk ink printing with cells and factors impact cell functions?

Acknowledgments

We thank the National Institutes of Health (NIH) (P41EB027062; R01EB021264; and U01EB014976), the Air Force Office of Scientific Research (AFOSR) (FA9550-17-1-0333) and the Army Research Office (ARO) (W911NF-17-1-0384) for support for the studies that in part led to this review. This work is part of the REGENERA project within the program Departments of Excellence 2018-2022 (DII-UNITN) - Italian Ministry of University and Research (MIUR). Figures 1 and 2 were created with Mind The Graph Platform (www.mindthegraph.com).

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