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Review article

A comprehensive review of silk-fibroin hydrogels for cell and drug delivery applications in tissue engineering and regenerative medicine

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ABSTRACT

Hydrogel scaffolds hold great promise for developing novel treatment strategies in the field of regenerative medicine. Within this context, silk fibroin (SF) has proven to be a versatile material for a wide range of tissue engineering applications owing to its structural and functional properties. In the present review, we report on the design and fabrication of different forms of SF-based scaffolds for tissue regeneration applications, particularly for skin, bone, and neural tissues. In particular, SF hydrogels have emerged as delivery systems for a wide range of bio-actives. Given the growing interest in the field, this review has a primary focus on the fabrication, characterization, and properties of SF hydrogels. We also discuss their potential for the delivery of drugs, stem cells, genes, peptides, and growth factors, including future directions in the field of SF hydrogel scaffolds.

1. Introduction

Tissue engineering (TE) stands at the crossroads of medicine, biological science, engineering and technology, thus implying an inter- and multidisciplinary approach to the field. In the late 1980s, the entire attention of medical science was focused on the genesis of the very concept of TE at the National Science Foundation workshop held in Washington, DC. TE demands a multidisciplinary attention to overcome the challenges and complexity of generating functional tissue substitutes consisting of cells, scaffolding and biomaterials [1]. While TE has made substantial progress in the field, the functional regeneration of damaged tissue and organs [2] remains challenging. TE and regenerative medicine reproduce the same idea, but with a different approach in which the former builds new tissues, while the latter restores functions using combinations of factors, both with the aid of foreign biological materials [3,4]. A basic requirement for this is an engineered tissue construct combining scaffold, cells, and therapeutics or biomolecules to orchestrate and support the target tissue regeneration, either *in vitro* or in humans [5]. This template provides the necessary microenvironment, such as biological, chemical, or biophysical signals, to support cell proliferation and neo-tissue formation. TE constructs are cultured *in vitro* [1] for transplantation into the damaged site *in vivo* for repair and regeneration [6]. However, given that TE is deeply rooted in biomedicine, it could also be argued that it is still in its infancy as it is neither economical nor easy. To date, its clinical success is limited to small arteries, skin grafts and the trachea among others, while the liver, lungs and heart tissues are grown in laboratories. Although laboratory-grown tissues are far from clinical settings, they are of great help in studying basic mechanisms and drug screening, thereby significantly reducing animal experimentation.

Biomaterials play a key role in TE by providing structural support for the delivery of cells and therapeutic factors. The tissue environment is mimicked by these materials by engineering a suitable architecture for cell growth and proliferation [7]. Several classes of materials have been identified and developed for this purpose, such as polyesters, polyamides, polysaccharides, and their combinations. Among a multitude of structural systems, hydrogels are particularly interesting due to their versatility. They can be designed to support cell growth in a hydrated three-dimensional (3D) environment that permits oxygen and nutrient transport to replicate the microenvironment for regenerative tissue growth [8]. Their popularity is also credited to their ability to sustain high water content and retain a porous structure, thus allowing them to dispose of cellular waste and trade cell metabolites [9].

Hydrogels provide important structural and functional support in the

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field of tissue engineering and regenerative medicine by controlling the sustained release of the desired factors and by regulating cell behaviour. They can be used in different forms, such as injectable solutions, *in situ* gels, structured matrices, viscous gels and thin sheets. Hydrogels also facilitate an intricate cell-matrix interaction at the physiological length scale. Therefore, hydrogels with their hierarchical structures have gained much attention in the field. Furthermore, there is a growing interest in developing hydrogels for regulating the target cell behaviour in a spatiotemporal fashion.

However, conventional hydrogels fall short when it comes to mechanical strength, structural stability, and electrical and magnetic properties [10]. As a result, there has been a growing interest in multifunctional hydrogels to control cell functions. Different kinds of materials, both natural and synthetic, have been explored with and without crosslinking using metallic, inorganic, organic or polymeric materials [11]. Among natural polymers, protein polymers such as silk fibroin offer a bottom-up approach that helps to introduce novel features rationally.

Silk is still considered as an excellent material in many industries due to its versatile biological properties. The silkworm has been domesticated for centuries to obtain silk fibres for commercialization purposes. From the Chinese tales of the unexpected unwinding of a silk cocoon by the wife of the Yellow Emperor of China [12], silk has started rolling ever since through human evolution, ranging from curtains in the textile industry to sutures in medicine. Further, silk-based scaffolds have emerged for TE applications. Once identified, the particular properties of silk fibroin have offered the research world myriad opportunities to use silk in various industries (Figs. 1 and 2).

In the case of silk derived from the silkworm, the fibres consist of two primary proteins: fibroin which forms the fibre, and sericin which acts as a glue to bind the non-woven composite cocoon. A fibroin molecule consists of a heavy chain of 391 kDa and a light chain of 26 kDa. [13] that are connected by a single disulfide linkage, such as a covalent bond. The primary structure of fibroin has a crystalline domain of a few repetitive sequences of hydrophobic (GAGAGS and less conserved repeats of GAGAGX), aromatic residue, and polar amorphous regions (Figs. 3a, 3b). Taken together, the crystalline region forms an antiparallel β-sheet secondary structure interspersed by an amorphous region [14] (Fig. 3c). The amorphous region of silk is rich in glycine, less ordered with helical structures [15]. The stability of silk fibroin (SF) is due to the crystallinity of the material. Hence, altering the crystallinity would make silk a better and more approachable material for delivery systems. SF also possesses exceptional thermal stability, with a transition temperature to 175 °C. Above this temperature, the material is stable up to 250 °C due to stable β-sheet conformation [16]. Crystalline SF is insoluble in typical solvents. However, electrolyte solutions, such as lithium bromide or calcium thiocyanate, can disrupt hydrogen bonds and dissolve SF [17]. Aqueous silk of a higher concentration tends to aggregate given its inter- and intramolecular interactions. SF hydrogels have poor swelling properties, which can be pointed to as a major drawback in the hydrogel system. However, this issue can be tackled by blending with other polymers, including polyvinyl alcohol, gelatin, chitosan, and collagen.

SF is one of the strongest fibres and has a similar tensile strength compared to glass fibre or other synthetic organic fibres, allied with toughness, good resilience, and elasticity [17]. SF is more biocompatible than materials like polylactic acid and polyglycolic acid and is less immunogenic and pro-inflammatory than collagen. SF degradation is predominantly due to the response to proteolytic enzymes [17]. It is entirely unproblematic and products are non-toxic. The rate of degradation is directly associated with the β-sheet content, degree of crystallinity and molecular weight. Degradation of a lower β-sheet content SF is much more pronounced. Enzymes, such as protease XIV, αchymotrypsin, papain, and matrix metalloproteinases-1 and 11, specifically act on cleavage sites like Tyr, Phe, Trp, His, Lys, Arg Val, Ile, and Leu to break down the chains to small polypeptides and amino acids in due course [17]. Hence, with such an extensive understanding of the degradation process, control over the performance of SF is feasible.

SF is a versatile biomaterial due to its natural safety, biocompatibility, and biodegradation properties. Recent developments of SF for molecular engineering, multifunctionalities, injectable formulations, conductive, stimuli responsive and bio-inks have widened the scope in the field of regenerative medicine. In this review, we discuss the importance and application of SF as a biomaterial and shed light on the design of different forms of silk biomaterials for drug delivery and TE applications. Among the various forms, hydrogels stand out as the better candidate. Therefore, the focus of the review is on the production, characterization and property of SF-based hydrogels, including their recent use for the delivery of drugs, stem cells, genes, peptides and growth factors.

2. Methods

Literature search was conducted using PubMed, EMBASE and Medline databases according to the PRISMA guidelines for the articles

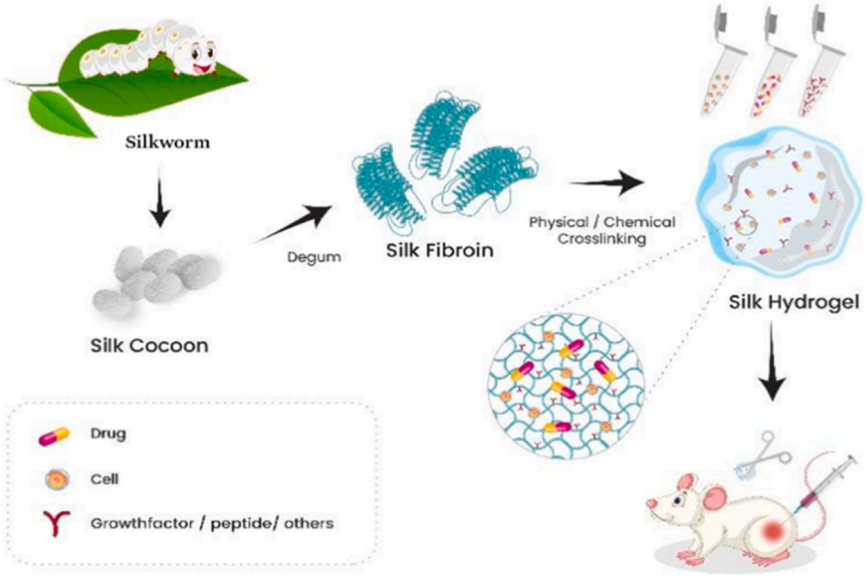


Fig. 1. Silk fibroin hydrogel, a versatile biomaterial for delivery application.

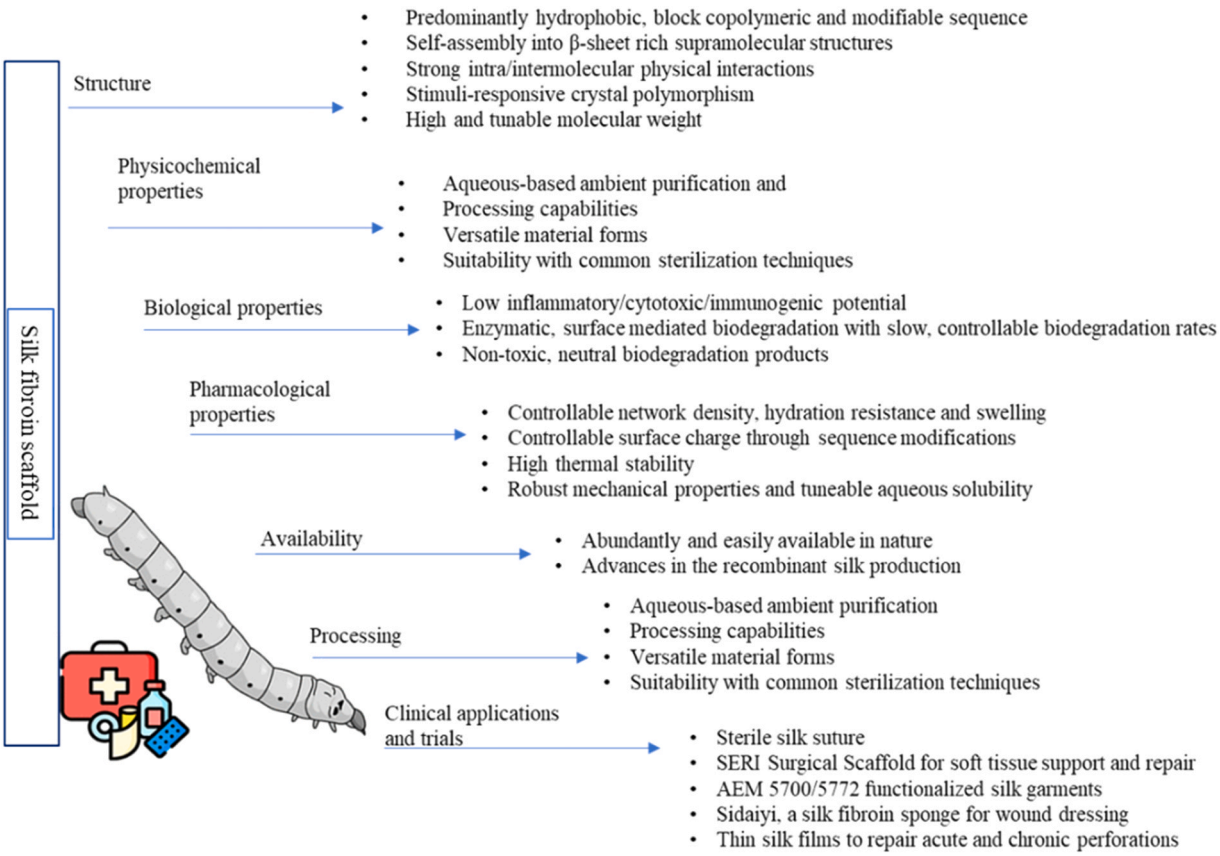


Fig. 2. Summary of the structure, properties and application of silk fibres.

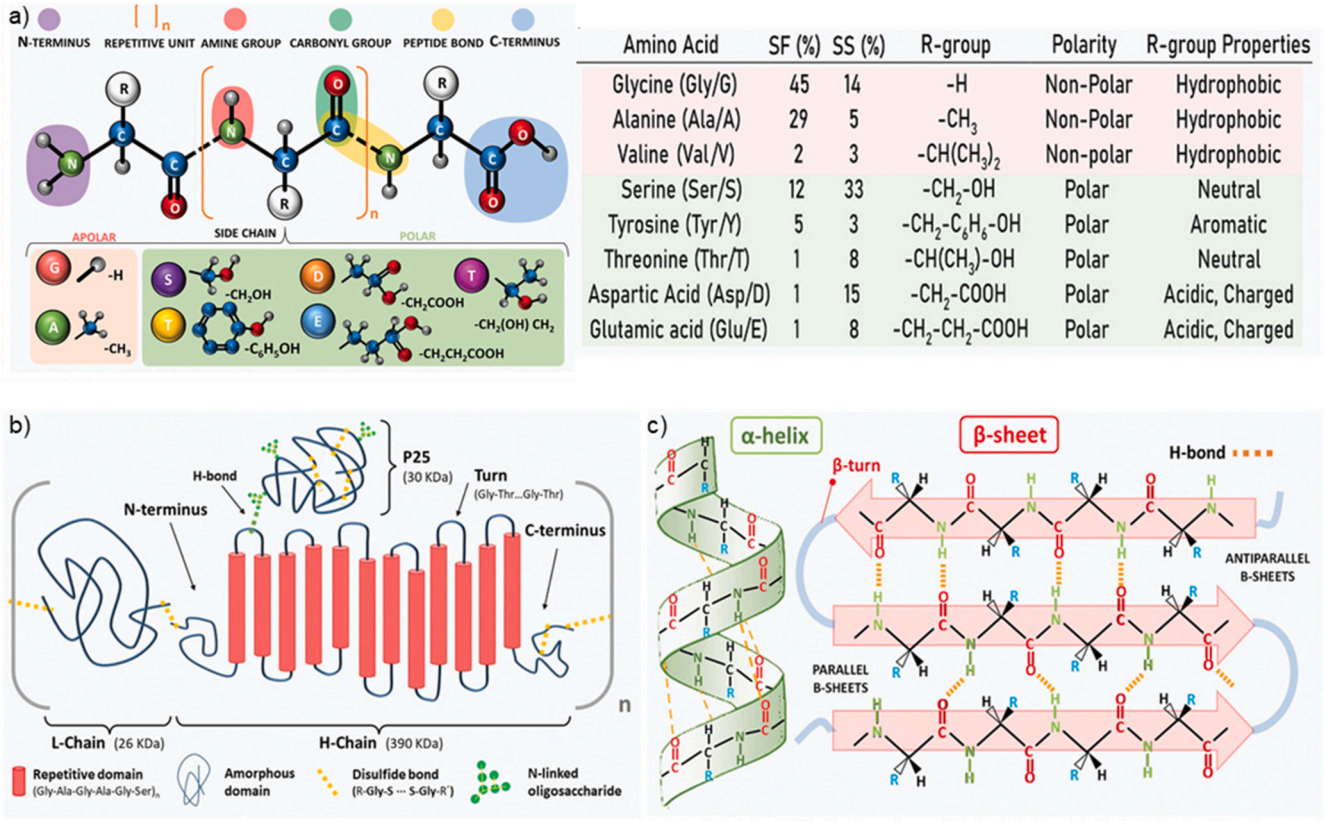
published in English until February 2023. The following search terms were used: “silk fibroin biomaterials”, “silk fibroin hydrogel”, “silk fibroin composite”, “tissue repair”, “tissue regeneration”, “drug delivery”, “cell delivery”, “growth factors delivery”, and “gene delivery”. Studies identified by the search outcomes were combined and duplicates were removed. Screening of the title and abstracts were performed before the extraction of full-text articles. Publications with inconclusive data and unrelated content were excluded. Selected articles were categorized as those primarily based on hydrogel fabrication and, subsequently, the resulting outcome was highlighted as described in the Methods. Fabrication methods were evaluated for required controls (verification) and structural characterization (validation). The search yielded 1021 studies and a total of 193 articles were selected. Critical review of the full text articles was conducted for those selected and further categorized by considering the scaffold fabrication, hydrogel properties and application of bioactive factors.

2.1. SF: a versatile material

2.1.1. Design of SF for tissue engineering applications

When designing a biomaterial, several elements should be considered: (i) the material should provide mechanical support parallel to the used tissue; (ii) no host immune response; (iii) the rate of degradation should be proportional to the tissue formation; and (iv) the material must guide cells with the help of physical, chemical and biological cues [19]. Silk is a distinctive family of proteins with a primary set of amino acids having varied functional differences, with different structural sequencing [19]. Silk has been amenable to the human body in all possible ways and has been successfully investigated for countless applications in the form of film, hydrogels, fibres and sponges, beginning with suture material that dates back several centuries [20]. Dry cocoons produced through a sericulture process are used as raw materials. These are then used in various applications after aqueous or organic solvent-processing methods. To achieve the dissolution of solid silk into a fibroin solution, a process called ‘reconstitution of silk’ was developed. Here, the sericin coating of the fiber is removed to enhance its biocompatibility as sericin can trigger immunological responses. Additionally, various methods, such as protein denaturation through chemical or thermal means and the modification of mechanical properties by altering crystallinity, can be induced during the reconstitution phase. This can be induced after the process of immersing in alcohol or through water annealing. The technique can enable the fabrication of the new and desired structure of the silk and helps to achieve the possibility of other fabrication techniques. The property of the resulting structures can be finely controlled and customized to meet specific needs. Regenerated SF exhibits exceptional versatility and biocompatibility, thus making it a valuable resource for a wide range of biomedical applications [21].

Non-woven mats are of general interest given their increased surface area and surface roughness for cell attachment. SF mats were prepared from reprocessed native silk fibre by solubilizing silk fibres in formic acid or calcium chloride prior to subcutaneous implant in the rat. The materials showed biocompatibility with little or no immune response. Vascularized, reticular and connective tissue formation was guided by the mat and growth was confirmed by histological and immunohistochemical characterization [22]. The homogenization technique made a 10–30 μm mat with a diameter of 300 μm. Endothelial cells proliferated when cultured for several weeks and micro-vessel-like structures were formed. Due to the low infiltration of cells, no degradation was observed during that time [23]. Electrospun fibres can range from a few nanometers to microns [24]. Human mesenchymal stem cells (hMSCs) were cultured on uniform fibre < 0.8 μm diameter of aqueous SF solution/polyethylene oxide (PEO) and a lateral modulus of 8 GPa was observed. A 100 nm diameter SF mat from formic acid recorded a 515 MPa Young’s modulus and 7.25 MPa tensile strength [25].



Amino Acid	SF (%)	SS (%)	R-group	Polarity	R-group Properties
Glycine (Gly/G)	45	14	-H	Non-Polar	Hydrophobic
Alanine (Ala/A)	29	5	$-CH_3$	Non-Polar	Hydrophobic
Valine (Val/V)	2	3	$-CH(CH_3)_2$	Non-polar	Hydrophobic
Serine (Ser/S)	12	33	$-CH_2-OH$	Polar	Neutral
Tyrosine (Tyr/Y)	5	3	$-CH_2-C_6H_6-OH$	Polar	Aromatic
Threonine (Thr/T)	1	8	$-CH(CH_3)-OH$	Polar	Neutral
Aspartic Acid (Asp/D)	1	15	$-CH_2-COOH$	Polar	Acidic, Charged
Glutamic acid (Glu/E)	1	8	$-CH_2-CH_2-COOH$	Polar	Acidic, Charged

Fig. 3. Detailed illustration of the chemical structure for silk fibroin. (a) Basic structure and composition of amino acid sequences; (b) primary structure of silk fibroin; (c) β-sheet secondary structure [18].

Electrospun non-woven mesh with random coils was prepared through methanol treatment, but adversely affected the structure by reducing porosity due to dehydration [26].

SF films can be prepared by many techniques, predominantly through casting. Silk structure is reformed by treatment with 50% methanol, which in turn alters the water and oxygen permeability of the film [24]. A nanoscale ultra-thin film made by layer-by-layer technique was shown to be stable due to the hydrophobic interactions and was able to support the adherence and proliferation of hMSCs. Silk films showed a better attachment to fibroblast and other mammalian cells than collagen films [27]. For example, SF film healed full-thickness rat skin in 7 days with a low immune reaction compared to porcine-based wound dressings [28]. Silk films modified with RGD or combined with bone morphogenic protein-2 [29] showed improved bone formation. In addition, SF films have been cast and blended with other polymers for improved or tailor-made properties. To increase the mechanical strength and for better hepatocyte viability, silk was blended with cellulose and recombinant human-like collagen, respectively [30].

SF has been utilized for the preparation of porous sponges through gas foaming and lyophilization or using porogen, which has great importance for cell growth. In a solvent-based method, porogens (salt, sugar) determined the pore size when added with a solvent and cast to molds [31]. A different porosity was achieved by controlling the varying stacking of salt/1,1 3,3 hexafluropropanol–silk solutions. RGD-modified SF sponge was able to promote growth and differentiation of hMSCs, depositing hydroxyapatite and regulated bone markers. When a 900 μm pore-sized sponge was used to culture hMSC in osteogenic media, the resulting structure had similarities to trabecular bone. In a study where aqueous-based SF sponges were used for cartilage TE, chondrocytes proliferated much faster than collagen and a higher content of glycosaminoglycan was observed [32]. Tables 1 and 2 covers some of the SF scaffolds with their properties and applications. Although SF mats, film and sponges have several advantages, a few disadvantages exist. For example, electrospinning of SF mats or films involves the use of solvents, which can adversely affect biocompatibility. In addition, they usually have poor cell encapsulation and penetration ability. As for sponges, there is a need for the removal of added porogens, thus making the production process tedious. The uneven pore size and interconnectivity can also influence cell growth. Therefore, a system with an ideal environment for cell survival that can mimic the microenvironment is required.

Hydrogels are now considered structurally intelligent with a wide

Table 1
General production methods of SF scaffolds for biomedical applications.

Type of scaffold	Fabrication method	Application	Characteristics	Reference
Non-woven mat	Electrospinning	Wound management	Anti-inflammatory, anti-adhesive and stretchable	[33]
Coaxial bead- on-string fibre	Electrospinning	DOX release	pH-sensitive drug release	[34]
PEDOT–PSS functionalized SF	Electrospinning	Axonal regeneration	Electroconducti-ve scaffolds	[35]
Iontophoresis-stimulated SF films	Casting	Neurotensin delivery	Sustained drug release	[36]
Avidin-adsorbed SF film/RGD-SF	Casting	hMSCs culture	Enhanced cell attachment and growth	[37]
SF sponge	Freeze-drying	Tissue repair	Tailored biomechanical properties	[38]
Regenerated silk fibre	Straining flow spinning	Axonal guidance	Enhancement of the guidance ability	[39]

Table 2
SF hydrogels with various crosslinking strategies.

Crosslinking method	Materials	Crosslinking agent	Properties	Reference
Enzyme	SF/gelatin	Transglutaminase	Enhanced mechanical properties	[70]
Enzyme	SF/mHA	Laccase	Improved pore structure and swelling behavior Modulus= 204 kPa Fracture energy= 56.6 kJ/m^3	[71]
Enzyme	SF/HA	Horseradish peroxide	High elasticity Storage moduli= 0.55 ± 0.03 kPa	[72]
Physical	SF/MA	UV Irradiation	Structural stability G′ is larger than G″	[73]
Physical	SF	Gamma irradiation	Improved gelation	[74]
Physcial	SF	Sonication	Controlled gelation	[75]
Physical	SF/gelatin	Electrostatic blending	Tailored biodegradation	[76]
Chemical	SF	Glutaraldehyde	Stiffness G′ and G″ increased by 10-fold	[77]
Chemical	SF/gelatin	Genipin	Improved mechanical properties	[78]
Chemical	SF/PVA	Dialdehyde starch	Enhanced swelling ratio	[79]
Chemical	SF/collagen/ chitosan	Glyoxal	Improved porosity Young's modulus 73.1 ± 3.4 kPa	[80]
Chemical	SF/chitosan	EDC/NHS	Enhanced compressive strength and porous architecture G′ increased with respect to SF concentration.	[81]

range of applications in the field of.

TE and regenerative medicine. They can be described as water-rich, 3D polymer crosslinked networks that can swell to an extent in an aqueous solution. Hydrogels derived from naturally occurring materials, such as SF, chitosan, alginate, and collagen, are particularly appealing given their intrinsic biocompatibility and biodegradability. Silk hydrogels can be formed through physical and chemical crosslinking methods where their β-sheet structures play an important role [40]. During hydrogel preparation, the pH concentration of the SF solution has to be kept in check as it can greatly impact gelation. While a 3% solution with pH 3–4 took only 2 days for gelation, a pH 5–12 solution took 8 days [41]. Gelation and pore size also depend on the concentration of the SF solution [40]. Supplementing SF with surfactants, such as poloxamer 407, induces desirable gelation, but was found to reverse sol-gel transition [42]. Similarly, a semi-interpenetrating polymer network was formed to increase the mechanical property of the system [43]. SF/gelatin-blend hydrogel affected the rheological and mechanical properties as the helix coil transition of the gelatin was influenced by temperature. In drug delivery applications, the drug release greatly depends upon the concentration of the SF and the blended material, if any, as in the case of benfotiamine in SF–glycerol hydrogels [44]. For TE application, osteoblast-like cells adhered to hydrogels and showed an enhanced proliferation rate with the addition of 30% glycerol. SF in bone TE is prominent as bone volume, thickness, minerals and the rate of bone formation were observed to be higher than in poly (lactide-co-glycolide) (PLGA) [45]. Compared to other structures, hydrogels can be more effectively structurally modified for controlled drug release and for facilitating tissue regeneration.

2.2. SF hydrogels and their fabrication

SF hydrogels emerged as an effective candidate for many biomedical applications given their excellent delivery properties for drug substances and cells. The popularity of silk has been ever-increasing for the past few decades and, recently, different functionalities have emerged to cement its properties and extend its application. Silk protein, together with natural/synthetic polymeric materials, usually forms hydrogel for biomedical applications. A structural change from a coiled structure to a silk II β-sheet conformation has been observed during the sol-gel transition [46]. Silk hydrogels are generally formed through physical or chemical crosslinking. In the former, silk molecules form a non-covalent bond through hydrogen bonding, hydrophobic interaction or electrostatic interaction, while in the latter, reactions are carried out with chemical crosslinking agents forming a special network. [47]. Physically crosslinked hydrogels formed without chemical reactions have poor mechanical properties [48]. Self-assembly and gel formation are generally influenced by the concentration, processing temperature, and pH of the solution [49]. Increasing temperature and concentration have an inverse relation to the time of SF gelation.

Temperature-induced gelation is a physical technique where increasing the molecular collision with temperature decreases gelation time [40]. Sonication induces pressure and temperature points leading to aggregation and gelation [50]. The self-assembly of SF results in the formation of different structures like microgels, micelles or vesicles due to the hydrophopic-hydrophilic combination in SPF. Bono et al. used ultrasonification to fabricated 200 μm microgels in a water-oil emulsion phase to encapsulate cells. [51] Applying shear force through vortexing impels molecule-molecule interactions to form a gel [52]. To decrease the pH, an electric field is applied across the solution for silk to agglomerate [53]. Photo-crosslinking falls under the better option of physical crosslinking. In SF hydrogels, gelatin methacrylate that forms hydrogels under UV light is commonly used. When SF (50 mg/Ml) was introduced into GelMA to form an interpenetrating composite hydrogel combining photo-crosslinking and alcohol treatment, compressive modulus was 300kPa, consequently improving mechanical strength. [54] Methacrylated silk fibroin/laminin-acrylate hydrogel was formulated *in situ* by Liu et al. to address spinal cord damage. The significant aspect of the hydrogel is its adhesion with native spinal cord that facilitates molecular scale hydrogel-tissue suturing, thereby aiding axonal growth and guidance [55]. Although physical methods are attractive, the resulting SF hydrogels are suboptimal due to a lack of controlled properties. Consequently, chemical crosslinking-based hydrogels evolved.

Chemical crosslinking techniques are employed to form a more stable hydrogel with better control over swelling and porosity, in addition to being able to regulate hydrogel strength and biodegradability [56]. Some fabrication techniques include salts like calcium (adding ions reduces gelling) [57], PEO or polyethylene glycol (PEG) to promote protein-protein associations [40]. Organic solvents like alcohol helps to form β-sheet structures [58] and surfactants help to bind the proteins to form aggregates [59]. Chemical coupling can functionalize amino acids on the SF chain to adjust the hydrophobic and hydrophilic properties and thereby control hydrogel formation [60]. Due to the presence of functional groups in silk, chemical crosslinkers can easily react with the –OH, $–NH_2$, and –COOH moieties to form networks. Some of the common chemical crosslinkers are genipin, glutaraldehyde and carbodiimide. Genipin react with the free amino groups in SF, whereas glutaraldehyde reacts with the amino group of lysine and phenolic group of tyrosine of the protein chain. Although the above-mentioned crosslinkers are widely used, they appear to exert a cytotoxic effect [61,62].

Thus, there is a growing need for biocompatible reagents. Rehman et al. synthesized citric acid crosslinked SF for biocompatibility. However, other crosslinking agents like gallic acid, ferulic acid and vanillin hold promise for the production of non-toxic SF hydrogels [63]. The safest and most commonly used chemical crosslinking methods are enzymatic crosslinking and radiation. The most used reagent is horseradish peroxidase [64], while carbonic anhydrase, tyrosinase and alcohol oxidase are some other reagents commonly used as crosslinking agents [65–67]. When SF was crosslinked to tyramine-substituted hyaluronic acid (HA) with horseradish peroxidase, a tyramine–tyrosine bond was formed with a relatable mechanical property to natural tissues [68]. The abundant tyrosine in the SF can form di-tyrosine crosslinks through two types of riboflavin (RF)-mediated oxidation mechanisms, direct oxidation and singlet oxygen. In the former, tyrosyl radical is generated through the direct reaction of photosensitized RF with tyrosine, while in the latter, dissolved oxygen is used. Here, both reactions are between the adjacent tyrosyl radical forming the di-tyrosine link [69]. Similarly, laccase crosslinked the same materials to attain better structural stability. Gelation kinetics, pore size and mechanical properties define the success of the hydrogel as a system for a tissue engineering application. Among these, a physical crosslinking technique such as temperature, shear force and ultrasound leads to a simplicity of operation without toxic agent involvement. However, gelation kinetics and pore size are not very easily controllable. Methods like using polar solvents and pH promote the gelation process, but are mostly cytotoxic. Usage of surfactants accelerated gelation, but their release leads to toxicity. Chemical crosslinking helps to alter the gelation kinetics by the suitable selection of crosslinking agents. This method can yield elastic and porous gel structures, which are highly recommended for tissue growth. New methods to improve functionalities are proposed very often by combining existing methods and this diversity can impart different characteristics according to needs.

2.3. SF composite hydrogels

Single and homopolymers may not be ideal for advanced TE applications as there may be a need for enhanced structural and functional properties in order to address the complex requirements of effective tissue repair. Similarly, SF alone has its own limitation, which can be met by forming a composite, *i.e.*, crosslinking a secondary material where the advantages of both materials increase the properties of the final product. Proteins/SF scaffolds are a type of SF composite in which collagen, keratin, or other proteins help to improve cell viability, proliferation, differentiation, and metabolism [82,83]. Polysaccharide/SF composites (chitosan/SF or HA/SF) alter surface properties, such as roughness, to promote cell growth [84,85]. Synthetic polymers are already well established in the biomedical industry and hence a synthetic polymer/SF composite is used to fine-tune any desired property. As an example, polymers such as polyethylene (PE), poly(methyl methacrylate) (PMMA), polylactic acid (PLA) and polyacrylamide are already in use [86].

In a biocomposite, the important influences on mechanical properties like stiffness and strength are the fibre volume fraction and the fibre orientation. Aharonov et al. formulated a laminate of long silk fibres of different fibre volume fraction and its orientation in alginate hydrogel. Tailoring the matrix could mimic the tissue structures such as knee meniscus. [87] Inorganic material like graphene can impart toughness to silk and integrate conductivity. Graphene oxide nanosheet was incorporated into SF hydrogel to improve the mechanical and structural integrity of hydrogel [88]. Graphene oxide/Fe3O4/carboxymethyl cellulose/SF bionanocomposite was developed to address hyperthermia in cancer treatment. [89] Wu et al. produced a hydrogel by reducing HAuCl4 salt *in situ* to incorporate gold nanoparticles into chitosan and SF hydrogel with a much faster gelation time, thus indicating the strong intermolecular interaction of the polymers [90].

SF/nanocellulose composites are being studied extensively as nanocellulose crosslinked with different mechanisms affects the properties, thus varying the final outcome of the composite. Physical crosslinking is mainly employed for fabricating SF/nanocellulose hydrogel, but has exhibited poor mechanical and water absorption properties [91]. Mechanical properties can be improved by chemically crosslinking with different polymers like polyacrylamide to refine swelling behavior, rheological property and morphological changes obtained by varying polymer ratios [92]. With a rapid gelation in approximately 3 min at 37 °C with 84% porosity and 25–66 μm pore size, semi-interpenetrating SF/polyacrylamide hydrogel was developed. These features enabled migration of keratocytes with no celluar toxicity for corneal tissue regeneration [93]. Various other factors (stimuli sensitivity and reversibility of sol-gel transition) can be manipulated to improvise existing features. Collagen crosslinked with SF was studied for thermal, viscoelastic, swelling, morphological properties and biocompatibility. The material proved to be stiff and thermally stable with the extended mechanical property [94]. A variety of materials, such as collagen, hydroxyapatite, carbon nanotube and hyaluronic acid, have been added as a reinforcement with SF for specialized applications and are summarized in Table 3.

Two-dimensional (2D) approaches by far could only poorly keep up with the complex tissue environment. With the possibilities brought by 3D models, the field has advanced for mimicking the physiological microenvironment by using the emerging 3D bioprinting technique. Hydrogel bioinks are an emerging source for tissue biofabrication. However, the precision, stability and reproducibility of the 3D-bioprinted structures are limited due to the lack of appropriate bio-inks. Recently, multiple approaches through physical and chemical methods have been used to formulate robust bio-inks combing ionic, photo- and enzymatic-induced gelation processes for 3D bioprinting [95]. After consolidating according to previously mentioned information, it can be observed that SF alone cannot be 3D-printed, but should be blended with other components to make a printable bio-ink [96]. The bio-ink should have the desired rheological property for it to be a successful 3D-printable compound. Since the bio-ink formulations including biological cues are still under development, developing bio-ink with a natural material like SF will boost its application in biofabrication. SF/gelatin after enzymatic crosslinking was developed for a cartilage TE application with a suitable mechanical strength and with the added benefit of cell adhesion [97]. For materials like SF, it is also important to choose the printing technique accordingly. Previously, inkjet printing, extrude printing and digital light printing were employed for the fabrication using silk bio-ink [98–100]. The commonly used bioprinting technologies are based on digital light processing and laser-printing. Inkjet bioprinting offers the advantages of cost-effectiveness and a moderate printing speed similar to conventional 2D inkjet printing. However,

Table 3
Properties of improved materials of SF composite hydrogel.

SF composite	Application	Properties	Reference
Graphene oxide nanosheet	BMSCs delivery	Improved mechanical and structural properties	[33,103]
Nanocellulose	3D-printing	Micro-porous biomimetic hydrogel	[91]
Polyacrylamide	Nerve regeneration	Controlled biomechanics	[62]
Collagen	Cell encaptulation	Improved biological functions G' > G''	[104]
Hydroxyapatite	Bone tissue engineering	Highly porous scaffold	[105]
Carbon nanotube	On-demand DOX release	Enhanced drug circulation	[106]
Hyaluronic acid	Vitreous humor substitute	Reduction of hydrogel stiffening G' increased proportional to silk concentration	[107]

highly viscous materials cannot be used due to nozzle clogging. The extrusion bioprinter is a modification of inkjet technology that uses an air pump or screw plunger to dispense bio-inks, this allowing the adaption of a range of viscous materials. The disadvantage is the mechanical stresses on encapsulated cells when dealing with thicker hydrogels. However, these issues can be overcome by digital light processing (DLP) bioprinters. The ultraviolet (UV) light-induced photopolymerization technique allows a layer-by-layer model formation with approximately single micron resolution and a printing speed of 30 mm/s, irrespective of size and complexity. DLP printing significantly enhances cell viability (85–90%) due to the short printing duration and nozzle-free technique [73]. Tao et al. formulated a regenerated SF solution functionalized with gold nanoparticle and inkjet printed to be used in applications ranging from photonics to bioimaging and therapy [95]. SF was photo-crosslinked with methacryloyl using lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP) photo initiator at 365 nm with an UV DLP printer. Kim et al. were able to successfully print several structures that could mimic heart, lungs, trachea and blood vessels. The researchers were also able to compose human chondrocytes and human turbinate-derived MSCs with tissue engineered trachea made of silk-methacryloyl hydrogel for use in a rabbit trachea damage model [101]. Xu et al., 3D-printed a hydrogel with gelatin, SF and methacrylate. A prepolymer, photo-crosslinkabe SF-gelatin was first formed and formed a hybrid hydrogel with the reactive methacrylate group. The matrix was used as a skin patch, which had adjustable mechanical behavior that improved the fibroblast growth [102].

2.4. Recombinant SF-based hydrogels

Traditionally, harvested silk has many limitations to be used in such broad and commercial industries as biomedicine. The structural (functional) variation of each batch, impurities and the threat of other disease transmissions always persist [108]. An alternative to tackle these risks is to use biotechnological advances and genetic engineering to develop a safe and consistent quality protein. In addition, incorporating the most modern computational methods to model and analyze structure-function relations enable predictions. Production of recombinant protein is now well established, although it is a massive process to follow from DNA sequencing, designing recombinant DNA, cloning, host transformation (a commonly-used host is *Escherichia coli*) and induction to purification of the protein [109]. The entire framework can be considered to be a hybrid system. With recombinant technology, silk–collagen, –laminin and –reflectin constructs have been explored to understand the various options of combinational and structural designs [110,111]. This synergistic approach enables SF to become a tailorable versatile material that can be turned into films, capsules, particles, foams, hydrogels, microfibres and other therapeutic agents [112].

Silk-elastin-like protein (SELP) is a genetically engineered polymer with amino acid motifs from silk (Gly-Ala-Gly-Ala-GlySer) and elastin (Gly-Val-Gly-Val-Pro) that could control solubility, gelation, stimuli-sensitivity, material strength and biodegradation [113]. Variants of a SELP hydrogel chemoembolization agent results in blocking blood vessels, thereby shrinking the tumor and delivering chemotherapy drugs. It was observed that the sol-gel transition occurred at the point of interest when tested *in vivo* in the rabbit. Hatefi et al. studied the prolonged release of the bioactive adenoviral vector by carrier SELP hydrogel where the controlled release profile was an impressive 28 days. Hydrogels could also keep the infectivity of the cargo intact. Of interest, SELP can be designed to be temperature sensitive [114]. Minimally invasive injection of the liquid polymer *in situ* showed that the firm and irreversible hydrogel was formed when stimulated by body temperature, later releasing the encapsulated therapeutics upon gradual degradation of the matrix. When the SELP hydrogel carrying DNA *in vitro* for 28 days was studied, no significant loss in molecular weight or bioactivity was observed, thus confirming the potential of this material for controlled gene delivery [115]. In other studies, transgenic silkworm was genetically modified to express sequences of collagen and fibronectin where the cell adhesion and calcium-binding activity were higher than natural silk [116], but further studies to investigate its hydrogel formulation are necessary.

2.5. Characterization of SF hydrogel

The determination and characterization of hydrogel network parameters are crucial in the design of hydrogels in order to obtain the desired structure and performance. Characterization methods aim to quantify swelling, mesh size, bound and free water content, pore structure, chemical composition, the strength of chemical bonds, and mechanical strength. Hydrogel characterization can be carried out by physiochemical methods (solubility, swelling measurements, rheology, ultraviolet absorption spectroscopy, infrared spectroscopy, mass spectroscopy, nuclear magnetic resonance [NMR], small-angle X-ray scattering [SAXS], X-ray diffraction [XRD]) as well as by morphological/structural methods (scanning electron microscope [SEM], transmission electron microscopy [TEM], differential scanning calorimetry [DSC], atomic force microscope [AFM], and dynamic mechanical analysis [DMA]).

The most explored characterization technique in hydrogels is used to understand their structural and functional properties. SEM is the exemplar of structural analysis in microscopy to analyze surface morphology, roughness fracture [117], porous/non-porous architecture, pore size [105,118], interconnecting network structure, [74] crosslinking status [119] and effects of loading compounds [119,120]. Scanning probe microscopy is also used to observe the mechanical properties. Hydrogel microstructure is specifically assessed using the SAXS technique [121]. Similarly, laser scanning confocal microscopy (LSCM), together with fluorescent dye, generates Z-stacks to evaluate similar features [122,123]. Structural conformation in SF hydrogels can be determined using Fourier transfer infrared spectroscopy (FTIR), while functional groups can be analyzed by FTIR and NMR. Absorbance at different infrared structural regions confirms the secondary and crystalline structures of silk I and silk II. DSC can confirm structural properties (silk I, silk II) through thermal degradation, which can also provide insight into thermal stability. Moreover, DSC allows a perception of the glass transition behavior of gel [124]. DSC and DMA contribute to characterizing the molecular chain dynamics. DMA was also used to analyze the effect of hydration on mechanical properties [125] (Table 4).

Rheological properties are equally important as mechanical and biological properties for injectable hydrogels. To comprehend fluid behavior, rheological measurements are performed to determine the crosslinking density and viscoelastic properties [124,126]. Water absorption influences the mechanical property and chain dynamics. The stability, hydration capability and tunability of the mechanical property of the formed hydrogel have been studied with respect to water intake. A water uptake study of hydrogels was performed in a controlled chamber at specific conditions including temperature and percent relative humidity (% RH) [125]. The swelling ratio (Q) and water uptake (h) were determined by the following equation:

$$Q = \frac{W_s}{W_d}$$

$$h = \frac{W_s \quad - W_d}{W_d}$$

W_s - weight of swollen SF gel at varying time intervals and conditions.

W_d - dry weight of crosslinked SF gel.

Functional analysis is a significant characterization segment where the effect of solutes or external stimuli, such as absorption-related parameters, solute/monomer levels, and photostability, are examined. In a hydrogel delivery system, drug loading, diffusion and retention are

Table 4
Silk fibroin hydrogel characterization technique.

Characterization technique	Parameters measured	Characteristics and properties	Reference
Fourier Transform Infrared Spectroscopy (FTIR)	Molecular vibration and rotation information	Secondary structures of silk proteins	[127]
Raman Spectroscopy	Vibration and rotation of molecules	Secondary structure content in silk	[128]
Circular Dichroism (CD)	Conformational transition kinetics	Secondary structure and conformational transitions of SF	[129]
Wide-Angle X-Ray Diffraction (WAXD)/ Small-Angle Angle X-Ray Scattering (SAXS)	Crystallite orientation and intensity of diffraction patterns	Crystal size, density and ordering parameters	[130]
AFM Force Spectroscopy	Degree of deflection of the cantilever *versus* piezo movement	Mechanical information and the internal nanostructure	[131]
Imaging techniques 1. Scanning electron microscopy (SEM) 2. Transmission electron microscopy (TEM)	Detect reflected or knocked off electron Transmitted electrons	Morphological, topological and compositional information	[132]
Mechanical technique 1. Differential scanning calorimetry (DSC) 2. Dynamic mechanical analysis (DMA)	Enthalpy Deformation	Mechanical characterization (storage modulus (E') and tan (δ)) and hydration	[133]

equally significant. Drug concentration in respective media is dynamically probed to measure the parameters (plotting concentration) against time/percentage. The degradation property of the materials also shadows the delivery system, thus aiding in controlled release. The material itself, or the added molecules, can bring about degradation. Cell differentiation and migration are related to the elastic modulus of the material and the degree of crosslinkage of the hydrogel.

Given the large number of processing, crosslinking and characterization techniques, SF can be crafted into an ideal delivery vehicle to carry drugs, cells and other molecules as discussed below (Table 5).

3. SF hydrogel as delivery vehicle

3.1. Drug

The basic necessity of a drug delivery system is the controlled release of the therapeutics at the targeted region in a predetermined course of time. Hydrogels in general are considered as an efficient model for drug release, enabling controlled release approach for which SF is found to be more suitable. SF hydrogel has been considered as pH-responsive drug delivery material as it controls the passage of molecules with SF acting as an amphoteric ion-exchanger. The chemotherapy drug, doxorubicin, was delivered with injectable silk hydrogels for a pH-dependent release, which lasted 8 weeks and provided a good antitumor effect [134]. The release profile can be controlled by the molecular weight of SF or of the blended material, if any. Pore size essentially influences the release property by slowing down the release with smaller pores and can be measurably formed by adding materials like glycerol to SF. They can also reduce gelation time by increasing interchain interaction [135]. An analysis showed that a silk composite offers a more sustained release than SF alone and this was confirmed by a study of the physiochemical property of SF/polyacrylamide hydrogels [136]. SF has also shown a potential control release in cancer treatment by reducing the dosage while releasing the chemotherapeutic agent (bevacizumab) [137]. For pulp regeneration, a complex injectable hydrogel was formulated in which initially Tideglusib was released followed by melatonin release. The hydrogel has electrospun fibers of melatonin (Mel)-PMMA/Tideglusib (Td)-SF carried by GelMA-thiolated pectin hydrogel [138]. The sequential release of Mel and Td was studied *in vitro* and the biological performance of the released factors was confirmed by the proliferation and differentiation of dental pulp stem cells into odontogenic differentiation. These results encourage further studies in animals to promote dental pulp regeneration and hold great promise for the future. Song et al. addressed articular cartilage repair by forming SF/pullulan hydrogel embedding etanercept. The drug was dissolved in tyramine-SF/carboxymethylated pullulan and enzyme crosslinked. The system could regulate catabolic and anabolic dynamics in the

Table 5
SF hydrogel as a delivery vehicle for therapeutic factors.

Delivery system	Active substance	Application	Fabrication method	Experimental model	Remarks	Reference
pH responsive SF hydrogel	DOX	Breast cancer	Self-assembling	Nude mice tranplanted with MDA-MB-231 cells	Long-term antitumor efficacy	[33,134]
SF hydrogel releasing anti-VEGF	Bevacizu mab	Ocular drug delivery	Sonication	Intravitreal injection into the eyes of Dutch-belted rabbits	Release over 91 days	[137]
SF-SWCNT-FA/DOX	DOX- loaded folic acid	Cancer therapy	Blending	KB cell line	pH and temperature-dependentDOX release	[106]
SF hydrogel for photo-thermal therapy	Biliverdin	Glioma tumor	Ultrasonication	Balb/c nude mice with subcutaneous glioma	Photo-acoustic and photo-thermal properties	[141]
SELPs 815 K	Thymidine kinase and luciferase	Oral cancer	Direct loading	CD-1 mice with squamous cell carcinoma	Prolonged release	[148]
SF-PEGDMA hydrogel	TGF-β1 and bFGF	Articular cartilage regeneration	Entrapment	Dental pulp	Site specific release	[154]
SF hydrogel	VEGF and BMP-2	Sinus floor augmentation	Entrapment	Rabbit sinus cavity	Slow release of dual factors	[176]
Injectable SF hydrogel	Insulin	Diabetes	Direct loading	Diabetic T1DM Wistar rats	Controlled release from porous scaffold	[160]
SF and phenylboronic acid /acrylamide hydrogel microneedle	Insulin	Diabetes	Polymerization	Mouse skin	Transdermal insulin delivery	[161]
SF hydrogel	BMSCs	Bone regeneration	Blended and sonicated	Calvarial defects in rats	Prolonged survival of BMSCs	[167]
SF/pullan composite hydrogel	MSCs	-	HRP and HP enzyme-mediated polymerization	-	Improved mechanical properties,	[170]

neighboring chondrocytes and were able enhance bone marrow-derived SC chondrogenesis in a rabbit osteochondral defect model [139].

In a very recent work, SF was blended with single-walled carbon nanotubes-folic acid/doxorubicin (SWCNT-FA/DOX) to form a nanocomposite injectable hydrogel. Here, silk was the carrier and the hydrogel was made into a near-infrared (NIR), light stimuli-responsive system by SWCNT-FA/DOX. The NIR-stimulated low pH tumor area helped to release SWCNT-FA/DOX into the environment. Consequently, the folate receptor-positive cancer cells will take up the content and undergo programmed cell death (Fig. 4a) [106]. In a similar study, drug release was controlled through the application of an electric field and NIR laser. Additional heat was also produced in parallel with the exposure to the radiation. The localized accumulation of the chemotherapeutic drug, apoptosis induced by external stimuli, and the sufficient heat generated by the system combined to form a synergistic strategy to eliminate the tumor (Fig. 4d) and pave the way for clinical application [140].

SF/chitosan-based hydrogel formed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemical crosslinking carrying chlorhexidine digluconate (CHD) was developed against bacteria and fungi for wound healing applications. The high rate of drug loading ensured long-term drug release. The combination of materials promoted antibacterial activity and reduced cytotoxicity [143]. In most solid tumors where surgical resection and multidrug chemotherapy is essential, the local and perioperative delivery of chemotherapeutics opens up a favorable route. A bioinspired biliverdin/SF hydrogel was developed for anti-glioma photo-thermal treatment (PTT) and subsequent tissue regeneration using photo-thermal effects. Under NIR irradiation, the temperature of hydrogel rises above 45 °C due to the presence of biliverdin, killing cancer cells *in vitro* and subsiding further growth *in vivo*. Alongside, wound repair and tissue regeneration is supervised by the system Fig. 4b [141]. To induce magnetic hypothermia and for cancer elimination, a nanobiocomposite was prepared consisting of GO and Fe3O4 MNPs along with chitosan/SF crosslinked hydrogel [144]. A recent *in situ* synergistic robust cancer treatment study reported the development of an intrinsic multi-stimuli-responsive hydrophilic SF hydrogel. The hydrogels were able to perform reversible thixotropic gel−sol transition with a cycled shearing stress and resting procedure. In addition, the drug release was guided by the acidity and reactive oxygen species (ROS) of the tumor environment and hyperthermia from the external NIR irradiation. DOX/Cy7 demonstrated a long-term retention characteristic on intra-tumoral injection. This synergistic treatment showed a better outcome and has great potential as a multi-responsive drug delivery system (Fig. 4c) [142]. In a recent study, silk sericin is also being explored and has showcased a wound healing capacity with no inflammatory responses and an excellent cell internalization profile. The chitosan/alginate-silk sericin hydrogel was orally administered to locally accumulate in the colitis tissues.

3.2. Genes

For the last several decades, gene therapy has emerged as a potential treatment strategy for treating genetic disorders. Furthermore, gene therapy also holds potential to address the issues related to the clinical tissue reconstruction of the traumatic form of injuries. The major issue with this delivery system that still needs to be effectively addressed is the targeted delivery and controlled gene expression. SF-based systems carrying biological cues, drugs and genes can be effectively manipulated to form a more stabilized vehicle required to achieve the desired therapeutic effects and targeted release [145]. However, without a carrier-cargo stable interaction, it is almost impossible to achieve the stability of active ingredients. In addition, apart from a few growth factors, the stability is entirely dependent on the entrapment of the cargo into the matrix [146]. SF-based biomaterials can withstand temperature, humidity and physiological variations. Hence, antibiotics like erythromycin with low stability in water are stabilized in SF sponge [147].

SELPs with a controllable sequence and composition are soluble in an aqueous solution at room temperature. Viral gene carriers are mixed with the material to form an insoluble hydrogel on intra-tumoral

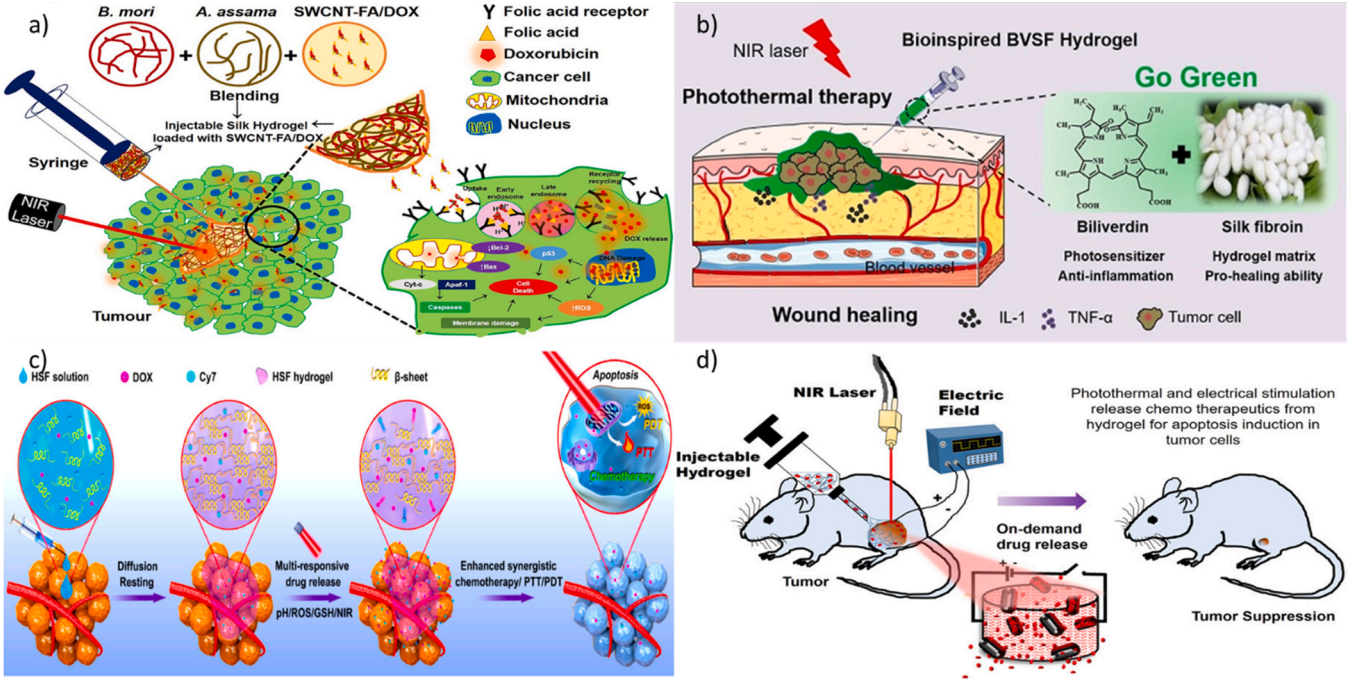


Fig. 4. (a) Schematic representation of injectable hydrogel consisting of a carbon nanotube/silk fibroin for the localized release of an anticancer drug [106]. (b) Representation of biliverdin/silk fibroin as a bifunctional hydrogel with photothermal and pro-regenerative properties [141]. (c) Injectable silk fibroin hydrogel with a multi-responsive capacity for effective tumor therapy [142]. (d) Silk fibroin hydrogel consisting of a photo-electro nanocomposite for the chemotherapeutic delivery of breast cancer treatment [140].

injection, which is apt for localized delivery. SELP 815 K was designed to carry and release adenoviral vectors in a head and neck tumor model based on studies by Price et al. Gene-directed enzyme prodrug therapy was the treatment method adapted by delivering genes for a therapeutic enzyme to targeted cells. In this case, the administered non-toxic prodrug was converted into a cytotoxic metabolite to kill transfected cells, which resulted in a remarkable reduction in tumor size, the longest time to tumor rebound, and prolonged survival in a mouse tumor model. Compared to the virus administered in poloxamer 407, SELP 815 K injection showed an increased duration of expression, thus avoiding possible immunological responses and lowered local toxicity caused by adenoviral administration, although it did result in an encapsulated hydrogel mass in the tissues [148].

Similarly, investigations on the spatial and temporal control over gene expression influenced by the concentration and structural conformations of SELP were conducted. In the series of studies conducted on a murine model of a head and neck tumor xenograft, the structure-dependent changes in degradation of SELP hydrogels were analyzed and 815 K at 4 wt% polymer concentration showed the highest transfection efficiency and prolonged gene expression for 21 days. Viruses disseminating to the liver were also minimized. Among the samples, SELP-815 K had the lowest degradation rate at equivalent concentrations of polymers and elastase. Further research is currently being carried out to evaluate the extent of the applications based on the polymer to deliver therapeutic adenoviruses [149].

3.3. Growth factor

The cells attune themselves and their function by communicating with each other through a series of molecular interactions, hormonal activity, and locally and systematically acting mediators. Growth factors are one such element regarded as a class of proteins that promotes cell proliferation and differentiation in response to the requirements [150, 151]. These factors have to be protected and carefully delivered as they are sensitive to proteolytic degradation. Due to their instability under high temperatures and altered physiological conditions, novel approaches are employed for the controlled release of growth factors for enhancing their therapeutic efficacy.

SC-based TE holds great promise in regenerative medicine. A typical cell proliferates, differentiates and survives in its niche, which is orchestrated by extracellular matrix (ECM). SF is a unique natural protein polymer that perfectly fits most criteria for such an approach. Kaplan and Zhang et al. assessed the *in vitro* and *in vivo* behavior of rat BMSCs by the lenti-green fluorescent protein tracking of porous silk scaffolds formed after gelation in cranial bone defects. An increase in the number of seeded cells from 2 days to 2 weeks after implantation was observed and a consequent moderate decrease at 8 weeks. Emphasizing the duration of cell survival, some cells also differentiated into endothelial cells and osteoblasts. Moreover, angiogenesis and osteogenesis were promoted by the addition of vascular endothelial growth factor (VEGF) and bone morphogenic protein-2 in the scaffolds, thus proving the efficiency of the system [152].

Hybrid hydrogels are a typically heterogeneous system that can ensure adequate cell organization, cell-cell interactions and adhesion. An *in situ* crosslinked hydrogel hybrid system modelled by Bragg et al. demonstrated controlled growth factor sequestering and release over time. The striking difference from other platforms was that sonicated SF was used to entrap bioactive gelatin or heparin-conjugated gelatin, thus employing a secondary crosslinking mechanism. Genipin was used to reinforce the design and gelation kinetics were systematically monitored. Notably, genipin additionally contributed to a ~40% increase in the sequestration of basic fibroblast growth factor (bFGF) from SF/heparin-conjugated gelatin (SSF-GH) to SF/genipin crosslinked heparin-conjugated gelatin (SSF-GH-GN) after 72 h. The crosslinks also slowed the release of bFGF by 15% from SSF-GH to SSF-GH-GN. In addition to its ability for the sustained delivery of growth factors, the platform can also serve as a vessel for *in vitro* cell culture [153].

For more efficient work progress, combination therapy with different growth factors can contribute to SC maturation following differentiation. For example, studies have been conducted with a focus on different growth factors acting simultaneously, mainly in 3D hydrogels mimicking natural tissue regeneration. To improve articular cartilage tissue regeneration, a semi-degradable SF-PEGDMA hydrogel system through a physically and photo-crosslinking mechanism for chondrogenesis was developed [154]. The system carried transforming growth factor (TGF)-β1 loaded PLGA nanoparticles, dental pulp stem cells, and bFGF. This dual delivery system helped cell viability, proliferation and chondrogenic differentiation at the defect site to form a cartilage-like structure. This synergy was brought into effect with the mechanical superiority of PEGDMA and the biological activity of fibroin, consequently controlling cellular responses. In parallel, the simultaneous delivery of growth factors (bFGF and TGF-β1) increased glycosaminoglycan (GAG) and collagen type II production, thus boosting chondrogenic differentiation. The system was attested as a cost-effective, target-specific, tuneable delivery vehicle for cartilage tissue regeneration [154] and *in situ*-forming SF-gelatin hybrid hydrogel was developed for the delivery of bFGF. However, at physiological temperature, the system was less stable and showed a significant release of growth factor. The sonicated SF hydrogel leached 50% of bFGF in 24 h, which was reduced to 42% after 72 h of incubation [153].

Combination therapies using inorganic materials have also been used to tune all properties, including the release of biological molecules. Magaz et al. designed a biohybrid composite in which SF was the continuous phase. The electro-responsive element of the composite was reduced graphene oxide (rGO), which was employed as the dispersed phase. With the conductive rGO, a long-term sustained release of nerve growth factor-β is made possible with the application of a pulsatile electrochemical stimulus. The application level of this system was very impressive due to its ability to support cell survival *in vitro* and tissue regeneration *in vivo*. At present, tailorable biohybrids (Fig. 5a) are a step ahead of other approaches currently in use in the field of TE [155]. The study by Cai et al. of GelMA/silk graphene-based double-sided tapes represents a novel approach for promoting diabetic peripheral nerve repair, where the tape was loaded with gradients of netrin-1 growth factor for directional alignment. A diabetic mouse model was used for the repair of a 7.5 mm sciatic nerve defect. Interestingly, animals treated with a gradient of netrin-1 resulted in enhanced directional axonal regeneration and concomitant myelin recovery and functional restoration as evidenced by the sciatic functional index. Thus, these results demonstrate the benefits of the gradient formation of netrin compared to all other animal groups for nerve repair applications. Notably, this system showcases many aspects of a potentially good design with good mechanical support, biocompatibility, quick curing, and extended growth factor delivery. The design also protected the animals from muscle atrophy [156].

3.4. Peptide

Peptides are profusely found in enzymes, hormones, structural elements and antibodies. They are unable to easily move across cells due to their poor penetration capability and thus need assisted transport for therapeutic strategies. Given their structural network, porosity, sustainability and stimulated responses, hydrogels play a significant role as the carrier of peptides [157,158]. For the continuous supply of insulin, a self-regulated injectable hydrogel was developed with optimum mechano-physical properties. Such systems help in minimally invasive delivery with no surgical implications and accompanying risk [159].

Maity et al. prepared an injectable hydrogel for insulin delivery (Fig. 5b) and also investigated the effect of adding ethylene glycol (EG) and triethylene EG for the gelation process. By introducing the additives, the transition from the random coil to the β-sheet structure was facilitated. At varying concentrations, a quick gelation with retention of the

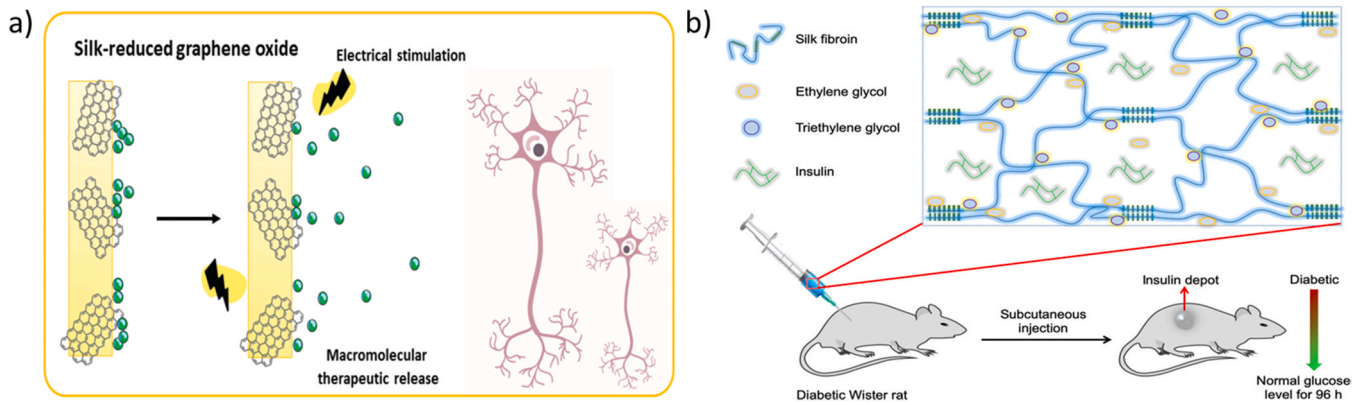


Fig. 5. (a) Electro-responsive materials consisting of β-NGF loaded silk fibroin and graphene oxide [155]. (b) Formation of injectable silk fibroin hydrogel as a sustained delivery system for insulin [160].

injectable property with a mesoporous nature was developed for subcutaneous injection. Human recombinant insulin was encapsulated in SF hydrogel and injected without affecting its structural and functional integrity in streptozotocin-induced T1DM Wistar diabetic rats. The insulin was slowly deployed from under the skin for 4 days to maintain glucose levels [160]. Chen et al. developed hydrogel-forming microneedles by a two-layer fabrication strategy to deliver insulin in a non-invasive manner transdermally. The base layer of microneedles was the stable and mechanically stiff SF and glucose-responsive SF combined with a semi-IPN network hydrogel formed the needle region. The system showed microporosity with interconnected structures. The hydrogels retained their structural integrity in an aqueous environment for 7 days and released insulin on demand accordingly, thus maintaining the glucose level. Their biodegradation addressed all safety concerns regarding the residues and could replace subcutaneous self-injection for diabetes management [161].

3.5. Cells

Researchers have made massive efforts to find cell-based solutions for a wide range of diseases and injuries (Table 6). However, advancements are impeded by the low rate of cell survival and the uncontrolled differentiation of the SCs. Hydrogels can be regarded as a scaffold system for cell encapsulation, which can even recapitulate the ECM with additional fine-tuning of the elastic modulus, chemical functional groups and other properties of the material. Apparently, non-modified silk hydrogels can easily be one such material, given their adsorption of proteins such as fibronectin and their ability to mimic endogenous ECM. Although silk does not possess integrin binding domains, the meticulously functionalized silk molecules can open up new possibilities for the use of silk with novel functions [162].

Table 6
Silk fibroin hydrogel for the encapsulation of therapeutic cells.

Hydrogel delivery system	Cell type	Application	Outcome	Reference
SF hydrogel	BMSCs	Bone regeneration	Prolonged survival of BMSCs	[167]
SF/pullan composite hydrogel	MSCs	Cell delivery	Improved cell survival	[170]
SF hydrogel	Muscle-derived MSCs	Angiogenesis Muscle regeneration	Enhanced tissue regeneration	[174]
SF-Alginate	MSCs Neural progenitor cells	Tissue engineering	Enhanced cell survival	[194]
SF-DMPG	L929 NIH/323 SaOS-2 CaSki	Tissue engineering	Biocompatible Cytoprotective	[175]
SF-DMPG	SaOS-2	Bone tissue engineering	Osteogenic differentiation	[173]
SF-chitosan	Hepatocytes	Tissue engineering	Enhanced functional phenotype	[172]
SF microgel	L929 Myoblast cells	Tissue engineering	VEGF release	[51]

Davis et al. entrapped islets and mesenchymal stromal cells in silk hydrogels to study how the combination improves islet transplantation for type 1 diabetes. In the *in vitro* studies performed, an improved insulin response was noted, due to the increase in gene expression for insulin I, insulin II, glucagon and pancreatic and duodenal homeobox 1 (PDX-1) [163]. In a related work, an *in vivo* evaluation on a streptozotocin-induced diabetic mouse model was conducted where marginal pancreatic islets and pelleted pancreatic islets were co-encapsulated with MSCs in a SF matrix. Both were able to control the blood glucose level, the former in 4 days and the latter within 15 days. The combination of the two types of islets with MSCs required 9 days to control blood glucose. Additionally, the system was able to induce the expression of VEGF, which helped the hydrogels to appropriately function and survive. MSCs aided in the expression of trophic and angiogenic factors, in turn supporting the function of the graft. Without the added SCs, the grafts failed to control the blood glucose [164].

MSCs can self-renew and differentiate to specialized cells and hence are used frequently for regenerative purposes. MSCs have a unique cross-talk with hematopoietic cells, which empowers them with immunosuppressive characteristics. Therefore, MSCs blend in perfectly for autologous and allogeneic transplantation, which is also due to their low immunogenicity. Martín-Martín et al. proved that 3D SF hydrogel matrix releases neuroprotective and neuroregenerative factors, although the release rate was higher for non-encapsulated MSCs. Encapsulated MSCs survived for several days in the matrix, but an over-secretion of TFG- β1 (anti-inflammatory factor) was observed. Thus, further investigation is necessary to understand the MSCs secretome in functionalized SF, as well as in their composites with organic and inorganic materials and polymers (Fig. 6c). When taking a step forward to pre-clinical and clinical analysis, it is imperative to contemplate the therapeutic effect of the desired therapeutic molecule to know to what extent these molecular groups are responsible for secretions and to identify their release profiles in order to open doors for their sustainable application [165]. To cater for two distinct functions: sustained release of the drug and adipogenic inducer, an injectable and bioresorbable SF was developed. Interestingley, DOX had a sustained release to 21 days, which contributed for

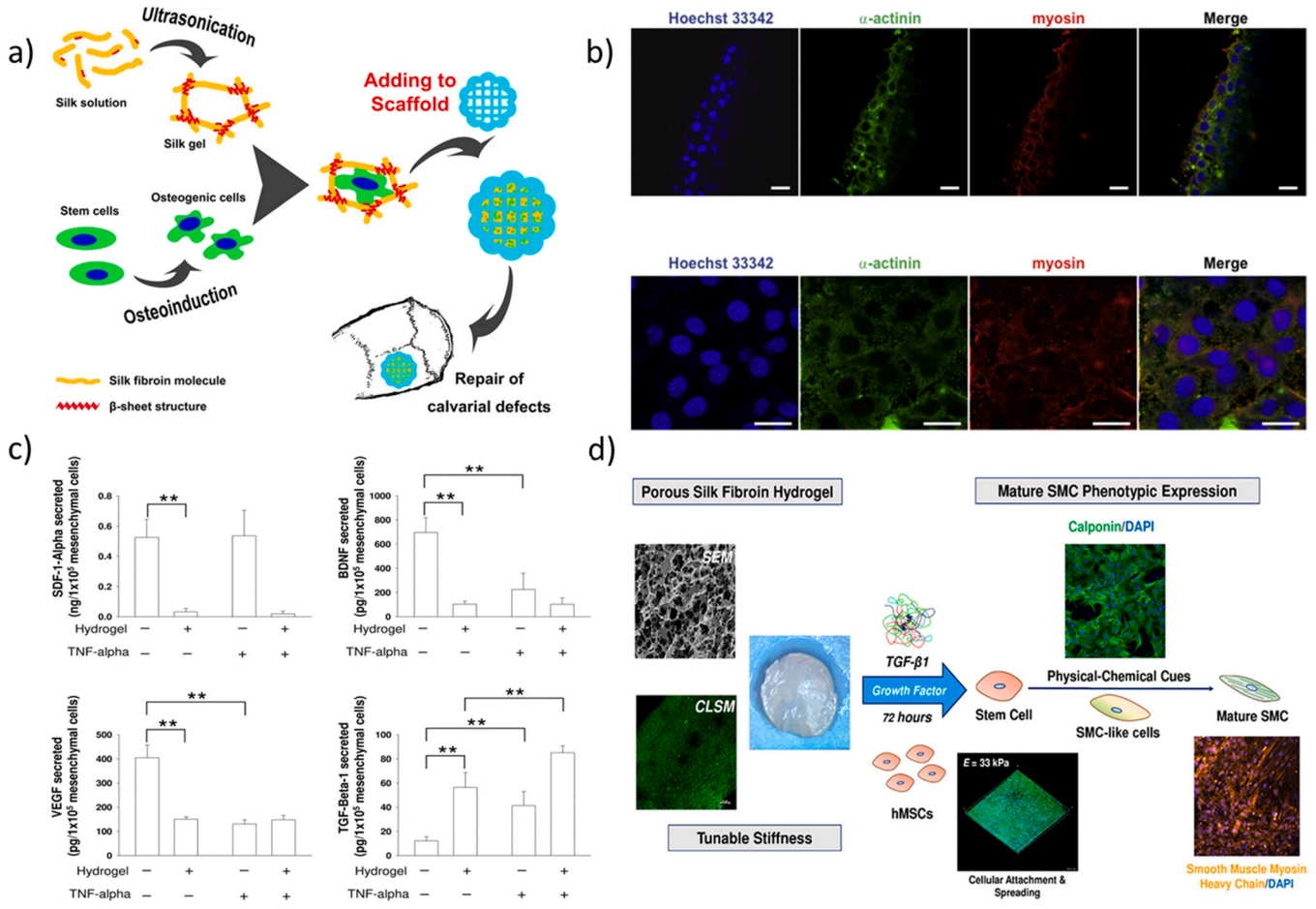


Fig. 6. (a) Preparation of porous silk fibroin hydrogel for the delivery of bone marrow SCs for bone regeneration applications [167]. (b) Microscopic data showing the cardiac mesenchymal SCs cultured on PEGylated silk fibroin hydrogel substrate over 14 and 21 days. Scale bar = 10 μm [168]. (c) Secretome profile resulting from silk fibroin hydrogels carrying hMSCs [165]. (d) Differentiation of hMSCs into smooth muscle cells using silk fibroin hydrogel features with different mechanical properties [169].

tumour supression and overcame recurrence, while dexamethasone was released for 30 days, which helped in the adipose tissue-derived SC differentiation for tissue reconstruction. The molecules retained their stability and bioactivity during the slow and sustained release [166].

Floren et al. studied the effect of SF stiffness and TGF-β1 on the vascular commitment of hMSCs. Providing the required stiffness and TGF-β1 and the upregulation of myosin heavy chain expression was demonstrated in 72 h. Additionally, by modulating stiffness, a directional hMSC differentiation was made possible. Hence, a well-defined and specialized biomaterial can be made out of SF that fits the TE portfolio [169]. Ciocci et al. developed a PEGylated injectable SF hydrogel with PEG diacrylated (PEGDa) through a sol-gel reaction. Molecular characterizations of the SF and PEGylated SF (molecular weight of 20 and/or 6 kDa) were carried out by reverse-phase high--performance liquid chromatography circular dichroism, and FTIR). During the mild photochemical sol-gel reaction, a pliable encapsulation of resident human cardiac MSCs (cMSCs) was possible, demonstrating the cytocompatibility of the system. The albumin-perfluorohexane microspheres were embedded inside the matrix of SF and this bio-functionalization encouraged biological cues to promote cell attachment, growth and function. This natural micro-environment helped in the expression of myosin and actinin (Fig. 6b). Microspheres increased the viability of the stem cells and helped in their proliferation in the microsphere modified PEGylated SF 3D hydrogel matrix [168]. BMSCs were delivered using an SF hydrogel, which was earlier sonicated for β-sheet structure into a specifically shaped 3D system for their growth. The transportation of molecules and proteins into the gels was monitored. Furthermore, after the gelation process, the inner seeded cells with the scaffold were implanted in calvarial defects in rats after osteogenesis (Fig. 6a) [167]. Chena et al. developed a pullulan-SF copolymer hydrogel for MSC delivery to provide cell-specific epitopes that are lacking in SF alone. The advantages of both materials highlight the importance of hydrogel as a biodegradable *in situ* forming an injectable carrier system. This hydrogel formed through enzyme-mediated polymerization was studied for its gelation time, swelling behavior, rheological properties and the mechanical property of different pullulan concentrations used. The composite increased fracture strength, breaking elongation and compressive reproducibility when individual materials were considered. The potential of this cell delivery system in musculoskeletal TE was assessed *in vitro* with rabbit BMSCs [170]. Ni et al., 3D-printed hydrogel with SF and hydroxypropylmethylcellulose of methacrylation (HPMC-MA) embedded with BMSC. The material had a modulus of 0.1–10 MPa, similar to that of natural bones. As for any 3D-printed hydrogel, SF and HPMC-MA ensure a sufficient nutrient supply, great biochemical supportabilit, and excellent mechanical properties. The survival and proliferation of BMSCs in the printed scaffolds have been studied to understand the chondrogenesis-related gene expression for their application in cartilage TE [171]. Tan et al. studied the effect of SF hydrogel stiffness and TGF-β1 in upregulating myosin heavy chain expression (Fig. 6d). hMSC differentiation for TE applications was also investigated [169].

Gholami et al. developed a 3D delivery vehicle for hepatocytes using SF and chitosan. This injectable system is also thermoresponsive due to the addition of glycerophosphate that can further undergo sol-gel

transition. Hydrogels with varying amounts and compositions of polymer and gelling agents were formed and evaluated for mechanical properties and cytocompatibility. The entire structure of gel composition of 1.3 and 1 wt/volume percentage chitosan and SF, respectively, with 0.05 M sodium hydrogen carbonate and 30% glycerophosphate, were layered and featured with 100 μm pores, which makes it ideal for a variety of tissue engineering applications. Modulus was increased by 6 kPa for this composition, while the addition of genipin also further increased the modulus. The sample did not compromise the viability of cells and had a modest haemolytic effect. More importantly, the liver-specific activity was studied to understand the functioning of the hepatocytes used. A significantly enhanced synthesis of urea in hydrogel encapsulating HepG2 was confirmed using quantitative analysis by 3 days after cell encapsulation [172].

To promote angiogenesis and muscle regeneration, a SF scaffold was prepared with 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG) sodium salt with comparable mechanical properties to natural soft tissue. The encapsulated muscle stem cells were extracted from sheep. The resulting scaffold was tested in an adult sheep model. The seeded cells formed new muscle cells with a faster growth rate until day 9 and then at a slower rate. They also promoted angiogenesis when a 10% DMPG SF solution scaffold was used and confirmed by immunohistochemical staining of CD34. However, the study was conducted only for a limited time of two weeks. In brief, the scaffold holds the potential to be a viable substitute for ECM [174]. Similarly, DMPG and SF were used to form a hydrogel with controlled gelation varying the lipid concentration (5,10, and,15 mM DMPG/3% SF). As the concentration of DMPG increased, the degradation profile decreased. Four different cell types were encapsulated in the matrix: L929, NIH/3T3 (fibroblastic cell lines), SaOS-2 and CaSki (cancer cell lines). The fibroblast had a normal proliferation rate, but cell adhesion was of concern. SaOS-2 maintained their shape and proliferated for 7 days and remained viable for 21 days. CaSki cell growth decreased with a higher concentration of DMPG [175]. Subsequently, DMPG-induced SF hydrogel was prepared and loaded with dexamethasone and osteoblastic SaOS-2 cells. The drug loaded underwent a burst release in the first 6 h. The remaining drug was released gradually over 28 days, indicating the capability of hydrogels for the prolonged release of entrapped molecules. Cell growth increased steadily during the first week and then plateaued at 28 days with cells mostly in S phase. The APL (alkaline phosphatase) activity test confirmed the osteogenic differentiation of the cells. The study also confirmed calcium production through alizarin red staining. Importantly, this study reveals that hydrogels can carry drugs and cells efficiently with their functionalities intact [173].

L929 was also encapsulated in SF microgel by Bono et al. using the batch emulsion method. Their proliferation and VEGF secretion were evaluated. Survival of myoblasts was confirmed by the expression of Ncam1 and Pax3 proliferation markers by qRT-PCR. VEGF levels were found to increase up to 22 pg mL^{-1} by day 7. Similar to a previous work, the shape and size were well maintained, thus meaning that SF microgels are stable and can exchange metabolites [51]. It is evident from the above-mentioned studies that SF is an excellent candidate for cell encapsulation for cell structural and functional preservation. With different approaches, SF can be fabricated into matrices for cell growth and delivery.

3.6. SF hydrogel and its drug release profile

The mastery of polymers that artistically deliver films, gels, capsules, tablets, creams and other therapeutic agents as carrier systems is key to the most important aspects of controlled drug release, such as long-duration sustained drug release, low toxicity, manipulation of the carrier and easy administration of cargo. Polymer stands as an experienced and well-qualified delivery system and has been abundantly studied since its well-deserved recognition [177]. Each of these materials should be developed and reinforced to cater to the needs for controlled delivery and TE scaffold. The primary factors are: 1) load-bearing capacity; 2) porosity; 3) cell-matrix interactions; and 4) the possibility to adjust the release profile to achieve the therapeutic concentration of the drug [178]. SF is such a protein fibre and can be considered as an efficient controlled delivery material in view of its controllable crystallinity and molecular conformation.

SF hydrogels have been highlighted for a variety of applications due to their excellent physical and chemical properties [75,179–182] and much effort is being made to comprehend the drug release mechanism of different concentrations and formulations. To perceive an exact point of release is almost impossible, leaving only near-zero order guidance to elucidate the kinetics. However, given the number of studies carried out on SF delivery systems, the factors affecting release can be narrowed down to diffusion, swelling and degradation. Fick's laws of diffusion describe drug mass transport in diffusion-controlled release through the following equation:

$$J_A = -D\frac{dC_A}{dx}$$

$$\frac{dC_A}{dt} = D\frac{d^2C_A}{dx^2}$$

J_A - Diffusive flux of the drug.
D - Diffusion coefficient.
C_A - Concentration of drug in the release medium.
x - Position.
t - Time.

Here, the drug diffusion coefficient is assumed to be constant and the initial drug concentration is observed to be lower than drug solubility. Among other parameters, an ideal sink condition should be established for this model. The conditions to be satisfied can vary with different geometrical shapes and edges. Swelling behavior, polymer dissolution/degradation and chemically-controlled delivery have been studied in complex mechanistic models [183], but these models have yet to be fully implemented for silk-based delivery systems.

The Peppas model (or power law model) has been adapted to compare the resulting release kinetics of different formulation parameters, although the model is based on empirical or semi-empirical mathematical models and not on true release mechanisms [184]. The Peppas equation is as follows:

$$\frac{M_t}{M_\infty} = kt^n$$

Mt - Cumulative amount of drug released at time 't'.
M∞ - Cumulative amount of drug released at infinite time.
k - Constant for the drug delivery system.
n - Release exponent.

As a matrix for drug delivery, SF hydrogels were prepared by de Moraes et al. by accelerating the gelation kinetics of fibroin by adding a varying content of ethanol. Ethanol acts on SF by dehydrating the fibroin molecules, thus preserving the chemical and conformational properties of fibroin hydrogel, despite the amount of ethanol. When evaluating the release profile of diclofenac sodium dissolved in (a) ethanol and (b) water, the former presented a more sustained drug release from SF hydrogel [117].

A double-release study investigated SF hydrogels containing fluorescent dye-incorporated SF nanoparticles, but the release of nanoparticles from hydrogels showed a prolonged release, while the dye was released quickly and sustained the release for 5 days [182]. However, although the dye was incorporated, its influence over the material interaction was overlooked in the study. Tomado et al. also studied the properties of dye-incorporated SF microparticles containing SF hydrogel. A study of the evaluation of matrix interaction and release aimed at using different dyes and properties. Dyes such as methylene blue (MB), rose bengal (RB), rhodamine B (RhB), and neutral red (NR) with a different charge, hydrophilicity, molar mass, physicochemical

properties and interaction were studied to expand knowledge on their application as a delivery vehicle (Fig. 7) [185].

In a further study, the influence of hydrophilic and electrostatic interactions over the common hydrophobic interactions was evident in the loading of dyes, thereby performing a more effective fusion with microparticles. Indeed, a 10-fold longer release time was observed in a hydrogel containing SF microparticles with dye (~900 min) compared to dye release from microparticles (~90 min). In contrast to the Fickian diffusion observed in the release of dyes from SF microparticles, the anomalous mechanism and transport of Case II mechanism were predominant, thus suggesting that the matrix degradation directly influenced dye release. The study confirmed that the scheme of double release prolonged the release and a better understanding of the influence of other factors, such as load, hydrophilicity and the size of the molecules in dye release from the matrix [185].

In another release study, Fang et al. characterized the drug release pattern and the physicochemical properties of low (18 kDa) and high molecular weight (76 kDa) silk protein (SP) hydrogels. Silk protein hydrogels are composed of hydrophilic amino acids with both beta-sheet and random coil conformation; formed hydrogels had interconnecting pores. Hydrogen bonding is important for the linkage of hydrogels and the study showed a structural conversion from crosslinks to sheet shape when SPL was incorporated into SPH hydrogel [179].

4. Challenges and future perspectives

The fundamentals of silk in itself stand out with its overall appeal, thus making state-of-the-art research of silk still relevant. Notably, the curiosity revolving around the performance of silk in cross-disciplinary research has never ebbed throughout several decades. These abundantly available fibres, once used as garments, have evolved impressively to heal wounds and treat cancer and more. Regardless, thus hinting that no material is perfect, silk comes with disadvantages. The necessity for purification to remove type 1 allergic sericin residues is a requisite for it to be used for clinical applications. For instance, SF sutures perform better than sutures made by synthetic materials [186]. Silk-based products have the tremendous potential to be an ideal healthcare material, mainly due to their ability to mimic extracellular matrix [187]. However, despite numerous clinical translations and research products, its real world application has yet to be endorsed by regulatory authorities.

Processed silk fibers molded to diverse forms and designs, including fibers, films, scaffolds and hydrogels, have emerged as an inevitable representative in biomedical science. 3D printing being a revolution *per se*, bioprinting is the immediate future in creating human tissue and body parts. Silk hydrogel 3D-printing is carried out primarily through extrusion and inkjet printing among other methods that are recently being investigated [188,189]. The shape fidelity of the extrusion 3D-printed silk cannot be guaranteed due to silk viscosity. Silk nanofibers in combination with several other materials can improve the mechanical outcome of printed silk and modify rheological properties to make silk desirable for printing [190–192]. Devising new formulations, characterizing and standardizing them can give access to different aspects including shape fidelity, avoiding cytotoxicity and other consequences from the additives used if any, to allow their use in clinical application [193].

Among other forms, SF hydrogel is the “pick of the litter” for delivery applications for drugs, cells and growth factors to name but a few, due to its biofriendly nature, multifunctionality and mechanical superiority. Certain aspects that add to their extensive use include injectability, self-healing property, adhesion, environmental responsiveness, antibacterial activity and 3D printability. SF hydrogels have already been used in established areas (adhesives, sutures, wound healing, myocardial patches, wearable biosensor) and unmet needs with existing technologies and thus adding novel approaches. From delivering and releasing single components to a complex multi-component system controlled spatially and temporally, SF hydrogel systems have evolved to respond to internal triggers and external stimuli, with localized controlled release corresponding to enzymes and cellular events. SF could nest and nurture therapeutic drugs, SCs and other cargo mimicking extracellular matrix and allow the release of optimal dosage by modulating the concentration over time. Moving forward at this rate of precision, SF hydrogel can optimize the implant structure, maintain material property, and control release behavior conveniently and safely, which otherwise cannot be achieved through traditional approaches.

The near future of the use of SF hydrogels will certainly be the surge in clinical applications. Although recognized as an exceptional material, SF hydrogels have yet to be moved towards clinical trials and US Food and Drug Administration approval. Although the materials lack certain aspects, such as poor attachment of certain cell types and mechanical/physical properties, crosslinking, blending or the conjugation of other moieties help to eliminate these issues and enhance functionality. The challenges posed by the significant toxicity of chemical crosslinking can be overcome by various physical crosslinking techniques. As highlighted, silk possesses the great property to be a perfect biomedical material. However, to reach clinical studies, many hurdles remain. Cost-effectiveness has still to be achieved and bulk-produced SF with a consistent property has yet to be accomplished and therefore new solutions should be explored. To satisfy the demands of commercialization, SF should meet criteria such as prolonged shelf life, and ease of

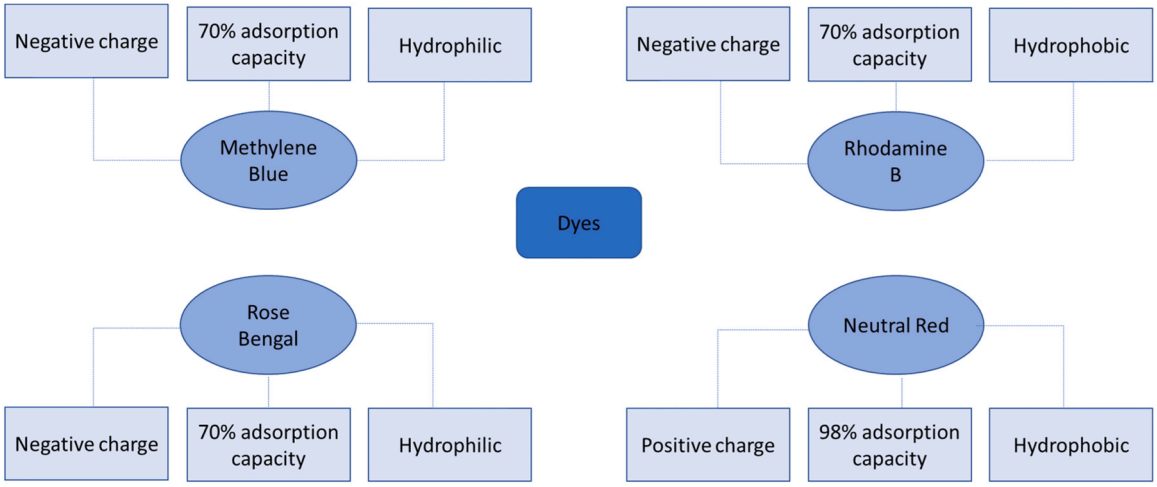


Fig. 7. Inference of the investigation conducted by Tomado et al. on assessing the interaction within silk fibroin matrix and its subsequent release properties.

application according to need (*in situ* gelation for wound healing, injectability for tissue regeneration/drug delivery).

5. Conclusion

This review provides an overview of the emergence of SF hydrogels as a drug carrier system in biomedicine, including their evolution, fabrication, characterization and application in tissue engineering. Compared to other materials, SF has a strong potential regarding biodegradability, cytocompatibility and ease of availability. As a natural material, silk has some drawbacks, but a focus on the improvement of its properties has significantly widened its range of applications. SF can be considered as a safe biomaterial from an excellent natural source that can be gathered, processed, manipulated and developed to meet a wide range of medical needs. Future *in vivo* studies should be conducted to further explore the potential of SF in drug delivery and tissue engineering applications in order to connect the bridge between laboratory success and the clinical application.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contribution

SM conceived the idea and designed the study. APM performed a review of the literature, and wrote the manuscript. SM critically reviewed the article, revised the article and provided the feedback and edits on all aspects of the manuscript. SM and APM read and approved the submitted version. SM acquired the funding and supervised the project.

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