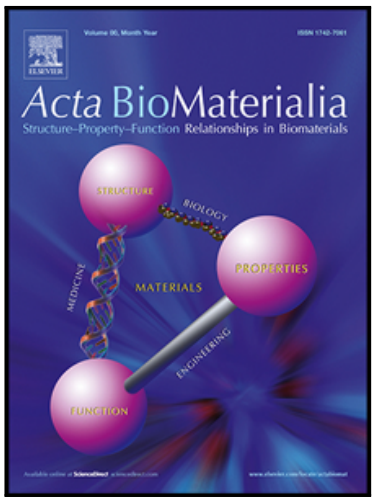

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Silk Biomaterials in Wound Healing and Skin Regeneration Therapeutics: from Bench to Bedside

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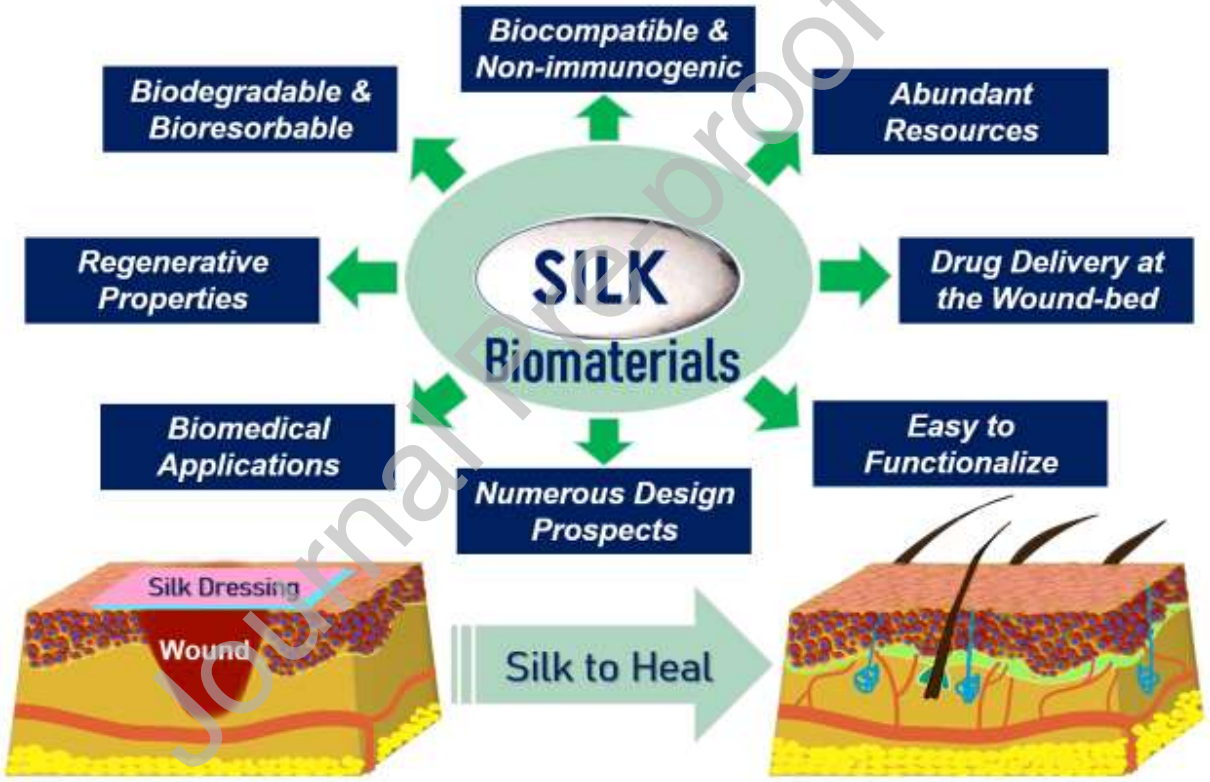
Graphical abstract



Abstract

Silk biomaterials are known for biomedical and tissue engineering applications including drug delivery and implantable devices owing to their biocompatible and a wide range of ideal physico-chemical properties. Herein, we present a critical overview of the progress of silk-based matrices in skin regeneration therapeutics with an emphasis on recent innovations and scientific findings. Beginning with a brief description of numerous varieties of silks, the review summarizes our current understanding of the biological properties of silk that help in the wound healing process. Various silk varieties such as silkworm silk fibroin, silk sericin, native spider silk and recombinant silk materials have been explored for cutaneous wound healing applications from the past few decades. With an aim to harness the regenerative properties of silk, numerous strategies have been applied to develop functional bioactive wound dressings and viable bio-artificial skin grafts in recent times. The review examines multiple inherent properties of silk that aid in the critical events of the healing process such as cell migration, cell proliferation, angiogenesis, and re-epithelialization. A detailed insight into the progress of silk-based cellular skin grafts is also provided that discusses various co-culture strategies and development of bilayer and tri-layer human skin equivalent under *in vitro* conditions. In addition, functionalized silk matrices loaded with bioactive molecules and antibacterial compounds are discussed, which have shown great potential in treating hard-to-heal wounds. Finally, clinical studies performed using silk-based translational products are reviewed that validate their regenerative properties and future applications in this area.

Keywords

Silk; Fibroin; Sericin; Recombinant Silk; Wound healing; Skin tissue engineering

Statement of Significance

The review article discusses the recent advances in silk-based technologies for wound healing applications, covering various types of silk biomaterials and their properties suitable for wound repair and regeneration. The article demonstrates the progress of silk-based matrices with an update on the patented technologies and clinical advancements over the years.

The rationale behind this review is to highlight numerous properties of silk biomaterials that aid in all the critical events of the wound healing process towards skin regeneration. Functionalization strategies to fabricate silk dressings containing bioactive molecules and antimicrobial compounds for drug delivery to the wound bed are discussed. In addition, a separate section describes the approaches taken to generate living human skin equivalent that have recently contributed in the field of skin tissue engineering.

1. Introduction

Silk is a protein biopolymer that is produced by a variety of insects such as silkworms, flies, spiders, mites, and scorpions [1]. Silk produced from silkworms and spiders is extensively studied for various biomedical applications. Silk is a broad term and often referred to as protein fibers spun by insects; however, it varies from species to species and function to function [1-3]. Silkworms develop silk cocoons by spinning silk fibers that shelter them during their period of metamorphism. Spiders produce silk fibers to build webs, capture preys, and for their movements. All the varieties of silk fibers differ from species to species due to significant changes in their amino acid sequence. Silks also vary in their physico-chemical and biological properties owing to the variation in composition [2].

Being a versatile biopolymer, silk is widely explored in the applications of tissue engineering and regenerative medicine [4]. Silk fibroin (SF), the fiber portion extracted from the cocoons of silkworms. SF isolated from the domesticated silkworm, *Bombyx mori*, is the most extensively studied variety of silk (**Figure 1-I,II**). It is well characterized in terms of physical properties, chemical composition, and biological properties [5-7]. The feasibility of extracting the fibroin component directly from silk cocoons using green technology has endorsed it as an ideal structural biomaterial. Silk is considered as a biocompatible material because it does not evoke a long-term or persistent inflammatory response, and it allows tissue ingrowth [7-11]. It induces mild or minimal initial inflammatory response when implanted *in vivo*, which subsides with time, indicating immunocompatible properties of the material [10]. Recently, scaffolds made up of reconstituted or solubilized silk protein, namely Silk Voice, are given the FDA approval for the first time in 2019 and the product is handled by Sofregen Medical, Inc. Medford [12]. Similarly, silk-based injectable fillers were applied for vocal cord augmentation [13]. Previously, a mesh construct (with the product name SERI) was given the 510(k) clearance by the FDA, which is fabricated from the silk fibers isolated from silkworm cocoons [14].

Apart from the fibroin component, there exists silk sericin protein in the cocoons that act as glue while the silkworms spin silk cocoons [15, 16]. Sericin holds the fibroin fibers together and contributes to almost 30 % weight of silk cocoon [15]. Both the fibroin and sericin components of silkworm cocoons are individually explored for wound healing, drug delivery and other biomedical applications [7, 15, 17]. In the context of silk biomaterials, it is worth

mentioning that spider silk has also been explored for tissue engineering and wound healing applications in recent times [3, 18]. In addition, there are recombinant silk varieties such as recombinant dragline spider silk and silk-elastin-like protein (SELP), which are developed artificially through recombinant DNA technology (RDT) [3, 19]. Technological advancements in genetic engineering have enabled successful integration of silk specific genes and expression of silk proteins through a range of host systems. For instance, 4RepCT partial dragline spider silk is recombinantly produced through *Escherichia coli* bacteria that can be easily used to make matrices like foam, film and mesh (**Figure 1-III,IV**) [20]. The 4RepCT is generated using a part of gene taken from the natural spidroin protein from *Euprosthenops australis* spiders, and comprises of four sequential repeats of poly-alanine/glycine-rich moieties and a non-repetitive C-terminal domain. Furthermore, functionalization of such recombinantly produced silk proteins is easy at the genetic level through RDT wherein the gene encoding a functional peptide sequence can be fused to the silk gene [3].

Silk, being identified as a biocompatible, non-immunogenic and bioresorbable material, has been extensively used in suturing of incisional wounds since the ancient times [1]. With the emergence of modern medicine, silk has widely been explored for wound healing applications in recent times [21-23]. The inherent property of silk to stimulate cell migration and proliferation has been found to be directly linked with the accelerated wound healing properties [24]. Numerous studies have proved silk as a good choice of biomaterial for the development of wound dressings and bioartificial skin graft [21, 22, 25, 26]. Skin, the largest organ of our body, performs the most essential function of protecting the internal organs by providing barrier properties towards the external environment and harmful pathogens (**Figure 2**) [27].

A cutaneous wound is basically a disruption in the healthy structure and function of skin tissue that creates a cavity, which needs to be repaired and regenerated. Although skin tissue holds self-repair property, specific types of wounds such as diabetic ulcers, burn injuries and the large surface area or deep wounds, fail to heal [27]. Such non-healing wounds require surgical interventions (plastic surgeries) or bioactive dressing materials that aid in the healing process. Matrices applied in the form of wound dressings or artificial grafts provide a platform over the

wounds, which restore the barrier properties and stimulate the self-repair mechanism of the wound healing cascade [27-29]. In this context, silk as a biomaterial has been extensively explored and utilized for the development of bioactive matrices for wound healing applications [1, 4]. Silk-based matrices developed so far have shown potential in treating various types of wounds ranging from diabetic wounds, third-degree burns, donor-site split thickness wounds, and pressure sores in animal studies [8, 22, 30, 31]. In addition, numerous strategies have been applied for the functionalization of silk-based matrices using growth factors, antibiotics, and other bioactive molecules since last few years [32].

Herein, we critically review the research efforts taken using the silk-based technology for wound healing applications. The review begins with the introduction of various types of silk biomaterials utilized for the development of wound dressings and skin grafts. It provides an updated overview of the intrinsic wound healing properties of silk materials, contributing to the overall skin regeneration process. Various composite matrices with other natural and synthetic polymers are briefly described. We have also touched upon the functionalization strategies and highlighted the drug delivery approaches taken using different bioactive molecules for wound healing applications. A separate section of silk-based cellular skin substitutes and artificially developed viable skin grafts is also included, where we have critically appraised the research efforts concerned with skin tissue engineering. Finally, we aim to provide an insight into the clinical studies performed using silk-based matrices along with future directions and perspectives of the improved wound healing technology using silk biomaterials.

2. Silk biopolymers: structure and properties for wound healing applications

2.1. Silkworm silk fibroin

Among the different varieties of silkworms, based on their feeding habits, they can be classified as: mulberry and non-mulberry silkworms [2, 33]. The host plants that give shelter and food to the silkworms are considered behind their classification. Silkworms that feed on the mulberry leaves are mulberry silkworms, for example *Bombyx mori*. Other varieties that do not feed on mulberry leaves are categorized under non-mulberry silkworms. There are numerous non-mulberry silkworm varieties worldwide; for instance, *Antheraea mylitta* (Indian Tasar silk), *Antheraea assama* (Indian Muga silk), *Antheraea pernyi* (Chinese Oak Tussah silk), *Antheraea yamamai* (Japanese silk) and *Philosamia ricini* (Indian Eri silk) [2, 33]. The minor or

major differences in the sequence of silk proteins are responsible for variations in their physical, chemical and biological properties [33]. The *B. mori* silk fibroin (BmSF) include two polypeptide chains: a heavy (H) chain of 391.367 kDa and a light (L) chain of 25 kDa [6]. Additionally, BmSF contains a glycoprotein known as P25. Assembly of H-chains, L-chains and P25 is in the ratio of 6: 6: 1, which is also considered as a signature of BmSF protein [6]. Non-mulberry silk fibroin (NMSF) consists of only the heavy chain, as it lacks both the L-chain and P25 glycoprotein [2, 33]. This marks the prime difference in the composition of BmSF and NMSF proteins. Other differences between BmSF and NMSF include presence of polyalanine blocks, RGD (Arg-Gly-Asp) tripeptide and Arg-rich motifs in NMSF [2].

The SF protein remains in α-helix and random coil conformation in the silk glands of silkworms, which transforms to mechanically strong silk fibers or threads during spinning [6, 34]. Stability of silk fibers at the time of construction of silk cocoons is due to the transition of the SF protein from random coils to β-sheet conformation [6, 34]. The β-sheet structures are majorly formed due to the presence of repetitive stretches of (GAGAGS)n and (GAGAGY)n in the protein sequence of BmSF. Silk fiber contains hydrophobic repetitive domains of glycine and alanine amino acids, which contribute to more than 50 % of total fibroin and thus confer crystallinity to the overall protein structure [1, 6]. The crystalline structures of silk are majorly responsible for their high mechanical strength and structural properties. Differences in the crystalline structures due to varied amino acid sequence among various silkworms also lead to different physical properties. For instance, NMSF consists of repetitive blocks of polyalanine (AA)n and polyglycine (GG)n, whereas BmSF consists of (GAGA)n repeats [2, 33]. The repetitive hydrophobic domains are responsible for the β-sheet structures in silk fibers [1, 6]. Due to the structural differences, non-mulberry silk has outperformed mulberry silk fibers in terms of mechanical properties [35-37]. It has been found that the crystallinity of (AA)n repeats present in NMSF is much higher and requires higher energy to break than that of (GAGA)n repeats of BmSF [38, 39]. In a comparative study, *A. assama* silk showed 40 % elongation at break, which was found to be comparable with *Nephila clavipes* spider silk (40 %); however, *B. mori* silk showed only 15 % elongation at break [38, 40]. Another non-mulberry silk variety, *A. pernyi* demonstrated stronger and tougher mechanical properties (Young's modulus = 9633 MPa) in comparison to *B. mori* fibers (Young's modulus = 7456 MPa), which showed comparatively weak and brittle behaviour [36].

Variation in the protein sequence of SF varieties is also a major reason for discrepancies in the protein dissolution methods [41]. The dissolution method of BmSF from silk cocoons is well established using 9.3 M LiBr, as the degummed *B. mori* silk fibers readily dissolve in the LiBr solution [5]. The LiBr solvent system disrupts the intermolecular hydrogen bonding of fibroin chains, thereby dispersing the fibroin polypeptides for proper dissolution. This readily works for *B. mori* silk fibers; however, this solvent fails in complete dissolution of non-mulberry silk fibers and results in significantly lower yield [42]. It is speculated that due to the presence of a hydrophobic core of A-motifs and excessive hydrogen bonding among the alternated hydrophilic sequences, the NMSF fibers do not completely dissolve in the chaotropic reagent like LiBr [42]. Therefore, researchers have resorted to an alternative method of isolating NMSF from silk glands of mature silkworms using surfactant like sodium dodecyl sulfate [41]. This method works for a wide variety of non-mulberry silkworms like *A. mylitta, A. assama* and *P. ricini* [21, 43]. Conclusively, the unique polypeptides of SF and their (intra/inter)molecular structural organizations are cumulatively responsible for different dissolution process and various extraordinary properties. Furthermore, SF is easy to modulate in various formats by controlling the crystallinity of β-sheet structures by physical and chemical treatments [23]. This also provide a way to achieve desired and tunable physical properties in terms of mechanical strength and degradation rate [23]. The details of SF properties that ultimately help in the healing process have been extensively discussed in section 3.

2.2. Silk sericin

Silk sericin (SS), another component of the silk cocoon, is also considered as a protein biomaterial containing inherent bioactivity, suitable for wound healing applications (**Figure 3**). Sericin owns beneficial properties for wound repair such as moisture retention ability, biocompatibility, biodegradability, antibacterial, and antioxidant activities [15, 44, 45]. Sericin is a glycoprotein present in silkworm cocoons that acts as a glue to hold the fibroin fibers during the cocoon fabrication. Sericin is extracted from silk cocoons through the process of degumming by boiling in 0.02 M sodium carbonate (Na_2CO_3) solution, which is the conventional extraction process [5, 45]. Previously considered as a waste product from silk textile industries, sericin is now considered as a potential biomaterial for tissue engineering and drug delivery applications [15].

Sericin proteins have been characterized and utilized in various biomedical applications since the past decade. Sericin from *B. mori* mulberry silk variety consists of 30 % serine content and approximately 15 % glycine, 15 % aspartic acid and 6 % threonine content [15, 48, 49]. It is believed that the protein is called sericin due to a high amount of serine residues in the amino acid sequence. The molecular weight of sericin ranges from 10 to 400 kDa with varied protein biochemistry depending on the extraction and processing methods [45]. Depending on the silkworm variety, amino acids content varies, and so the structural conformation of the protein also varies. For instance, *B. mori* silk sericin (BMSS) has 36.1 % random coils structure that predominates other conformations such as turns (35.1 %) and helix (28.8 %). *A. assama* silk sericin (AASS) has turns (37.1 %) as the predominating structure, whereas *P. ricini* silk sericin (PRSS) has β-sheets (40.8 %) as the predominating conformation [45]. Sericin plays various roles in the wound healing process due to cell stimulating properties, which have been discussed separately in section 5.

2.3. Spider silk

The silk protein produced by numerous spiders differs from species to species in composition and structure. Varieties of spider silk proteins exist depending on the differences in their amino acid sequence. Their compositions also differ based on particular movements and functions such as dragline movement, orb-web formation or cocoon construction [3]. The unique physical properties of spider silk have led to an increasing interest in harbouring the protein for various biomedical applications. However, harvesting large numbers of spiders for obtaining native spider silk fibers is difficult in comparison to silkworms. Besides, the yield obtained from spiders ranges from 12 – 137 meters, which is far lesser than the cocoon fiber from a single silkworm (600 – 900 meters) [50, 51]. Therefore, researchers have resorted to using the RDT for the production of recombinant spider silk artificially. Technological advancements in genetic engineering have enabled the integration of spider silk encoding genes and successful expression of spider silks through a range of host systems [3, 52]. Both the native and recombinant spider silk proteins have been used to fabricate dermo-epidermal skin constructs, as the platform of spider silk supports cell encapsulation and cellular co-culture (**Figure 4**).

Dragline silk is the highly explored spider silk variety owing to the unique tensile and light-weight properties [3]. Spiders store the soluble form of dragline silk in a sac of their major ampullate glands and spontaneously produce silk fibers through the duct during their spontaneous movements. Moreover, dragline silks hold supercontraction property under hydration conditions and are considered to be a tougher material in comparison to steel and Kevlar [53]. Numerous varieties of recombinant spider silks have been generated over the years that mimic the native structural and functional properties of dragline silk. For example, MaSpI and MaSpII - Major Spidroin dragline types I and II of *Nephila clavipes*, ADF-3 and ADF-4 dragline silks of *Araneus diadematus* and 4RepCT partial dragline silk of *Euprosthenops australis*. [3, 52]. Till date, micro-organisms like *E. coli* bacteria are the most exploited host organism owing to the simple bacterial system, easy purification process and high yield [3]. However, recombinant spider silks have been produced from a wide variety of other host systems like potato, tobacco, mammary glands of mice and goats [3]. Transgenic mice carrying genes of spider silks was a successful attempt, wherein the mice produced ADF-3 and MaSpI silk proteins in their milk over many generations [54, 55]. Mammalian cell systems are also efficient in the expression of recombinant spidroins (ADF-3) secreted from mammalian cell lines cultured under hollow fiber bioreactors [56].

The spider silk is composed of an assembly of spidroins, which contains crystalline repetitive regions along with non-repetitive N-terminal and C-terminal domains [3]. Spider silk protein is abundant in glycine and alanine blocks in its repetitive region similar to silkworm silk fibroin protein; however, the overall protein sequence is quite different. Another feature of spider silk is its high elasticity due to the presence of short repeats of semi-amorphous regions like GPGXX (β-spirals or β-turns) and GGX (helix) in the non-crystalline regions [50]. The β-sheet crystalline regions serve as connecting links to the amorphous chains and form a network structure, thereby maintaining the tensile and elastic properties of spider silk. The method of producing recombinant spider silk holds another advantage of synthesizing silk with desired biophysical properties. For example, two types of bioengineered spider silk were produced; one containing 2 repeats of individual blocks and another containing 12 repeats [58]. The silk protein thus obtained were referred as H(AB)2 (11.6 kDa) and H(AB)12 (43.7 kDa), where ‘A’ block

comprised of a β-sheet forming poly(alanine) hydrophobic sequence and the 'B' block consisted of four repeats of GGX hydrophilic sequence. By varying the number of repeats of (AB) blocks, silk proteins with different degradation rate and mechanical strength were successfully obtained [58]. The study thus revealed that by controlling the sequence and number of repeat motifs, programmable silk materials could be artificially synthesized with desired physical properties [58].

Significant progress in the field of genetic engineering has made it possible to produce recombinant spider silk with high yield. In a recent report in 2018, mass production of spider silk was demonstrated in transgenic *B. mori* silkworms, where the fibroin heavy chain (FibH) was replaced with major ampullate spidroin-1 gene (MaSp1) of *N. clavipes* [59]. In another recent report, researchers were successful in producing large size dragline spidroin of *N. clavipes*, which consisted of 192 repeat motifs (highest till date) [60]. Through this, the spider fibers completely replicated the mechanical strength of native silk fibers, achieving a tensile strength of 1.03 ± 0.11 GPa with modulus 13.7 ± 3.0 GPa and extensibility (18 ± 6%). Such high mechanical properties in recombinant spider silk were previously not achieved due to significantly short repeats of spidroins (96-mer) [60, 61]. Such research outputs indeed demonstrate the future prospect of spider silk protein as a next generation material in various applications.

The unique and diverse biomechanical properties of spider silk proteins have encouraged its application in regenerative medicine to a great extent. Both the natural as well as recombinant spider silks are biocompatible and biodegradable; therefore, considered as a potential candidate in the field of tissue engineering [18, 62]. Spider silk has shown great potential in wound healing applications when examined *in vitro* and *in vivo* [18, 63, 64]. The woven mesh of native spider silk fibers demonstrated significant development of epidermal layer over the fibers, as the silk frame supported keratinization of cultured cells [18]. Spider silk has also demonstrated wound healing properties when applied on burn wounds in an animal study [63]. Such studies have motivated many researchers to develop simple strategies to use spider silk protein for biomedical applications. The recombinantly produced 4RepCT spider silk and 4RepCT-based fusion proteins have been used to functionalize bulk materials by simple coating procedures [65, 66]. The 4RepCT proteins hold inherent property to self-assemble at ambient temperature and

develop a thin coating over bulk base materials like titanium implants and silkworm SF matrices [65, 66]. A real-time study through quartz crystal microbalance with dissipation monitoring (QCM-D) confirmed that the spider silk fusion proteins tend to continuously self-assemble onto surfaces by forming nano-fibrillar structures [65, 66]. This leads to the formation of a homogenous thin coating of spider silk proteins over base materials. The stable interaction between 4RepCT fusion proteins and SF bulk matrices was smartly utilized to generate functionalized constructs containing bioactive domains attached to 4RepCT proteins. This also led to a chemical-free coating method to functionalize silkworm SF matrices for developing bioactive wound dressings and bioartificial skin graft [26]. The spider silk coated nanofibrous matrices and microporous scaffolds thus developed demonstrated faster wound healing outcomes when examined under diabetic and burn wound models *in vivo* respectively [67, 68].

Similar to 4RepCT, recombinantly produced eADF spidroins also hold self-assembly properties upon shear stress or in the presence of different ions to form self-assembled nanofibrils and stable structures [52, 69]. The self-assembly property of recombinantly produced eADF led to the facile formation of hydrogels and 3D printed constructs for tissue engineering applications [69, 70]. The highly concentrated eADF4(C16) (3 % w/v) and its RGD motif containing variant readily formed hydrogels at ambient conditions, which could be applied to develop cell-laden 3D bioprinted constructs [70]. The recombinant spider silk when printed with human dermal fibroblasts demonstrated cell viability under the layered structure, indicating potential applications of such 3D printed constructs in skin tissue engineering (Figure 4-III) [57].

2.4. Silk-elastin-like protein (SELP)

Like recombinant spider silk, another variety of recombinant silk is silk-elastin-like protein (SELP), which contains properties of both silk and elastin proteins [71, 72]. The recombinantly produced silk proteins hold an advantage over naturally available silk materials because material properties and functionalization can be easily controlled and modulated at the genetic level. SELP is a good example of the recent development in the field of material science and biotechnology. SELPs are *de novo* biomaterials that are designed to combine the biological and mechanical properties of silk and elastin based on their individual repetitive motifs of amino acids [72]. SELP holds self-assembling property, which makes pH- and thermo-responsive materials and can be easily processed into nanofibers, nanoparticles, hydrogels, fibers, and thin

films [19, 71-77]. SELP is made up of silk-like and elastin-like repetitive blocks that together render pH- and thermo-responsive properties [71, 74]. The presence of glutamic acid in the silk-like repetitive octapeptide GAGAGAGE imparts pH-responsive properties. The pentapeptide of elastic-like repetitive VPGXG motif are responsible for temperature induced phase transition and thus impart thermo-responsive properties to the SELP. The random coiled structures are transformed to β-spiral structure (similar to β-sheet in SF) upon lowering the pH and changing the temperature, thereby forming aggregates. The (GA)n repeats further help in self-assembly of the protein [71, 74].

Further, there are many varieties of SELP materials depending on the choice of X residue of elastin block, the ratio between silk and elastin repetitive blocks and length of the overall protein chain [74, 78]. Tunable materials can be easily generated by changing these parameters. For instance, the thermal responsive properties of SELP were tuned by increasing the silk-elastin ratio, which led to higher inverse transition temperature [74]. In another example, by varying the amino acid in X residue of elastin block, SELPs responded differently to various stimuli like temperature, pH, ionic strength, redox, enzymatic stimuli and electric fields [72]. SELP is a biocompatible material with low cytotoxicity and holds the potential to promote proliferation and migration of fibroblasts [79]. The phase changing properties of SELP was utilized in forming thermo-responsive injectable hydrogels. Besides this, other properties like biodegradability of SELP materials is easy to tune by simply varying the ratio of silk to elastin blocks. High elastin content in SELP demonstrated faster degradation and *vice versa* [80, 81]. Moreover, the combination of silk and elastin did not compromise the biocompatibility of the final material. The electrospun mats fabricated using SELP biomaterial demonstrated high swelling degree (570–720 %), water vapour transmission rate in the range of 1083 g/m^2/day and mechanical properties with the elastic modulus of ~126 MPa, illustrating the material as a potential candidate for wound healing applications [82]. The nanofibrous SELP mat supported adhesion and proliferation of normal human skin fibroblasts, proving biocompatible properties [82].

SELPs are largely explored in the controlled delivery of macromolecules, drugs, plasmid DNA or adenoviral vectors [80]. There are a few reports on the application of SELP in tissue regeneration and wound healing as well. The current state of the art demonstrates an immense potential of SELP in wound healing applications. The advanced genetic engineering has shown

facile ways of producing engineered biomaterials artificially and functionalizing them at the genetic level. Successful functionalization and generation of spider silk materials fused with bioactive moieties has already been demonstrated with the help of RDT [3, 50, 52]. Similarly, SELP if functionalized with bioactive moieties such as growth factors, cell binding peptides or antibacterial moieties, can also contribute to advanced next-generation wound dressings. In a study, SELP based thermo-responsive hydrogel accelerated the wound healing rate in diabetic mice model [83]. A study revealed the migratory effect of SELP protein on fibroblast and macrophage cells [79]. The study also showed that fibroblasts cultured on SELP matrix helped in producing a higher level of collagen, thereby indicating the potential of SELP in skin tissue engineering [79]. Being recombinantly produced, there is a lot of research on the production of SELP with high yield. In a recent study, Collins et al. developed a strategy to increase the volumetric productivity of SELP up to 4.3 g L^{-1} post-purification, which is the highest reported yield till date [84].

3. Suitability of SF as a biomaterial for wound healing – an update on the properties of silk aiding in skin regeneration and wound healing.

Wound healing is a sequential process that begins with haemostasis to stop bleeding and prevent excessive blood loss [85]. The next phase includes inflammation and proliferation, which comprises recruitment of various types of cells like neutrophils, macrophages, endothelial cells, fibroblasts, and keratinocytes [85]. Multiple cells play numerous roles of secreting chemokines, producing growth factors, forming blood vessels, developing granulation tissue, and re-epithelializing wounds to ultimately seal the wound cavity. Once the wound is completely closed, the final phase of healing process starts, where the previously formed matrix of the wound cavity is gradually remodelled into a new matrix, leading to either a completely regenerated skin or a semi/non-functional scar tissue [85].

Application of silk in healing the cutaneous wounds commenced decades ago through silk sutures. Silk fibers, directly isolated from the cocoons, were used as sutures in the ancient time. With the advent of modern surgery, silk sutures were commercialized considering the mechanical properties of silk threads, as the knots formed by silk threads were strong and easy to handle [1, 86]. However, the threads directly isolated from the cocoons consisted of both fibroin and sericin protein, which elicited an immune response in patients. Sutures made up of such silk

threads were known as virgin silk sutures, which were later modified to remove the sericin content [1]. Sutures made up fibroin protein after complete removal of sericin component are referred to as black braided silk [1]. On further processing the black braided silk variety, silk threads were developed having a coating of wax or silicone materials (Perma-Han dTM). Virgin silk has been reported to cause type I allergic reactions, induce asthma, and upregulate IgE levels in some cases owing to the immunogenicity elicited by the presence of sericin glue in the silk fibers [1]. On the other hand, black braided silk sutures were reported to be relatively safe, and no cases of allergy or upregulation of IgE antibody were found. Following the immunogenic reports, sericin and fibroin components of silk are not used in combination for the fabrication of matrices for tissue engineering applications [5].

Both silk sericin and silk fibroin are separated from the silk fibres and processed separately to fabricate individual matrices. Although both the materials are considered suitable for wound healing, the physical properties of SF give additional advantage in developing matrices. SF, being a bulk biopolymer, has structural properties, which provide benefits in fabricating self-standing matrices through numerous strategies designed so far. On the other hand, sericin doesn't hold structural properties, and hence, pristine sericin based matrices are challenging to fabricate. Due to its highly hydrophilic nature, sericin based constructs are designed by taking the help of blending materials and cross-linking agents [87]. If we look deeply into the healing process supported by a silk-based matrix, we find that the platform of silk is involved in every major step of the healing as described in the details in the following sub-sections. Briefly, the basic properties provided by SF for cutaneous wound healing applications are as follows:

- **Haemostatic property** – By interaction of SF with fibrinogen and blood platelets.
- **Cell migration and cell recruitment –** By NF-κB signalling pathway.
- **Exudate absorbing capacity** – by high water and moisture retention capacity of SF protein.
- **Mechanical strength and elasticity** – due to the high mechanical strength of SF as a biopolymer, which renders integral stability to a dressing material, prevent wound bed disruption and ability to conform to the wound size and shape.

- **Cell – material interaction** – SF supports cell attachment, migration, proliferation and differentiation
- **Wound healing action** – due to the intrinsic bioactive properties of SF in cell migration, helping in neo-vascularization, enhanced re-epithelialization, and tissue ingrowth.
- **Skin friendly (non-toxic, non-allergenic and non-sensitising) –** due to biocompatibility property of SF protein.
- **Ease of application and structural support** – easy to fabricate different format designs (film / nanofiber / hydrogel / porous sponge / 3D printed construct).
- **Easy functionalization –** cross-linker free functionalization of SF-based matrices with a range of antibiotics, growth factors, and other bioactive molecules.
- **Cost effective** – due to the inexpensiveness of silk as a raw natural material.
- **Easy to commercialize** – *B. mori* silk fibroin-based products: SERI scaffold (mesh of silk threads) and regenerated silk-based scaffolds are FDA approved with 510(k) clearance.

3.1. Haemostasis by SF

There are reports on the thrombogenic effect of black braided silk sutures during implantation, which is attributed to the binding properties of silk with blood platelets and fibrin [88]. The study showed thrombus formation during the initial days, which later declined and depicted signs of new endothelium layer and complete endothelialization by 28 days of implantation [88]. The haemostatic activity of silk fibers was also found to be attributed to the surface properties of silk threads and the ability to bind with proteins, helping in clotting cascade [89]. This was validated by the wax coated silk threads, which modified the surface properties of silk and significantly reduced the thrombotic response. Sulfation and heparinization of SF are commonly used methods to make the silk an anticoagulant material because the non-treated silk was reported to coagulate the blood [90-92]. In another study, thin films made up of regenerated silk solution demonstrated binding with fibrinogen (a component of fibrin clot), confirming the haemostatic property of SF even in the regenerated form [93]. This phenomenon of natural interaction or binding of fibrinogen with silk was utilized in a recent work, in which the coagulant supplements, namely, fibrinogen and thrombin were mixed with silk solution and casted to form porous sponges. The haemostatic matrix thus developed acted as a carrier to slowly deliver

fibrinogen and thrombin [94]. The interaction of silk with clotting proteins like fibrinogen not only offered haemostasis, but could also serve as a cargo that releases inflammatory factors to lead the healing process toward its next phase.

3.2. Effect of SF on cell attachment, migration, and proliferation

Quick adhesion, migration, and proliferation of cells are crucial factors that decide the rate of the wound healing process. A wound dressing acting as a supporting platform over the wounds should enable cells to attach and migrate, and provide a conducive microenvironment for cellular growth, proliferation, and differentiation. Cell adhesion, as the name implies, refers to the attachment of cells on the matrix. Moreover, the initial binding of cells is a critical factor for wound healing, because the sooner cells adhere to the dressing material, the faster they migrate and get recruited to the wound site to aid the healing cascade [26]. Once the cells are attached, they tend to migrate from higher cell-density region to lower cell-density region. This migratory behaviour of cells actually helps in recruiting the cells from wound edges towards the wound cavity. To study the migration behaviour of cells, a scratch assay is considered as a standard experiment under *in vitro* conditions [95]. Herein, a cell monolayer is developed and a scratch is created in the form of a thin line by removing the cells from a particular region. With time, the cells present in the wound edges try to fill the gap created in the cell monolayer. In order to find the efficacy of any biomolecule or biomaterial, this experiment is performed by taking the biomolecule in the foetal bovine serum (FBS) depleted media.

Migration of keratinocytes studied through the scratch assay revealed that the SF protein stimulates cell migration by activating MEK, JNK, and PI3K signalling pathways [96]. Keratinocyte migration was also observed through a different approach using agarose gel drop assay in our recent study. Herein, a small drop of cell-laden agarose gel was placed on top of an acellular silk gel matrix; the experiment showed the cells migrating out from agarose gel towards the silk gel [22]. The study thus indicated that silk holds inherent property of helping the cells to migrate and therefore recruiting them towards the wound site. A recent study proved the wound healing property of BmSF attributed to the NF-κB signalling via microarray analysis and scratch assay [24]. The study by Park et al. not only proved the impact of silk on cell migration, but also demonstrated that it promotes healing by regulating the expression of vimentin, cyclin D1, VEGF, and fibronectin, which are well-known markers of cell proliferation (**Figure 5**) [24]. In

another study, peptide fragments derived from chymotrypsin digested *B. mori* fibroin heavy chain demonstrated bioactivity in promoting the proliferation of skin fibroblasts [97].

3.3. Immune response to the SF biomaterial

Our body has an inbuilt mechanism of the immune system that regulates the prevention of foreign substance invasion. The foreign substances not only include pathogens and infectious organisms, but also materials that are implanted in case of organ transplant. The body's immune system does not recognize the implants as self and therefore respond to it by eliciting an initial foreign body response [98]. Once a graft is implanted in the host system, the inflammatory response is evoked and depending on the type of material, the reaction either subsides in a later phase or becomes chronic due to long-term immunogenicity. Such long-term inflammation against allogenic transplants is often the common cause of graft rejection, as the host system fails to accept the transplanted graft [98]. Various *in vivo* and clinical studies have demonstrated that silk is minimally immunogenic [8, 10, 22]. Silk does not provoke long-term inflammation as evidenced in various studies. Silk-based matrices elicit an initial immune response that subsides in the later phases, showing signs of graft acceptance [10, 22]. In a study, artificial dermis fabricated using SF material demonstrated low inflammatory response along with showing signs of cell infiltration, neovascularization, and extracellular matrix deposition [9].

An initial inflammatory phase is already a part of the wound healing process after the haemostasis. Therefore, in the context of wound healing, silk dressings, or silk grafts might help in accelerating the healing process because of overlapping inflammatory responses [10, 22]. A mild inflammatory response provoked against implanted matrices during initial time-points of dressing application or implantation help in the activation of macrophages and thereby stimulated them to secrete chemokines and growth factors [10]. The activation of mild foreign body response includes the formation of multinuclear giant cells and recruitment of immune cells and fibroblasts [10]. It was observed that implanted silk scaffolds induced the formation of foreign body multinucleated giant cells (FBGCs) soon after implantation, but the response was significantly reduced in the later stages of implantation [10, 99]. FBGCs is beneficial if formed temporarily at the early phase of wound healing because it could destroy the pathogens and recruit cells like macrophages, fibroblasts and blood capillaries to grow a granulation tissue at

the boundary of the implant [10, 100]. However, presence of FBGCs for prolonged duration often leads to the formation of a fibrotic capsule that might turn into a permanent scar tissue [100]. Long-term implantation of silk sponges revealed that silk material does not induce FBGCs for prolonged duration; and hence, are considered safe as an implantable material [101]. In a study, porous silk sponges demonstrated a significantly lower population of immune cells post 4-weeks implantation in comparison to 2 weeks [102]. In another study, quantification of inflammatory cells post 12 weeks implantation of silk constructs showed negligible inflammatory response with no inflammatory cells in comparison to that of 4 weeks post-implantation [103]. Regression of fibrosis was observed in a study, in which silk scaffolds were implanted in heart tissue to treat myocardial infarction [104]. Herein, fibrosis observed after two weeks of implantation was completely disappeared after 8 weeks, thereby indicating negligible long-term immunogenic reaction by silk biomaterial [104].

3.4. Degree of neovascularization and angiogenesis supported by SF matrices

Neovascularization or angiogenesis during wound healing is often referred to as the formation of blood vessels in the newly developed granulation tissue at the wound site [105]. This event is a part of the proliferation phase of the healing process, where new blood capillaries sprout in the neo-tissue to provide nutrient supply. Silk matrices have shown extraordinary properties in helping the angiogenesis of wounds in various studies [22, 30, 106]. Nanofibrous matrices of different silk varieties demonstrated the formation of blood capillaries at an early onset of healing in both acute and diabetic wounds in our study [30]. In our recent study, silk hydrogel treatment for 3rd-degree burn wounds demonstrated 10-fold higher blood vessel density in comparison to untreated wounds post 7 days of treatment [22]. This might also be attributed to the property of silk to evoke an immune response at the initial time-points [22]. This clearly indicates that the platform of silk over wounds support the formation of neo-tissue and help in recruiting cells for the formation of blood capillaries.

In the context of skin grafting, neo-vascularization within the graft becomes critical, because the newly formed blood vessel must merge with the previously present blood vessels at the wound boundaries, the process known as anastomosis [107]. A detailed study of silk-based artificial skin grafts supporting anastomosis under *in vivo* conditions is not available in the current literature. Anastomosis using pre-vascularized silk-based matrices seems very

challenging because it requires permanent integration of the graft with the native tissue. Moreover, the growth of blood vessels within the scaffolds also depends on the pore size and pore interconnectivity [108]. However, such comparative studies are yet to be investigated using silk matrices in the skin engineering applications under *in vivo* conditions. Successful permanent implantation of silk grafts in the form of artificial skin is a challenging task. By significantly modulating the physical properties of silk, development of pre-vascularized skin grafts using silk as a biomaterial can be an interesting and revolutionary topic of research in the field of skin tissue engineering.

3.5. Re-epithelialization supported by SF matrices

Re-epithelialization is considered as a major event in the wound healing process, as it seals the wound cavity with a fully-grown epidermal layer on the top. Silk has shown cell migratory effects via NF-κB signalling pathways, which attributes to improved re-epithelialization as previously described [24]. The platform of silk nanofiber matrix was found to promote the formation of epithelial layer over an *in vitro* wound model made up of collagen gel [109]. In the follow-up work, it was also evidenced by the expression of cytokeratin 10 (CK10) and cytokeratin 14 (CK14) markers in the regenerated epithelial layer under *in vivo* wound model [110]. CK10 expression demonstrated the differentiation of keratinocytes in the newly formed epidermis in the wounds treated with silk matrices [110]. Such observations were also found in our recent study, which demonstrated suprabasal and basal expression of CK 10 and CK 14 respectively in the wounds treated with silk hydrogel after 3 weeks of healing in a burn wound model [22].

In another study, a thin epithelial tongue could be visible in the histological stainings of wounds treated with silk-based matrices [30]. The study showed a budding epithelial layer progressing towards the wound cavity during the mid-stage of the healing process [30]. The inherent bioactivity of aiding epithelialization present in the SF is also utilized in corneal wound healing. Herein, silk was used in the solution form (0.5 % and 2 % w/v) as a liquid eye drop under rabbit models denuded of their epithelial surface. The study demonstrated regeneration of corneum epithelium and epithelial cell proliferation as marked by Ki-67 and focal adhesion kinase markers [111]. Conclusively, it can be said that the platform of the silk provides an

artificial cell conducive microenvironment that helps in the migration of keratinocytes, thereby leading to early stage re-epithelialization of wounds.

3.6. Biodegradability of SF *in vivo* and *in vitro*

SF, being made up of polypeptide chain, holds biodegradability in the proteolytic conditions. The degradation rate also depends on the concentration, format, and type of protease treatment [112]. Under *in vivo* conditions, degradation of silk depends on the implantation site and host system. Under the non-proteolytic environment, silk is known to be very slowly biodegradable (taking months or years) [112, 113]. The high stability of SF is due to the (GAGA)n repetitive hydrophobic domains present in the fibroin heavy chain [113, 114]. Subcutaneous implantation of silk-based constructs is often considered as a standard method to examine biodegradability and immune response *in vivo* [101]. Under *in vitro* experiments, significant degradation of silk-based constructs is observed using proteases such as protease K, alpha-chymotrypsin, collagenase, and matrix metalloproteinases (MMPs) [112-115]. The slow degradation behaviour of silk also demonstrates the protective effect of β-sheet regions in the overall stability of the protein, because most of the proteases act outside the β-sheet regions, thereby leaving the bulky hydrophobic core intact and stable [115].

In vivo degradation study of silk constructs provided interesting outcomes, revealing the role of macrophages and immune cells in slowly degrading the silk matrix when implanted subcutaneously [99, 101, 116]. The silk scaffolds remained intact and showed significantly slower degradation rate when implanted in immune-compromised nude rats in comparison to healthy Lewis rats [101]. This indicated that silk is highly stable under *in vivo* conditions and is slowly degraded by the phagocytosis action of macrophages [101]. In another study, the stability of silk fibers was examined by measuring the tensile strength of subcutaneously implanted fiber after 70 days of implantation in an animal model. The results indicated a significant reduction in the tensile strength after 70-days of implantation [117]. Being a slowly degradable biomaterial, the sutures made up of silk threads are also considered as long-term absorbable or permanent sutures [1]. Slower degradation rate might be advantageous to fabricate wound dressing matrices or for temporary grafting applications, which holds long-term stability and provides sufficient mechanical strength. However, such matrices are not suitable for permanent grafting applications and should be precisely modified to match the wound remodelling rate.

Wound dressing, is basically a temporary platform provided on the wounds, to seal the wound cavity momentarily and aid in healing. Depending on the type and size of wounds, dressings are changed after a frequency of some days. Thus, a stable matrix is required for dressing applications, so that it does not disintegrate in the wound bed. However, for permanent graft implantation applications, the rate of degradation should match with the rate of formation of neo-tissue [112]. Since the platform of silk supports cell migration and growth, it has been observed that it integrates well with wound bed during initial time-points; but as the underneath tissue grows with time, the silk matrix dries up and gets automatically removed in the later phase [22, 68]. To develop a silk-based artificial skin, the biodegradability of the material might be a big limitation, and needs to explored in future. One way to achieve a faster degradation rate is to use the material with low content of β-sheet structures, as the degradation rate is inversely proportional to the content of β-sheet structures [114]. Another approach might be the fabrication of constructs with a low concentration of the material or functionalizing it with high proteolytic sites. Such grafts having faster biodegradation rate might be capable of remodelling into skin tissue once implanted permanently.

4. Silk-based matrices and their design considerations

Easy processability of SF to cast into various shapes is an additional advantage of this material that makes it a suitable candidate for fabricating wide varieties of structural constructs. The bulk SF biopolymer is easy to transform into numerous types of constructs like thin films, hydrogels, injectable systems, porous scaffolds, 3D printed grafts and nanofibrous mats (**Figure 6**) [8, 25, 26, 118, 119]. Silk is extensively explored for engineering artificial organs ranging from soft tissues like pancreas to hard tissues like bone [4, 23, 120-123]. The tunable mechanical properties and biodegradability have attributed to the development of a wide range of organs using silk. Researchers have utilized this property to fabricate tissue-mimicking surfaces and structures. With the idea of 'form follows function', a variety of tissue-mimicking constructs have been developed so far. For instance, thin films with a patterned surface, lamellar scaffolds with aligned pores and nanofibrous mats with fibers having a diameter in the nanometer scale [30, 37, 122]. Herein, we discuss various design considerations and composite matrices developed for skin regeneration and wound healing applications.

The fibroin heavy chain of silk is the structural protein complex that consists of long crystallizable hydrophobic domains along with amorphous hydrophilic domains. The packed β-sheet structures of the crystallizable domains in SF play significant roles in manipulating the properties of the protein. Amount of crystallinity can be easily varied in a controlled manner by tuning the β-sheet formation induced by numerous well-established physical and chemical methods. For instance, changes in solution conditions like fibroin concentration, pH, temperature, solution aging, ionic strength or blending with other polymers lead to permanent crosslinking and an overall increase in β-sheet content in the SF [124-127]. Physical methods of β-Sheet induction include mechanical shear (vortexing), sonication, and electric field, which result in enhanced physical permanent crosslinking [127-130]. The most well-established and commonly used method for β-sheet induction in the pre-formed silk constructs includes immersion of constructs in ethanol/methanol bath or vapor annealing using water vapor or ethanol vapor [21, 37, 122]. Crosslinking of silk in the solution form is mostly performed by the horseradish peroxidase (HRP)-H_2O_2 enzymatic method, which is used to fabricate hydrogel-based constructs [131]. Hydrogels are formed by polymeric networks that hold a significant amount of water between their chains. Hydrogels possess an additional advantage over other construct designs, as they maintain hydration environment for a long time [132]. Therefore, hydrogel-based dressings and skin grafts might be considered as an ideal design for cutaneous wounds, especially for burn injuries.

Other highly utilized design formats include silk-based thin films and nanofibrous mats for wound dressing applications. Such matrices act as occlusive or semi-occlusive matrices because silk possesses sufficient water holding properties, and they also provide barrier properties [8, 30]. SF being a biopolymer, is easy to blend with other natural and synthetic polymers. Utilizing this property, several composite materials have been developed so far as listed in (**Table 1)**. Nanofibrous mats could be successfully developed from various silk varieties by using PVA as a blending material (Figure 6-I) [30]. Silk blended with alginate demonstrated significantly improved wound healing as compared to commercially available Nu Gauze™ in a rat model [133]. In a recent study, composite scaffolds made up of SF, and human hair keratin developed as a dermal substitute for skin regeneration demonstrated enhanced secretion of collagen type I (Figure 6-II) [123]. Crosslinked SF-elastin composite scaffolds were also designed to achieve desired elasticity for treating third-degree burn cases [134]. Biomimetic

nanofibrous mats by SF and chitin in 3:1 ratio exhibited better attachment and spreading of human keratinocytes, suggesting their potential in skin tissue engineering [135]. The encouraging results shown in such studies demonstrate great potential of silk-based matrices in wound repair and regeneration.

5. Wound healing properties of silk sericin and applications

Owing to the inherent bioactivity of SS, it has been directly used in ointments and dressing materials for wound healing applications [44, 136]. The dressings are designed so as to deliver the SS at a slow and sustained rate at the wound site [44]. Under *in vitro* cell culture conditions, SS can be used as a growth supplement in serum-free media, as it supports cell growth and differentiation [137]. SS is also considered as an alternative to FBS for the culture of bovine embryo [138]. The study demonstrated the development of blastocysts in the presence of 0.05 % sericin, which was similar to the *in vivo* blastocysts development stage [138]. Sericin is also considered as an albuminoid protein similar to bovine serum albumin (BSA), which is a supplement in commercially available serum-free media [139]. The mitogenic effect of sericin on mammalian cells is well-established in numerous studies, especially on fibroblasts and keratinocytes, which are majorly involved in the wound healing process [44, 140]. Apart from the biological efficacy of SS towards mammalian cells, SS also holds antibacterial activities [141]. SS promotes blebbing of the bacterial cell membrane, thereby inhibiting the bacterial growth and reproduction [141]. In a study, nanofibrous mat containing SS demonstrated zero microbial penetration when used as a cover over test tubes containing nutrient broth [44].

Other bioactive property of sericin includes antioxidant activity that helps in scavenging reactive oxygen species (ROS) [45]. Cell culture media containing sericin protein was found to be beneficial for cellular viability even under hydrogen peroxide (H_2O_2)-driven oxidative stress conditions *in vitro*. The free radical scavenging activity of sericin is attributed to the presence of hydroxyl group of the serine residues [45]. This property of SS provides an additional advantage to the biomaterial, as the ROS generated during chronic inflammation in cutaneous wounds can be scavenged using sericin based wound dressing. Oxidative stress and persistent inflammation are the hallmark of chronic cutaneous ulcers like diabetic foot ulcers [142]. Therefore, materials containing sericin might help in preventing prolonged inflammation of chronic wounds. Being rich in serine residues (with a higher number of hydrophilic groups), SS is responsible for natural

moisturization of skin and keeping the wound bed moist for accelerated healing outcomes [143]. In previous studies, it has been shown that SS helps in fibroblast proliferation and inducing collagen secretion, thereby aiding in wound contraction at a faster rate [48, 144, 145]. Sericin holds ultraviolet (UV)-light protection and anticancer activities as well [146, 147]. In another study, sericin in combination with silver sulfadiazine cream promoted healing of full-thickness burn wounds in animal model without provoking any proinflammatory or allergic response [148]. All these properties indicate SS as a multifunctional bioactive material and demonstrate huge potential in skin regeneration therapeutics.

On studying the effect of sericin on cellular migration and proliferation under *in vitro* conditions, it was found out that it stimulates migration of cells via upregulation of c-Jun and c-Jun phosphorylation [96]. The study revealed activation of MEK, JNK, and PI3K signalling pathways underlying the wound healing properties of silk sericin similar to silk fibroin. The results were also confirmed by inhibiting the above mentioned three kinases that prevented c-Jun upregulation and phosphorylation [96]. In another study, the role of sericin in angiogenesis was investigated through biomolecular pathways. The study demonstrated that sericin has a direct impact on the increased expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor–1α (HIF–1α) and (HIF–2α) [149]. The comparative study showed higher expression of VEGF in the presence of sericin via HIF and MMP-mediated pathways in the comparison to the sample without sericin.

Although, the presence of silk sericin with fibroin in virgin silk sutures showed adverse inflammation in patients; such inflammatory reactions were not observed when sericin was used in its pristine form. Sericin, in its soluble form, was found to be immunologically inert, as it did not induce long-term expression and tumor necrosis factor (TNF)-α secretion when mixed in the media in the culture of RAW 264.7 immune cells [150]. In order to further investigate the immunological response to silk sericin, inflammatory mediators were studied under both *in vitro* and *in vivo* conditions [144]. The study was performed using monocytes and alveolar macrophage cell lines to monitor the levels of interleukin (IL)-1β and TNF-α secreted in the presence of sericin at concentrations of 0.2–1.0 mg/mL [144]. The *in vitro* results indicated dose-dependent secretion of TNF-α and IL-1β from both the cell lines in the culture medium; however, the secreted cytokine levels were not high enough to begin a cascade that leads to

inflammatory effects. Animal studies demonstrated faster healing, and lower levels of inflammatory mediators in the sericin treated full-thickness excision wounds [144]. In another study, sericin based hydrogel developed via photo-crosslinking demonstrated scarless wound healing, as attested by the regeneration of hair follicles and sebaceous glands in the regenerated skin in an animal model [143]. The study revealed better healing efficacy of sericin hydrogel in comparison to the commercially available Pelnac and Tegaderm wound dressings [143].

Furthermore, a combination of sericin and fibroin was utilized to develop a bi-layered wound dressing [151]. Herein, porous sponge of sericin and glutaraldehyde-crosslinked silk fibroin/gelatin was fabricated that acted as a bioactive layer on top of a wax-coated silk fibroin woven fabric. The dressing was designed in such a way that both sericin and fibroin were used as components, but both the materials were in two separate layers. The bi-layered bandage thus developed supported cellular proliferation and adhesion, and promoted faster healing of full-thickness wounds in an animal model in comparison to the clinically used Tegaderm dressing [151]. However, the authors did not study the inflammatory response of the developed dressing. Hence, a detailed study is missing at the molecular level, which focusses the signalling pathways and long-term implantation of sericin-fibroin combination materials. It is worth noticing that the combination of sericin and fibroin has shown immunogenicity in the silk threads that are directly isolated from the silk cocoons. There is no study on the inflammatory response towards the scaffolds made up of combination of sericin with regenerated fibroin protein (post LiBr extraction method) as per the current state of the art. Therefore, more research is needed to explore detailed signalling pathways behind the immune response provoked by combining sericin and fibroin components of silk. Looking deep into the inflammatory reaction in response to the combination of silk materials and their impact on wound healing can be a major topic of research and may unfold numerous unknown properties of both the silk components.

Sericin has been used in combination with other natural biomaterials or synthetic polymers like chitosan, alginate, gelatin, collagen, bacterial cellulose, PVA, poly(L-lactide-co-ε-caprolactone) (PLCL) and many more for wound dressing applications [44, 87, 152-156]. Sericin along with supporting materials as mentioned above are fabricated in various formats like a porous sponge, nanofibrous mats, glue, hydrogels, bilayer sponge, thin films and 3D printed constructs (**Table 1**) [15, 44, 87, 151, 153-155]. In our recent study, we developed composite

nanofibrous matrices by blending SS with PVA, which generated nanofibers in the range of 130-160 nm in diameter (Figure 3-I) [44]. Another widely explored blending material is chitosan, which is used as a composite material along with sericin. In a study, sponge dressing was developed containing sericin and chitosan glutamate loaded with platelet lysate for enhanced fibroblast proliferation [157]. The dressings demonstrated a beneficial effect of platelet lysate loaded composite dressings for dermal matrix reconstruction under *in vivo* conditions. In another recent study, medical tissue glue was developed containing gelatin, sericin, and carboxymethyl chitosan blend solutions to obtain biological properties of all three biomaterials in the combination [158]. The study showed high bond strength of 2.50 ± 0.04 N within 10 minutes post-application of the glue, which was comparable to the alpha-cyanoacrylate biological glue (2.25 ± 0.05 N) currently being used in clinical practices. The sericin based tissue glue thus developed was biocompatible, non-toxic, cost-effective and suitable for wound healing applications [158].

Since the beginning of sericin utilization in the tissue engineering field, numerous types of sericin based wound dressings have been developed for the treatment of diabetic wounds and burn injuries. Sericin-based porous freeze-dried scaffolds demonstrated successful generation of bilayer skin tissue by co-culture of fibroblasts and keratinocytes (Figure 3-II) [46]. In a recent study, sericin was used in 3D printed dermal substitute in combination with GelMA [47]. The printed constructs were transparent and supported coculture of HaCaT and HSF cells to generate dermal and epidermal layers in the developed artificial skin, suitable for wound healing applications (Figure 3-III,IV) [47]. Sericin is also used in the dissolved form, where it is mixed with the ointment and applied on wounds [136]. In the study, wounds created in streptozotocin-induced diabetic rats when treated with an ointment containing sericin demonstrated enhanced wound healing rate [136]. Sericin was also used to develop an *in situ* forming hydrogel owing to the presence of a high number of hydrophilic residues in it, which could readily form a semi-interpenetrating hydrophilic network with polyacrylamide to act as a dermal sealant [159]. To further functionalize the sericin, Wang et al. applied genetic engineering to develop transgenic silk cocoons that contain sericin protein functionalized with FGF1 [160]. The injectable hydrogels fabricated using FGF1-functionalized sericin demonstrated long shelf-life of FGF-1 and enhanced cellular activities in terms of quick adhesion and viability [160]. This strategy led

to a cost-effective production of FGF containing biomaterial at a large scale for regenerative therapeutics.

[TABLE 1: List of silk-based composite matrices and design of the constructs].

6. Human skin equivalent and bio-artificial skin using cellularized silk biomaterials

With the growing global impetus on harnessing the regenerative properties of silk biomaterial, a few studies have shown the development of artificial cellular skin using stem cells and co-culturing various cells under *in vitro* conditions. Apart from the development of wound dressing, fabrication of viable skin grafts should be considered as a separate genre in the silk-based technology. Therefore, the review demands a separate discussion on the silk-based bioengineered living skin equivalents developed so far. Taking advantage of the biocompatible properties, silk matrices could be easily cultured with single or multiple cell types. This allowed co-culture of skin cells in silk derived constructs under suitable *in vitro* conditions to develop laboratory grown artificial graft. The most common practice of developing an artificial skin include fabrication of a bilayer structure using dermal fibroblasts and epidermal keratinocytes. Skin, being a layered structure, is often constructed artificially in the form of a bilayer or trilayer design [27, 187]. The bilayer structure is build-up of the dermal bottom layer (containing fibroblasts) and the epidermal top layer (containing keratinocytes) [188]. In the trilayer construct, an additional bottom-most hypodermal layer is fabricated, which contains adipocytes. Till date, only a few studies are reported on the trilayer skin construction concept using silk biomaterial.

As far as the fabrication of bilayer skin construct is concerned, the most common approach is applied with porous matrices, where fibroblasts populate the silk scaffold, on top of which keratinocytes are seeded and allowed to mature [27, 187, 189]. In order to generate a stratified epidermal layer, the co-cultured silk matrices are lifted to provide an air-liquid interface (ALI) and hence, tissue maturation takes place with time. This well-established practice of generating bilayer skin grafts is successfully demonstrated using few construct designs like woven/non-woven fibers, porous freeze-dried scaffold, nanofibrous matrix, and 3D printed hydrogels [18, 26, 47, 118, 190]. Co-culture of skin cells in silk matrix began more than a decade ago by Dal Pra et al., where the non-woven fibrous matrix was developed directly from the degummed silk fibers via formic acid crosslinking [191]. The fibrous 3D matrix supported long-term co-culture of human dermal fibroblasts (HDFs) and human epidermal keratinocytes (HEKs)

up to 75-97 days under *in vitro* conditions, thereby forming a dermo-epidermal bilayer equivalent. The study also showed *de novo* production of collagen fibers in the co-cultured constructs, thus indicating dermal tissue formation *in vitro* [191]. Similarly, Wendt et al. fabricated a woven matrix of cross-weaved native spider silk fibres on steel frames to co-culture fibroblasts and keratinocytes [18]. Herein, the metal frame offered an additional advantage by not only giving mechanical support but also provided an easy way for lifting the cultured matrix in the ALI conditions (Figure 4-II). The study demonstrated the generation of a bilayered skin model, comprising of dermal and epidermal equivalents in 5 weeks culture period [18].

Although the woven or non-woven fibrous architecture support the fabrication of bilayer skin equivalent, manual construction of fibrous matrices can be a cumbersome process. Smart technological advancements like electrospinning hold potential to venture large-scale production of a nanofibrous matrix. However, the electrospun nanofibrous matrix faces a significant drawback of porosity, which limits the migration of cells within the construct. To overcome this, Park et al. developed a novel 3D porous scaffold containing electrospun silk nanofibers by adding NaCl crystals [190]. Highly porous architecture could be easily obtained by adding NaCl crystals into silk nanofibers during electrospinning and subsequent salt leaching. The nanofibrous scaffolds thus developed contained large pores and supported fibroblast-keratinocyte co-culture under ALI to generate skin equivalent tissues [190]. Although the initial work on generating bilayer skin tissue using silk began with woven/non-woven fibrous matrices, the fibrous architecture was not explored much for skin tissue engineering applications in recent times. This might be attributed to the fact that a porous sponge or hydrogels provide better interconnectivity in pores in comparison to a fibrous matrix [192].

More commendable work in this area includes the utilization of porous freeze-dried silk scaffolds as a 3D matrix for developing tissue model. A highly porous sponge with suitable pore size is able to guide cells for migration and proliferation, especially when the target is to develop a vascularized dermal layer. In our recent study, we used freeze-dried porous silk scaffolds to simulate the dermal bed by co-culturing skin-derived HDFn and HDMEC [26]. The porous silk scaffolds used herein were coated with FN-4RepCT recombinant protein (containing fibronectin motifs) for enhanced attachment and proliferation of cells (Figure 4-I). Further, to generate bilayer skin construct, HaCaT cells were cultured on top of the simulated dermal layer, and ALI

was provided. The study demonstrated a facile fabrication strategy of living skin equivalent, expressing mature epidermal markers like keratin 5, keratin 10, and Involucrin under just 21 days of *in vitro* culture conditions [26]. Similar results could be observed in another study, where composite scaffolds made up of silk fibroin, and fibrin sealant provided a suitable environment for the co-culture of fibroblast and endothelial cells [193]. The addition of fibrin biomaterial to the silk scaffold provided a suitable microenvironment for the generation of capillary-like structures in the scaffold. However, the study did not focus on epidermal layer development.

In another study, development of bilayer skin construct using porous scaffold was performed using silk sericin biomaterial. Herein, sericin hope was extracted from the cocoons of *B. mori* mutant silkworms to obtain a high yield of sericin material, which was subsequently designed into a porous freeze-dried scaffold using genipin crosslinker [46]. The 3D scaffold co-cultured with fibroblasts and keratinocytes maintained prolonged cell viability and demonstrated stratified epidermal layer, indicating successful development of a bilayer living skin construct. The study also provided evidences that silk sericin can be a potential biomaterial for fabricating artificial skin grafts under *in vitro* conditions. The potential of sericin in skin tissue engineering was further observed in a recent study, where 3D bioprinted skin construct was fabricated [47]. The bio-ink consisted of sericin and methacrylic-anhydride-modified gelatin (GelMA) transparent hydrogel that sustained fibroblast-keratinocyte co-culture for bilayer skin development.

Another breakthrough study in the field of skin tissue engineering is recently carried out by Vidal et al., which demonstrated the fabrication of immunocompetent full-thickness human skin equivalents containing nervous system (**Figure 7**) [131]. Till date, the study is first of its kind that has successfully developed a complex skin tissue with co-culture of multiple cell types under laboratory conditions. Herein, a silk-collagen composite hydrogel system was fabricated, which provided long-term stable cultivation of cells with minimal gel contraction. The study targeted to overcome the major drawback of gel contraction in the pristine collagen hydrogel system. By blending mechanically strong silk bulk biopolymer with collagen hydrogel, the unique composite hydrogel not only prevented matrix contraction but also maintained cell-binding domains of collagen. The blend hydrogel was matured to form a neuro-immuno-cutaneous system (NIC) by co-culturing primary cells, neuronal cells, and immune cells [131].

The study was one-step ahead of the bilayer system, as the composite system showed a third layer of the hypodermis.

A three-layer artificial skin tissue better mimics the actual skin architecture; however, generating the hypodermis along with dermal and epidermal layers is very challenging and hence there are only a few reports on the generation of trilayer skin system *in vitro*. Previously, authors from the same group led by Prof. David Kaplan demonstrated the fabrication of trilayer skin construct by developing a bilayer (dermo-epidermal) of pristine collagen gel and subsequently placing it on top of a previously formed hypodermal layer made up of pristine silk scaffold [194]. The study demonstrated the generation of trilayer full thickness skin equivalent within 2 weeks; however, long-term stability of the graft was not studied. In comparison to the previously developed trilayer skin system containing different layers made up of different biomaterials [194], the NIC composite silk-collagen hydrogel proved to be a better system for long-term culture [131]. The authors also demonstrated a follow-up study one year later to study relationships between the skin and immune/nervous systems by RNA sequencing [25]. Comparison among various sample groups was performed through the established model of NIC to discern the effect of neural and immune components in the skin system. The detailed analysis proved that the cutaneous system containing both neural and immune cells resulted in upregulation of specific genes involved in key biological pathways, which could be further studied to understand various cell signaling pathways [25].

With the emerging 3D printing technology, silk-gelatin bio-ink is recently used to bioprint a bilayer living 3D printed skin construct [118]. The 3D printed silk-based graft mimicked the bilayer structures of skin tissue anatomically, showing mechanical and biochemical features resembling the natural skin (Figure 6-III). Migration of keratinocytes within the porous construct aided in epithelialization of the bilayer skin substitute with a cornified epidermal layer. The printed construct demonstrated epidermal-dermal junction upon tissue maturation, showing basement membrane structures at the interface. The detailed study also involved extensive transcriptomics and proteomics analysis using the 3D bioprinted skin construct, which depicted the involvement of pathways related to dermal development, keratinization, and organization of ECM components such as collagen fibril synthesis [118].

Therefore, the generation of a complex cutaneous system *in vitro* helped in understanding the necessary factors involved in skin development. Such a functional 3D model of full-thickness skin might be instrumental in studying wound healing pathways, and testing of drugs or cosmetic products on a large scale to avoid animal experiments or animal testing respectively. Another significant achievement using this technology may lead to the development of *in vitro* disease models in the near future; for instance, 3D melanoma model can be established to study cancer pathways and drug testing under laboratory conditions.

7. Bioactive factors loaded functionalized silk matrices for drug delivery to the wound bed

Silk has been used as sutures, tissue scaffolds, haemostatic, and drug delivery agents since decades [1, 110, 195]. Both physical and biological properties of silk material are easily manipulated simply by structural re-adjustments. Apart from the biocompatible properties of silk as a biomaterial, it holds extraordinary properties for drug delivery applications. The bulk material of SF can be utilized as a cargo, which delivers drugs, biomolecules, and growth factors at a slow and sustained rate [32]. This property of silk has been extensively exploited for wound healing applications as illustrated in the composite image showing functionalization of silk with EGF, antibiotic or DNA molecules (**Figure 8**). Various bioactive molecules that have been used to functionalize silk matrices are listed below that demonstrates the application of silk in drug delivery application for wound repair and regeneration (**Table 2**). In addition to the drug delivery applications, silk also helps in stabilization of biomolecules and preserve the bioactivity of drugs/molecules for more prolonged time [196]. For example, antibiotics like penicillin and tetracycline incorporated in silk films demonstrated higher stability in comparison to their storage in solution and powder form [197]. Silk films are largely explored for wound healing applications. Therefore, functionalization of silk films with antibiotic incorporation provides an additional advantage to cure infected wounds and prevent wound infection [197]. In another study, silk microneedle arrays containing tetracycline demonstrated sustained release and preserved bioactivity of the antibiotic against *Staphylococcus aureus*, which is the most commonly found bacteria in the infected wounds [198].

Drug entrapment and stabilization within silk biomaterial depend on various factors like hydrophobic or hydrophilic interactions, electrostatic interactions or hydrogen bonding. The aqueous solution of silk is generated by dissolving silk fibers in LiBr solution [5]. This process

breaks the highly stable hydrogen bonds in the β-sheet structures of fibroin fibers and the aqueous silk solution is obtained. While fabricating constructs using the silk solution, the hydrogen bonds are re-formed and the stable β-sheet structures are formed to render stability to the structure of silk construct [199, 200]. For functionalization, aqueous soluble drugs or biomolecules are directly added in the silk solution, and thereafter, the solution is processed into a particular design. For example, during the fabrication of a thin film, the hydrogen bonds are reformed in the drug loaded silk upon air-drying, causing entrapment of drugs or biomolecules in the film [201]. Hydrogen bonding with the biomolecules might be one factor behind their stability in silk constructs. As the drug-loaded silk constructs remain in wet condition, the drug gets slowly released from the cargo. In a study, drug release mechanism from silk films was examined by loading the films with different molecular weight FITC–dextrans ranging from 4 to 40 kDa [202]. The study revealed that the diffusion mechanism was the primary factor for sustained release rate, as also determined by the Peppas equation.

Antibiotic drug incorporation in silk can be done permanently by conjugation as well as temporarily for sustained release. In a study, functionalized spider silk fibers were generated that were conjugated with Levofloxacin antibiotic through click chemistry (Figure 8-II) [203]. In a detailed study, antibiotic release assays were performed from different material formats like films, microspheres, lyophilized porous sponge, hydrogels and injectable formulations fabricated from regenerated silk solution and also from degummed silk fibers isolated directly from cocoon [205]. The study revealed efficient drug loading and sustained release properties in all the design formats. The study determined the efficacy of ampicillin-releasing hydrogels under *in vivo* conditions in a murine model of wounds infected with *S. aureus* [205]. The lyophilized sponges loaded with gentamicin and cefazolin demonstrated sustained drug release for 3-5 days. Drug release saturation was achieved within 5 days from the silk matrix containing hydrophilic antibiotics like gentamicin [205]. In contrast, hydrophobic drugs like rifampicin and erythromycin showed longer release durations (9-31 days), which might be attributed to the hydrophobic interactions between the drug and the hydrophobic cores of silk [205]. Penicillin, ampicillin, cefazolin, and gentamicin loaded in various formats like films, hydrogel or microgel also demonstrated short release durations with preserved antibiotic efficacy. The

release behaviour of hydrophilic drugs might be attributed to the diffusion mechanism as also determined in the FITC-dextran loaded silk matrix [202].

The release mechanism of hydrophobic drugs was found to be non-diffusion-based, and hence, drug loading procedure was also different in comparison to that of hydrophilic drugs [205]. Loading of hydrophobic drugs into the silk constructs was performed by taking the help of methanol solution unlike hydrophilic drugs that were simply mixed in aqueous silk solution [205]. For encapsulating hydrophobic drugs, pre-formed silk constructs were dipped in the methanol solution saturated with the hydrophobic drug; thus, drug loading was done by adsorption or soaking mechanism [205]. The drug compound was entrapped in the silk polymer network during methanol soaking, which showed slow and constant release upon immersing in aqueous solution due to drug hydrophobicity. For example, porous silk sponges loaded with rifampicin via methanol soaking demonstrated longer release duration up to 9 days [205]. Therefore, drug loading strategies with silk material might be different depending on the nature and properties of the drug. The study not only validated the drug release efficiency of various silk constructs but also proved the capability of silk material to sequester and stabilize bioactive antibiotics in an encapsulation form.

Similarly, loading of growth factors is performed by various strategies for fabricating a biofunctionalized wound healing material (Figure 8-I) [109, 110]. In the case of epidermal growth factor (EGF), both adsorption and solution mixing strategies were analysed with silk films and nanofibrous mats [110]. There was no significant difference between EGF loaded film and EGF coated film. The method of pre-mixing the growth factor with aqueous silk solution prior to the fabrication of silk dressings was also utilized to deliver EGF and fibroblast growth factor (FGF) from silk-based nanofibrous mats [30]. Further, post-treatment of silk matrices that lead to β-sheet induction differs depending on the functionalization type and material format. For example, the drug/biomolecule loaded silk constructs were treated with vapours of ethanol/methanol or water vapour, instead of directly immersing the constructs in ethanol or methanol solutions [21, 206]. This procedure was applied to minimize the initial burst release, thereby preventing the drug loss in methanol solution.

To achieve precise control of drug release rate, modulation of the silk fibroin crystallinity using different processing conditions (e.g., ethanol/methanol, water vapour annealing, salt

treatment, silk concentration or design format) can be critical parameters. In another report, sulfonated SF solution was used by diazonium coupling chemistry to improve the binding of FGF-2 to fabricate functionalized silk films [207]. Thin films made up of silk decorated with sulfonic acid (70 groups per SF molecule) demonstrated 2-fold increment of FGF binding in comparison to non-sulfonated silk [207]. This mimicked the natural binding of FGF with glycosaminoglycan heparan sulfate, an ECM component present in skin, which is considered as a physiological storage site of FGF. Similarly, sulfonated silk was used for fabricating 3D bioprinted dressing patch containing FGF [186]. The sulfonated silk containing FGF aided in accelerated wound healing, as confirmed by the *in vivo* experiments in murine model [186]. Growth factor loading and release rate depend on various factors like charge and hydrophobic/hydrophilic moieties. SF, with a pI of 4.2, is mostly negatively charged. By adjusting the charge of SF, growth factor loading efficiency can thus be modulated.

Apart from ionic or electrostatic interactions, the presence of hydrophobic blocks in the silk might be an additional factor for sequestering growth factor and variation in their release rate. The hydrophobic cores vary among various silk varieties, which might interact differently with the amino acids of additive molecules (for example growth factor peptides). In our recent study, we observed that the release rate of EGF and FGF from various silk based nanofibrous matrices were different, thereby supporting the speculation [30]. The study revealed variation in the growth factor release profile between mulberry and non-mulberry SF matrices owing to the differences in their overall crystallinity and variation in the hydrophobic cores [30]. Further, differences in the EGF release and FGF release rates from the same type of silk matrix was attributed to the variation in the isoelectric point of EGF (pI 4.78) and FGF (pI 9.58).

Loading of growth factors via silk not only offers a platform of sustained release but also increases the stability of the bioactivity of loaded growth factors [196]. Local or topical administration of growth factors may lead to their short half-life and early degradation in the proteolytic wound environment [208, 209]. Combining the bioactive molecules with silk biopolymer helps in sequestration of the bioactive molecules within the bulky silk biomaterial and thereby keep them stable for a prolonged duration. The drug delivering potential of silk biopolymer indicates its full applications in delivering various active biomolecules for pharmaceutical therapeutics applications [32]. A study also proved that cultured cells provided

essential cell conducive cues to the silk matrix that benefitted the construct with secreted cytokines and bioactive molecules. Herein, human adipose-derived stem cells (ASCs) were cultured on silk nanofibrous mats for 7 days, which were subsequently decellularized to prime the silk patch with secretions of cultured ASCs [210]. The study showed that the primed silk mats (decellularized) promoted wound healing and demonstrated complete wound contraction within 10 days, compared with 15–17 days in the control group (non-primed acellular silk mats). The study indicated that silk constructs could be a housing material to grow allogenic cells, which can be later decellularized before grafting [210]. This strategy could indeed overcome the troublesome procedure of culturing autologous cells by using allogeneic cells and subsequent decellularization of the construct.

In the context of drug delivery, it is worth mentioning that silk is also explored for gene delivery applications. Silk scaffolds conjugated with cationic complexes of poly(ethylenimine) (PEI) and pDNA efficiently acted as gene delivery carriers [211]. The cell binding RGD motif inherently present in *A. pernyi* SF was found to be helpful in specific targeting to cells. pDNA encoding VEGF165 and Ang-1 packed in ApSF carriers could successfully transfect mammalian cells [211]. Cationized BmSF scaffolds were also successfully fabriced containing PEI/pDNA complexes for gene delivery of VEGF165–Ang-1 and wound healing applications (Figure 8-III) [204]. Another emerging therapeutic approach of treating highly infected wounds is phage therapy. Herein, bacteriophages are used as antibacterial agents for controlling some specific pathogenic bacteria. As a proof of concept, lyophilized silk matrices containing live viral vaccines of Measles, Mumps, and Rubella demonstrated improved viral activity retention owing to the vaccine–silk interactions [197, 212]. Silk with its ability to incorporate viruses might be explored in the future to develop phage-containing silk dressings for treating wound infections.

[TABLE 2: List of silk-based functionalized wound dressings loaded with bioactive molecules/antibiotics/antimicrobial peptides/growth factors].

8. An update on the clinical studies and silk-based translational products from bench to bedside

Clinical implementation of silk-based grafts or wound dressings is necessary to validate the proof of concept, which might be helpful in delivering such products in the healthcare market. In this context, a lot of silk-based technologies are patented that describe the inventions for wound

repair and regeneration. Some of the recent patents on silk-based materials are listed below that indicate huge potential of these products in the market in the near future (**Table 3**). Most of the patents are also owned by healthcare companies like Allergan Inc., which clearly demonstrate the ongoing translational route of silk-based products from bench to bedside. As listed in the table, the patented technologies include silk wound dressing patch, dermal fillers, tissue sealants, haemostats, antibiotic-loaded silk patch and scaffolds for tissue engineering applications. Apart from this, there are few reports on the clinical trials and pre-clinical studies using silk-based matrices for wound healing applications (**Figure 9 and Figure 10**). The FDA approved SERI scaffolds made up of *B. mori* silk threads are clinically used in surgical applications [14, 246]. The SERI surgical scaffold contains a mesh network of silk threads isolated directly from the silk cocoons [246]. The proprietary rights of the product were held by Serica Technologies Inc. (Medford, MA, USA), which made efforts to obtain 510(k) clearance from the FDA to commercialize the SERI Surgical Scaffold. The SERI surgical scaffold was accepted because it cleared the International organization for standardization (ISO) biocompatibility testing, proving the scaffold a non-toxic, non-pyrogenic, non-allergic and overall a biocompatible grafting material [14, 246]. The product is now owned by Sofregen Medical Inc., Medford and can be purchased by surgeons in the USA for surgical applications.

SERI scaffolds are examined in various surgical applications like abdominal wall reconstruction, body contouring, breast reconstruction, massive weight loss surgeries, and as an adjunct to lower body lift [247-251]. The multicentre retrospective clinical trial of SERI scaffold in the form of a supportive artificial tissue for abdominal wall reconstruction was performed on 172 patients. The study revealed success rates of SERI scaffold in the cases of abdominal wall fascial repair, abdominoplasty, reinforcement of abdominal flap donor site and ventral hernia repair after 18 months of the surgery with low or minimal postoperative complication rates [251]. In the clinical study of 2-stage breast reconstruction over 161 number of breasts, SERI scaffolds proved to be a good implantable material as observed by the patient satisfaction and 98.8 % success rate after 2 years. The study also demonstrated long term tissue stability analysed by breast anatomy measurements, thereby proving long lasting benefit and safety of the SERI scaffold in breast reconstruction surgeries [249]. In a case report of weight loss surgery, SERI scaffold was implanted as a supportive matrix to the abdominal fascia in order to prevent the loose and poor appearance of skin at the abdomen region [250]. The body contouring surgery

demonstrated that the silk scaffold was able to maintain flat shape of trunk and integrity of the abdominal fascia along with signs of circumferential body lift and no recurrent laxity after 24-months follow up [250]. All these clinical reports suggest long-term efficacy of SERI scaffold due to the bioresorbable nature of silk biomaterial. The body contouring and breast reconstruction surgeries also indicate that the silk scaffold integrates well with the native tissue. However, the clinical studies done so far lack a comparative study with control groups such as well-established silicone-based implants in case of breast reconstruction surgeries. By comparing the silk group with other commercially available implants could provide a better understanding on the regenerative properties of silk biomaterial. One advantage of silk scaffold over synthetic materials is that there is no need of secondary surgery to retrieve the implant due to the bioresorbable nature of silk and invasion of host cells in the scaffold. Synthetic implants such as silicone-gel filled breast implants remain as a separate entity and do not integrate with the host tissue. Leakage of silicone gel upon silent rupture of the implant is a common drawback of silicone-based breast implants because it requires additional revision surgeries and frequent follow up visits for rupture detection [252]. Further long-term study of silk-based scaffolds in comparison to the commercially available implants is definitely required to examine their efficacy, advantages and limitations.

The wound healing efficacy of SERI scaffolds examined in mice model using dorsal skinfold chamber (DSC) revealed early granulation tissue formation and neovascularization in the scaffolds on day 5 post-implantation [14]. Blood vessels were also observed to be penetrating the scaffold, thereby making a densely vascularized regenerated tissue at the wound site. The experiment not only demonstrated successful integration of SERI scaffold with the host tissue but also showed possible graft take potential of the silk scaffolds (Figure 9-I). Regressed inflammatory response in the later phase of wound healing observed by the decrement in neutrophils levels indicated minimal immunogenicity and long-term safety of the scaffolds. Both early and late stages of inflammation showed immunocompatible properties of the scaffold. Histological study of the implanted scaffold further revealed deposition of collagen fibers within the threads of SERI scaffolds on day 10 post-implantation [14]. The study thus demonstrated tissue bioresorbable and regenerative properties of the SERI scaffold that could be implanted

permanently at the wound site. The study also indicated an enormous potential of the resorbable SERI scaffolds for reconstructive surgery and skin regeneration applications [14]. In a long-term study, biodegradation mechanism of the scaffold was found to be macrophage-associated, with a granulomatous inflammatory response within the yarns, as evidenced in an intramuscular rat model [253]. Non-immunogenic response by the scaffold was demonstrated by measuring the average plasma IgE concentration, which was constant at baseline levels for 6 months post-implantation in rats. Similar tests of the scaffold performed in a large animal model (goat knee) demonstrated no signs of acute inflammation or adverse immunogenic response when implanted for 12 months [253]. The tests thus indicated slow biodegradation, negligible immune response, and absence of hypersensitive reaction.

Although the SERI scaffold proved to be a decent choice of matrix for cutaneous wound healing in animal studies, there are no reports of clinical trials on patients with skin wounds. Clinical trials on cutaneous wounds would be an interesting study to further examine its healing efficacy in comparison with commercially available wound dressings. Application of SERI scaffolds on split-thickness wounds could reveal how the matrix interacts with the host tissue when applied on external organ of the body. Although bioresorbable nature and tissue integration was observed when the scaffold was implanted in internal organs like breast and abdomen, host tissue response might differ at the dermal region. The animal study on SERI scaffold performed on DSC mice model used a metallic frame to prevent graft contraction, which helped in the integration of silk scaffold with the host tissue [14]. However, preventing graft contraction in case of human skin wounds is a challenging task. Cutaneous wounds tend to contract while healing and silk scaffolds might fail to permanently integrate with the skin tissue due to high mechanical strength and slow biodegradation rate of silk. In our recent work, we observed that the silk hydrogels and scaffolds integrated well with the native skin tissue at initial time-points (day 3 and day 7) in murine model; however, they could not remain permanently integrated at later time-points (after day 14) because a supportive metallic frame was not used in the study (Figure 9-II,III) [22, 68]. Human trials on silk-based grafts could further reveal interesting outcomes on how these matrices could be applied permanently in case of large full-thickness trauma wounds.

In this context, it is necessary to mention that silk-based matrices when used as wound dressings and applied temporarily on the wounds (with a timely dressing change schedule) have shown great potential in animal studies. The animal trials conducted with silk wound dressings have demonstrated better healing outcomes in comparison to commercially available products such as Tegaderm 3M tape, Tegaderm hydrocolloid 3M dressing and Duoderm dressing patch made up of synthetic materials [67, 68, 110]. Better outcomes observed by silk dressings in comparison to synthetic materials are attributed to the cell-interactive properties of silk biomaterials that stimulate cell migration and proliferation. Commercially available dressings made up of synthetic polymers mostly provide barrier properties to the wounds but they lack cell recognition sites. Silk, on the other hand, guide cellular ingrowth and tissue regeneration at the wounded region due to its inherent biocompatibility and bioresorbability (also discussed in details in section 3). However, clinical study on patients is necessary to confirm better healing efficacy of silk wound dressings in comparison to well-known synthetic wound dressings available in the market. In addition, wound microenvironment differs among various types of cutaneous wounds such as trauma wounds, diabetic ulcers, pressure sores, burn injuries and severely infected wounds. Clinical studies on different wound types in human cutaneous system are still missing in the current state of the art. This suggests that performing wound specific treatments using silk-based matrices under clinical trials are current difficulties in the application and translation of silk products.

Another SF based product, particularly for wound dressing applications, is Sidaiyi silk sponge that is attached to a silicone membrane. It is a two-layered scaffold dressing approved by the China Food and Drug Administration [8]. In a recent clinical study using thin silk film on donor site skin wounds, the silk films demonstrated better healing outcomes in comparison to Sidaiyi silk sponge (**Figure 10)** [8]. The comparative study between silk film and Sidaiyi sponge was performed on 36 and 35 patients respectively, which showed 9.86 ± 1.79 days as the average healing time by silk films in comparison to 11.35 ± 3.03 days by Sidaiyi sponges [8]. The study also revealed that none of the patients suffered from severe conditions when treated with silk films, indicating 100 % healing by 14 days. However, adverse events were observed in 4 out of 35 patients when treated with Sidaiyi, indicating 88.6 % healing by 19 days as calculated by continuity correction statistical method. Although both the matrices are made up of BmSF, different format and construct design might be a plausible reason for discrepancies in the clinical

results. For instance, the exudate handling capacity of silk film and Sidaiyi sponge was different. The wounds treated with silk film did not show accumulation of exudates; however, Sidaiyi scaffolds accumulated exudates at the wound-site and demonstrated empyema in 3 patients and excessive fluid leakage in 1 patient. Apart from the biological activity of SF helping in the wound healing process, better fluid handling capacity and gaseous permeability by silk films provided additional advantage as seen in this study [8]. Such clinical studies indicate an immense potential of silk-based matrices in wound healing applications and skin regeneration therapeutics. However, more clinical trials on other types of wounds and comparative studies with well-established commercial products may confirm healing efficacies of silk products for better clinical outcomes.

Silk patches in the form of thin silk films are also used in clinical studies for the treatment of tympanic membrane perforations [254]. A silk patch made up of regenerated *B. mori* silk fibroin is approved in the South Korea by the name Tympasil (Daewoong-Bio, Seoul, South Korea). According to the clinical trial, 40 patients suffering from chronic tympanic membrane perforation demonstrated better performance of Tympasil in comparison to the conventional perichondrium myringoplasty [254]. Another silk-based commercial product is provided by an Indian company 'Fibroheal^{TM}' that delivers 'bilaminated wound healing sheet' for surgical wound cover applications [255]. The Fibroheal silk dressings are made up of thin sheets of woven silk fibers having a coating of BmSF solution to better interact with the wounded tissue [255]. Application of silk is well-known in textile industries as a clothing material. Special silk clothing with the product name MICROAIR DermaSilk® was examined in a clinical study for the treatment of atopic dermatitis in 31 young children (mean age 2 years) [256]. Kids suffering from atopic dermatitis with acute lesions showed better outcomes with significant decrease in the severity of lesions after 7 days. The study conducted in comparison with cotton clothes indicated that silk material could be a better clothing option in such conditions, especially for children [256].

Apart from fibroin-based matrices, sericin has also attracted cosmetic and pharmaceutical attention. In a recent patent, the sericin-based invention describes the potential use of sericin in personal care or cosmetic formulations (Patent no. WO 2019/101524 A1, patented by Unilever

international company, 2019). The sericin-based compositions provide benefits of moisturization, anti-inflammation, anti-pollution, anti-oxidant, and anti-aging. Such cosmetic products patented by international companies indicate their expected translational in the market in the near future. In addition, the beneficial effects of sericin in wound healing applications have attracted many researchers to conduct detailed pre-clinical and clinical studies to further explore the benefits of this natural protein. In a study, porous scaffolds made up of sericin/PVA blend demonstrated a significantly higher rate of wound healing and re-epithelialization, in comparison to the PVA scaffolds without sericin biomaterial [165]. This inspired the researchers to further validate the wound healing efficacy of the developed scaffolds in patients [257]. The clinical trials on the treatment of split-thickness skin graft donor sites were performed in comparison to the clinically available "Bactigras®" wound dressing [257]. The results demonstrated healing properties of sericin based dressings, as the wounds were completely healed in 12 ± 5.0 days in comparison to those treated with commercially available Bactigras® (14 ± 5.2 days) in the patients [257]. In another clinical trial study, patients with burn wounds were treated with silver zinc sulfadiazine cream added with sericin in comparison with the control cream without additional sericin protein [258]. The randomized and double-blind clinical trials demonstrated that the burn wounds treated with sericin loaded cream were completely healed in 22.42 ± 6.33 days, in comparison to the control group that took approximately 29.28 ± 9.27 days. The study also showed that the incorporation of sericin was safe and beneficial because there were no signs of post-treatment allergies or severe inflammation in the patients [258].

[TABLE 3: List of the published patents that highlight the application of silk-based materials and uses thereof. Source: Data retrieved from Google patents (accessed 23.06.19)].

9. Conclusion and future perspectives

The complicated process of wound healing involves various events and interactions between different cells and ECM components. Cell-matrix interaction is one of the most important parameters that regulate the wound healing process. In this context, interactions between silk biomaterial and cells play a significant role in healing the cutaneous wounds. As discussed above, cell-silk interactions promote wound healing and aid various cellular events associated with faster wound repair and regeneration. From the clinical perspective, silk-based matrices are suitable as a dressing material as well as to develop a dermo-epidermal bio-artificial skin graft.

The results indicate that silk-based matrices hold great potential in plastic surgeries in the near future. Furthermore, easy and facile techniques to functionalize the silk material with various bioactive molecules and antibiotic drugs depict the prospect of multi-functional wound dressings for wound specific treatments. Different fabrication strategies and design formats are also of great interest from the wound management perspective. A particular wound type demands specialized dressing material, which is possible using silk biomaterial. Application of silk-based products in the healthcare section clearly depicts the progress of silk biomaterial from bench to bedside. However, more detailed clinical studies and pre-clinical studies are needed to validate the wound healing efficacy of silk-based dressing materials and implants.

Recently, smart dressings like flexible electronic skin (e-skin) are developed that can detect temperature and pressure through the assembled temperature- and strain-sensors using silk-based matrix [289]. Such research efforts demonstrate the potential of next-generation smart dressings and wearables that might have human-machine interfaces for human-health monitoring. Transdermal drug delivery using silk-based microneedles is another example of the developing technology using silk biomaterials [290]. With the scope of on-demand drug delivery approaches, smart dressings containing sensors and drug cargos can be easily developed using this bulk material [119]. Besides, utilization of the aqueous state of silk biopolymer through green extraction methods allow easy processing techniques in mild condition without the application of harsh organic solvents. Such facile methodologies have also made it possible to fabricate functionalization of silk-based materials with sensitive bioactive molecules.

Finally, the emerging technological advancements like 3D bioprinting may utilize silk-based bioinks for precisely controlled architecture, reproducibility, and large-scale production. Precise positioning of biomacromolecules and cells using silk-based 3D bioprinted construct may deliver functional and viable full-thickness artificial skin grafts. In addition, silk being an inexpensive natural material may bridge the gap between the need and the high demand by lowering down the cost of healthcare products. Improvement in the bioprocess engineering has already established large scale production of recombinantly produced biomaterials like spider silk, SELPs and their fusion proteins containing bioactive domains. The advanced genetic engineering techniques have also delivered transgenic silkworms that produce silk cocoons containing growth factor peptides [241]. All such innovations using recombinant DNA

technology may be fruitful in developing next-generation smart materials in the near future. Overall, we support the idea that technological advancements in basic science and engineering in combination with material science research hold great potential in the future. Further pre-clinical and clinical studies on the developed silk materials may open gates for efficient treatments and commercialize the silk-based products.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations:

SF, silk fibroin; SS, silk sericin; FDA, food and drug administration; BmSF, *B. mori* silk fibroin; NMSF, Non-mulberry silk fibroin; LiBr, lithium bromide; Na_2CO_3, sodium carbonate; AaSF, *A. assama* silk fibroin; 3D, 3 dimensional; FN, fibronectin; RGD, Arg-Gly-Asp; FBS, fetal bovine serum; BSA, bovine serum albumin; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; UV, ultraviolet; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; HIF, hypoxia inducible factor; MMPs, matrix metalloproteinases; PVA, poly(vinyl alcohol); PCL, poly(caprolactone); PEI, poly(ethylenimine); PLCL, poly(L-lactide-co-ε-caprolactone); PEG, poly(ethylene glycol); PVABM, PVA + *B. mori* SF; PVAAA, PVA + *A. assama* SF; PVAPR, PVA + *P. ricini* SF; AMP, antimicrobial peptide; COL, collagen; TNF, tumor necrosis factor; IL, Interleukin; TGF, transforming growth factor; MaSpI, major ampullate spidroin I; MaSpII, major ampullate spidroin II; FibH, fibroin heavy chain; RDT, recombinant DNA technology; HDF, human dermal fibroblast; HDMECs, Human dermal microvascular endothelial cells; HEKs, human epidermal keratinocytes; LPS, lipopolysaccharides; SELP, silk-elastin-like protein; ECM, extracellular matrix, ASCs, adipose-derived stem cells; FBGCs, foreign body multinucleated giant cells; CK10, cytokeratin 10; (CK10); CK14, cytokeratin 14; siRNA, small interfering RNA; ApSF, *A. pernyi* SF; AySF, *A. yamamai* SF; GelMA, methacrylic-anhydride-modified gelatin; ALI, air-liquid interface; NIC, neuro-immuno-cutaneous system; K10, Keratin 10; VIM, Vimentin; AgNPs, silver nanoparticles; SFFD, silk fibroin freeze-dried; SFFG, silk fibroin freeze gelled; SFKR, silk fibroin–keratin blended scaffolds; CAD, computer aided design; ISO, international organization for standardization; HE, haematoxylin and eosin.

Figure Captions:

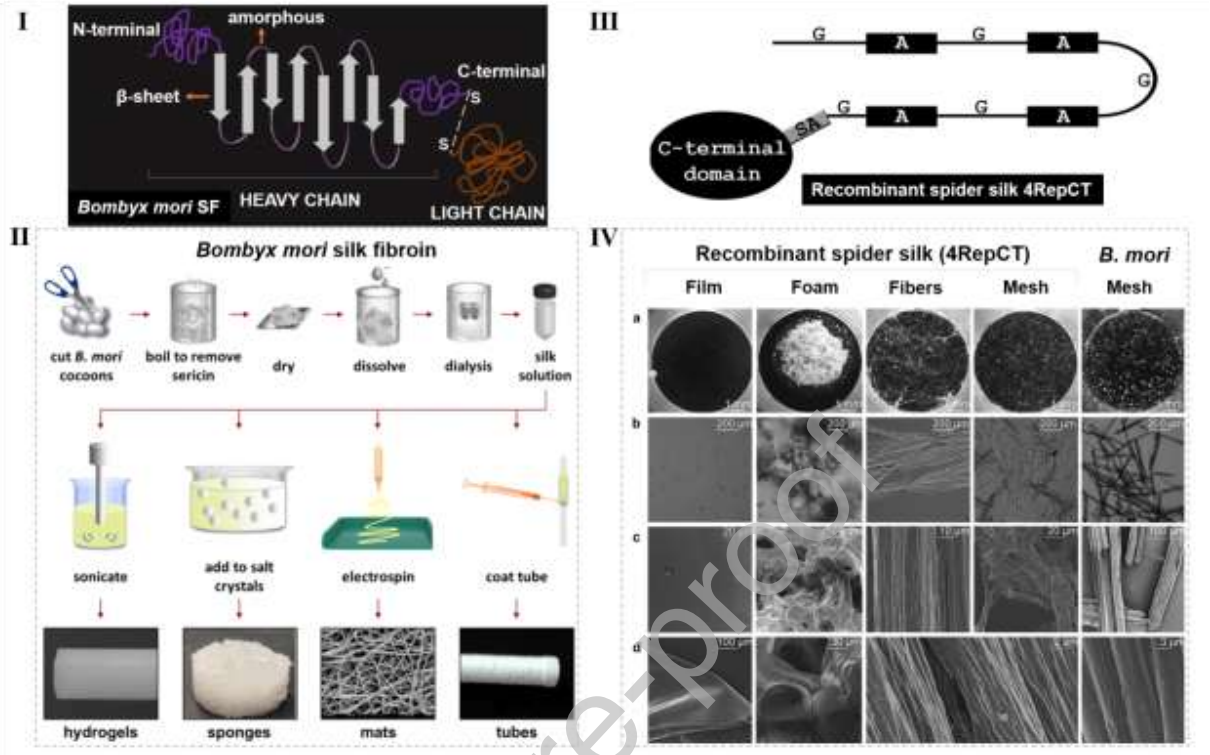

Figure 1: (I) Schematic diagram of the structure of *B. mori* silk fibroin (BmSF) showing heavy chain of the fibroin protein containing both amorphous and β-sheet structures; image adapted with permission from [4]. (II) Schematic design of the processing and fabrication strategies using regenerated BmSF; images reproduced with permission from [10] ©2015 Elsevier Ltd. (III) Schematic diagram of the structure of 4RepCT depicting repetitive poly(alanine) blocks alternated with glycine-rich segments along with a globular C-terminal domain; images reproduced with permission from [20] ©2010 Elsevier Ltd. (IV) Morphology of various types of matrices fabricated using 4RepCT recombinant spider silk like film, foam, fiber and mesh in comparison to the control matrices prepared from regenerated BmSF; images reproduced with permission from [20] ©2010 Elsevier Ltd.

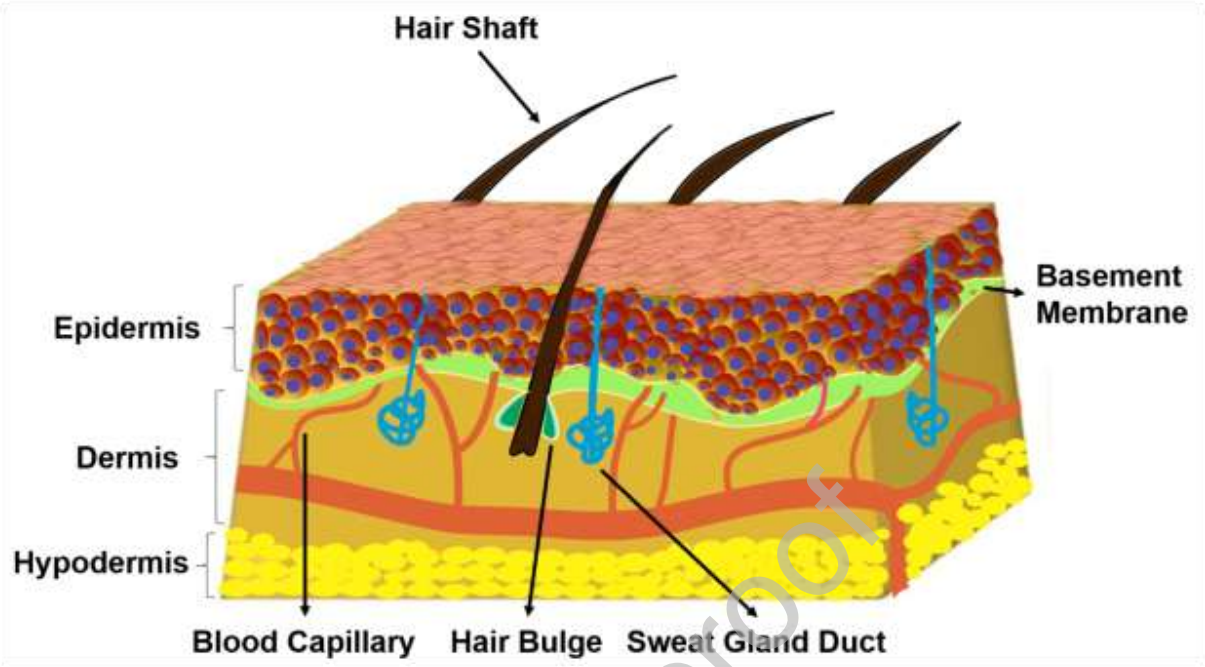

Figure 2. Diagram of a three-dimensional (3D) structure of the skin representing three layers, namely, epidermis, dermis and hypodermis. The anatomy of skin constitutes of various important structures like blood capillaries, sweat gland ducts and hair shafts that play major roles in protecting the body against external environment.

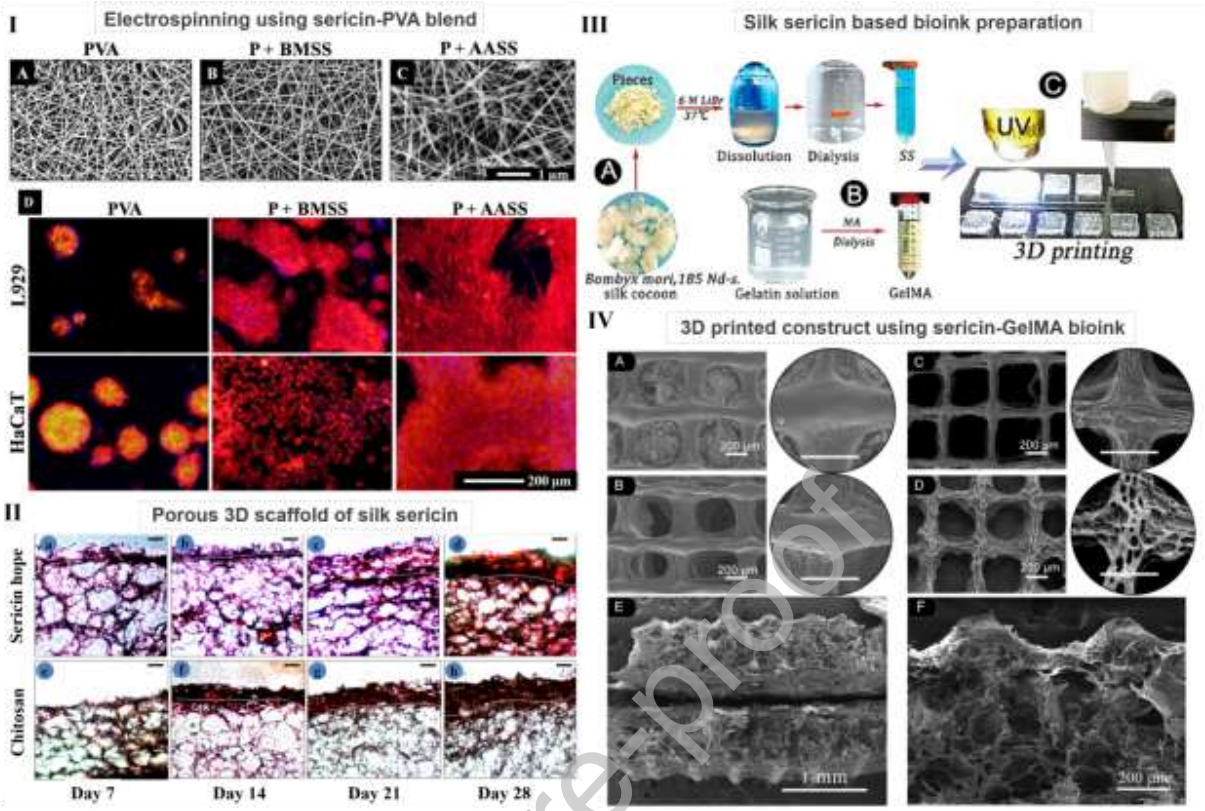

Figure 3: Sericin based matrices for wound healing applications: (I) Nanofibrous mats of poly(vinylalcohol) (PVA) - Sericin blend (A-C) Images of electrospun mats showing nanofibers of PVA, PVA + *B. mori* silk sericin (P + BMSS) and PVA + *A. assama* silk sericin (P + AASS) and (D) Morphology of cells cultured on the mats depicting spread out morphology of cells on the mats containing silk sericin; images reproduced with permission from [44] ©2018 Elsevier B.V. (II) Porous scaffolds made up of sericin extracted from hope cocoons demonstrating successful co-culture of fibroblasts and keratinocytes to develop a bilayer skin tissue in comparison to chitosan scaffolds used as control; images reproduced with permission from [46]. (III) 3D printed construct using silk sericin indicating the application of sericin in bio-ink for bioprinting and wound healing applications, and (IV) Morphology of the 3D printed constructs developed using sericin-based bio-ink showing grid-line structures and porous architecture; images reproduced with permission from [47] ©2018 American Chemical Society.

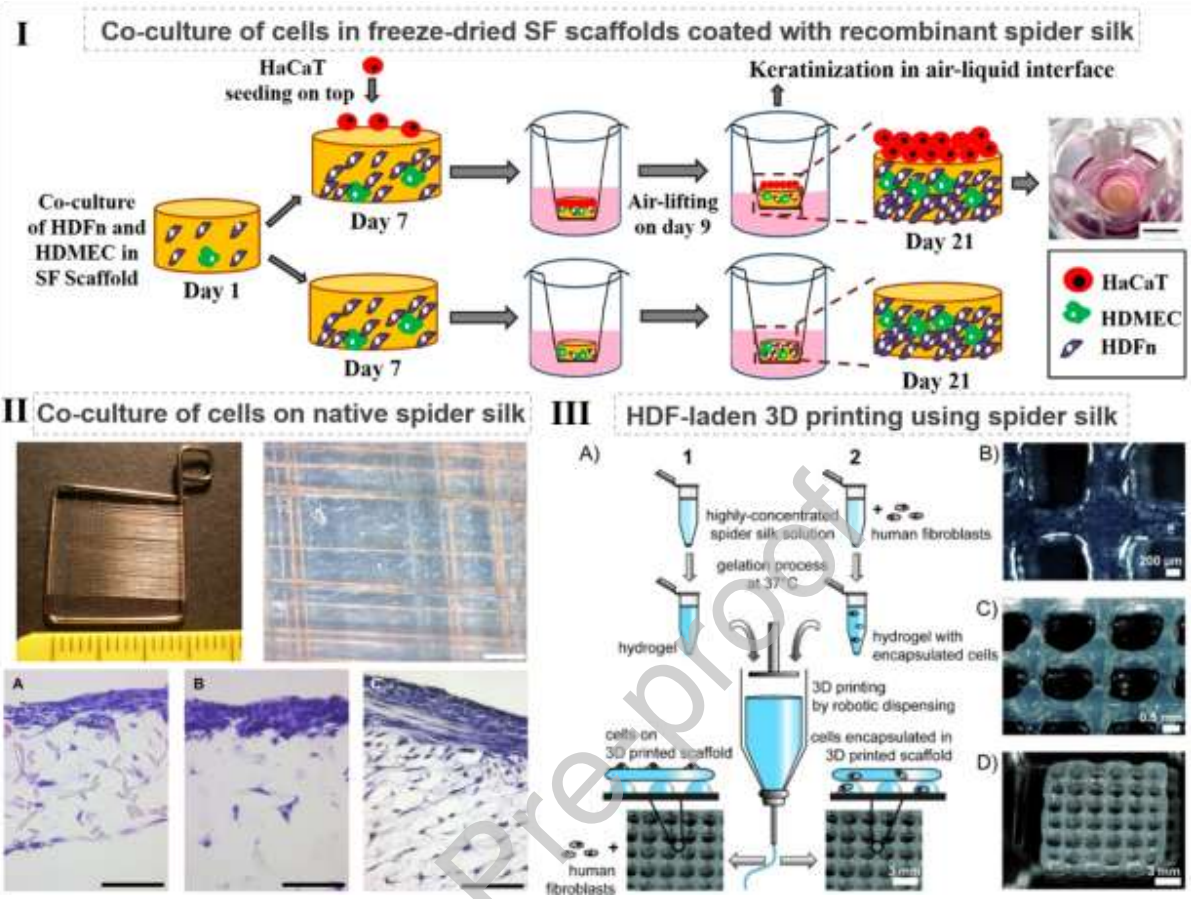

Figure 4: Application of spider silk generating dermo-epidermal bilayer skin constructs: (I) Schematic representation of the silkworm SF porous scaffold coated with recombinant spider silk (FN-4RepCT) for the development of a bilayer graft by co-culturing human dermal fibroblast of neonatal origin (HDFn), human dermal microvascular endothelial cells (HDMEC) and HaCaT cells under air-liquid interface conditions; images reproduced with permission from [26] ©2018 American Chemical Society. (II) Fabrication of a metallic frame made up of stainless steel containing cross-weaved native spider dragline silk fibers to provide co-culture conditions under air-liquid interface. The histological images represent successful generation of a bilayer model using co-cultured cells on spider silk cross-weaved frame; images reproduced with permission from [18]. (III) 3D bioprinting of cell-laden construct using 3 % hydrogel of recombinant spider silk eADF4(C16); images reproduced with permission from [57] ©WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. The hydrogel containing dermal fibroblast could be successfully 3D printed to develop living constructs of cell-laden layers.

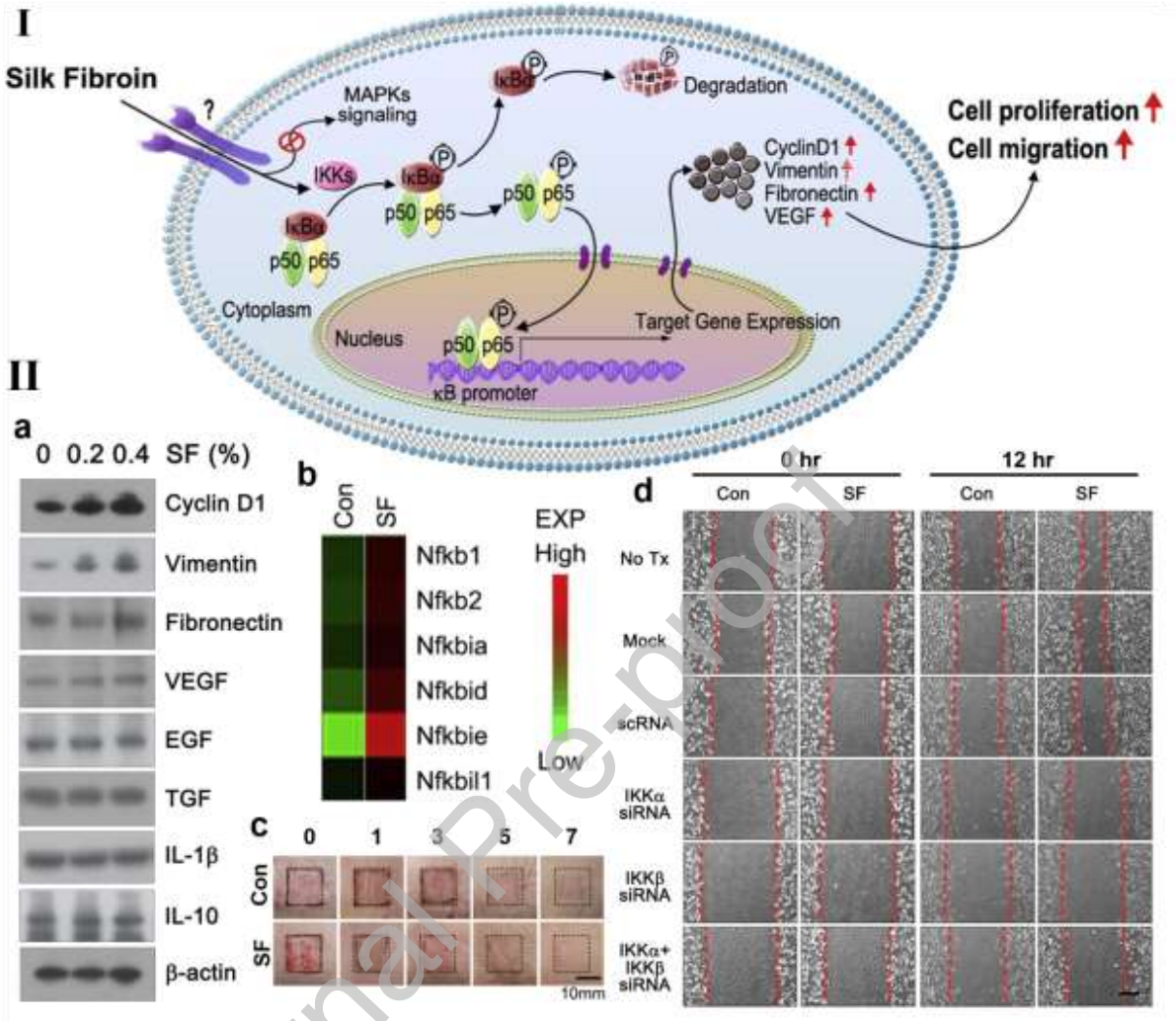

Figure 5: (I) Role of SF in wound healing as examined by NF-κB signalling pathway, which provided clues of the role of SF in enhancing the cell migration and cell proliferation. (II) The western blot analysis and other assays demonstrated expression of markers related to cell proliferation like cyclin D1, fibronectin, vimentin, EGF, VEGF, TGF, IL-10, and IL-1β in the fibroblasts cells treated with SF solution towards accelerated wound healing. The scratch assay confirmed the role of silk in cell migration through various types of transfected cells using small interfering RNA (siRNA) validating the role of silk in wound healing through NF-κB signalling pathway; images reproduced with permission from [24] ©2017 Acta Materialia Inc. Published by Elsevier Ltd.

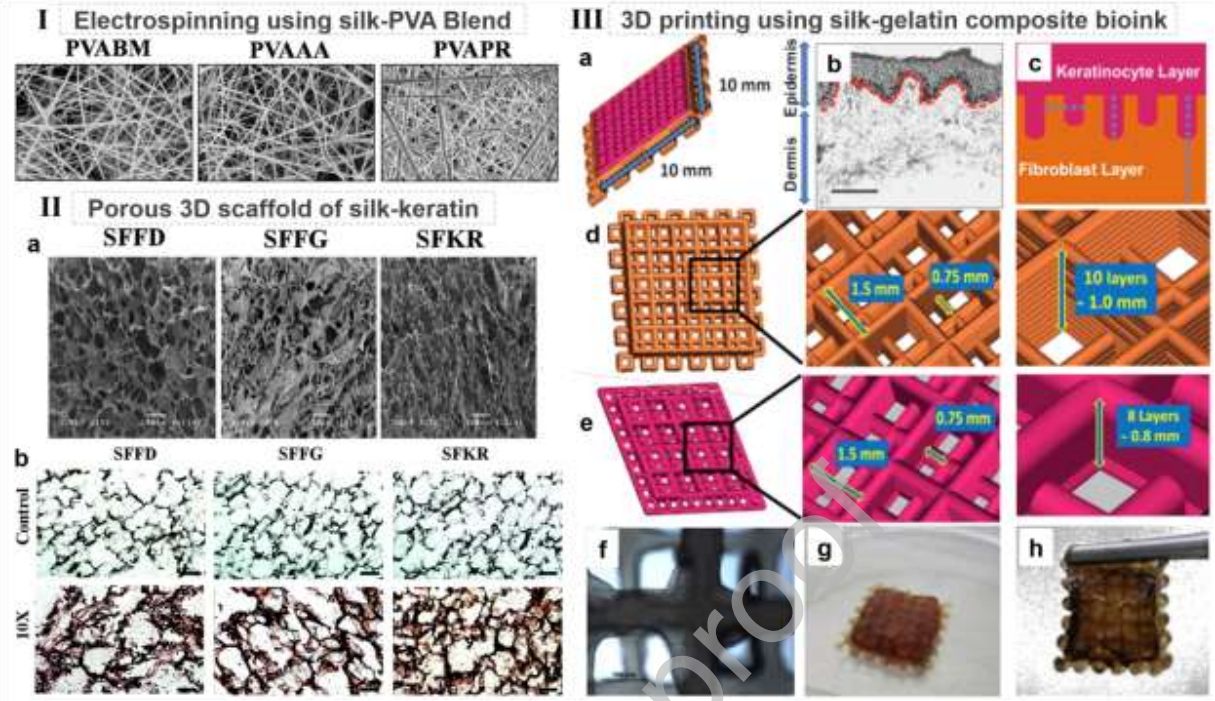

Figure 6: (I) Field emission scanning electron microscopy images of electrospun silk-PVA composite mats representing morphology of nanofibres of various blends: PVA + *B. mori* SF (PVABM), PVA + *A. assama* SF (PVAAA) and PVA + *P. ricini* SF (PVAPR); images reproduced with permission from [30] ©2017 John Wiley and Sons. (II) (a) Scanning electron microscopy images of (A) porous silk scaffolds and silk-keratin composite scaffolds: Silk fibroin freeze-dried (SFFD), silk fibroin freeze gelled (SFFG) and silk fibroin–keratin blended scaffolds (SFKR); (b) The porous architecture of the fabricated scaffolds remained intact after culture of cells for 14 days as depicted by the images of immunostained sections, which demonstrate staining of Collagen type I homogenously distributed throughout the scaffolds, images reproduced with permission from [123] ©2014 Oxford University Press. (III) 3D printed skin graft using silk-gelatin bioink representing the design strategy, Computer-aided design (CAD) model, detailed layer design, dimensions of the epidermal and dermal layers and macroscopic view of the 3D printed construct; images reproduced with permission from [118] ©2019 Elsevier B.V.

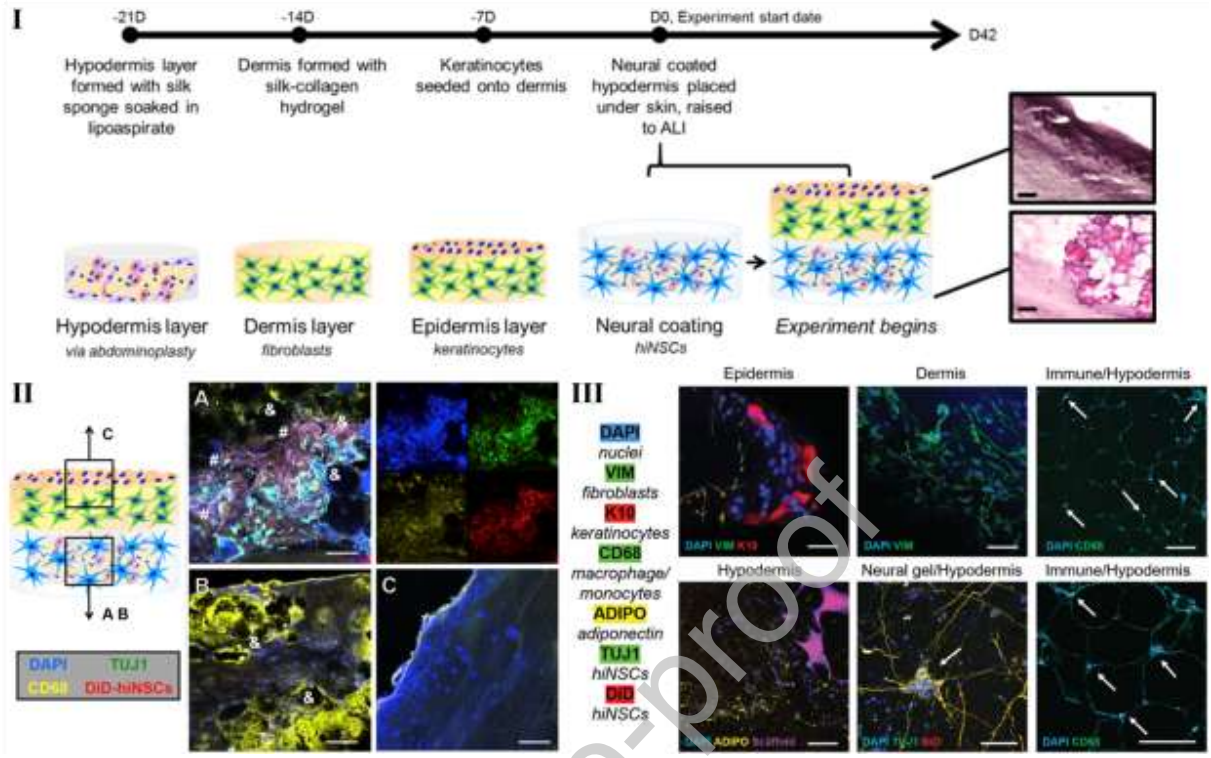

Figure 7: (I) Schematic representation of the fabrication process of a viable full-thickness tri-layer immuno-competent skin equivalent containing nervous system components. The composite human skin equivalent consisted of hypodermis, dermis, and epidermis by using silk sponges and blend of silk-collagen hydrogel; images reproduced with permission [131] ©2018 Elsevier Ltd. (II) Immunostaining of developed human skin equivalent confirming the specific markers of neural (TUJ1 and DiD) and immune cells (CD68). The hypodermis consisted of macrophages, and the dermal layer consisted of neural cells [131]. (III) Immunohistochemistry of the developed skin equivalent indicating various markers of epidermal, dermal and hypodermal layers in the tri-layer skin graft: Keratin 10 (K10) – keratinocytes, Vimentin (VIM) – fibroblasts, ADIPO – adipocyte, TUJ1 and DiD – hiNSCs, CD68 - macrophage and DAPI – nuclei; images reproduced with permission [25] ©WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

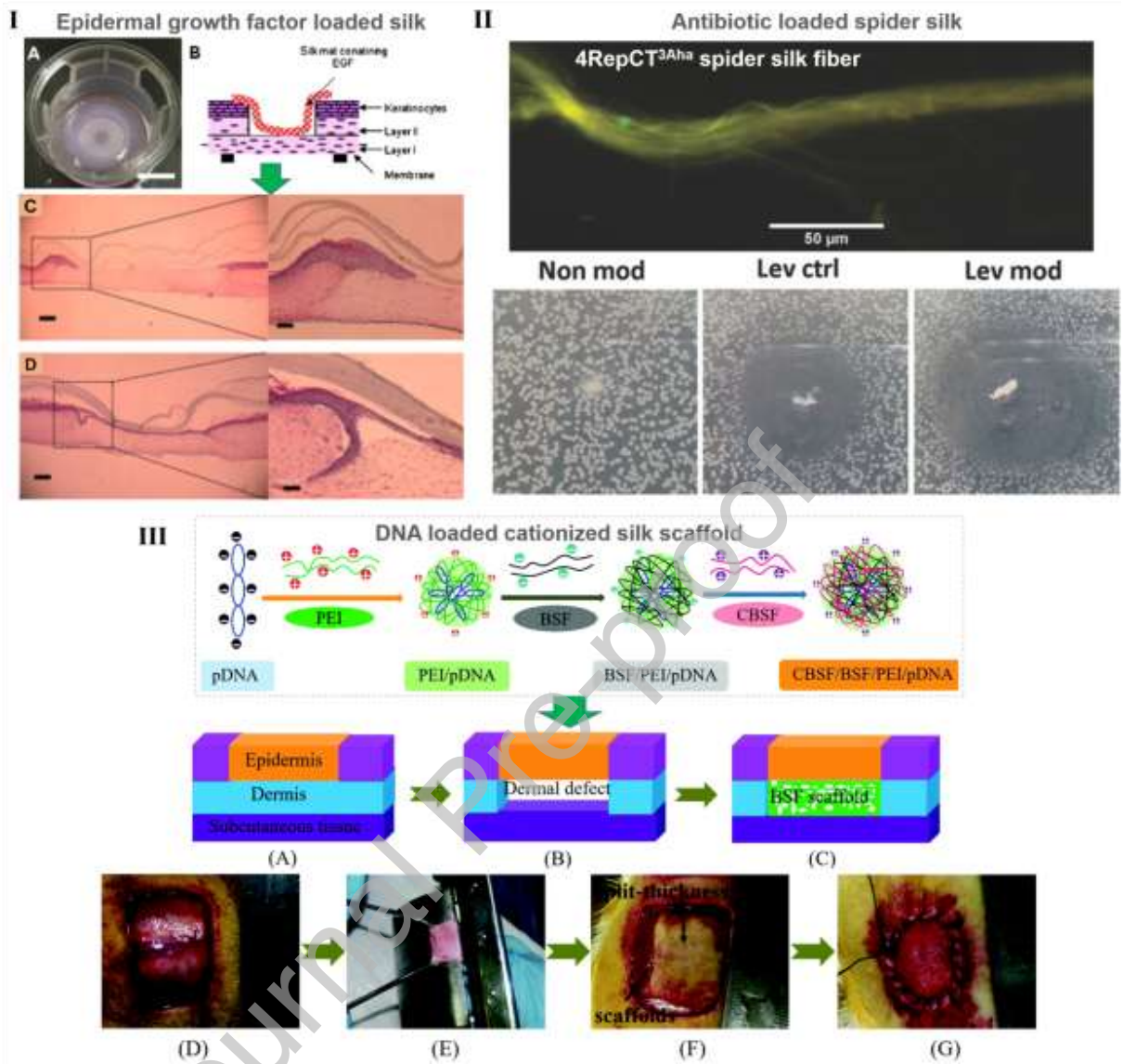

Figure 8: (I) Functionalization of silk nanofibrous mat with EGF showing (A,B) schematic images of analysing the healing efficacy of EGF-loaded silk matrix under *in vitro* skin model. Histological images of the *in vitro* wound model representing extent of re-epithelialization after 48 h when treated with (C) silk mat without EGF and (D) silk mat with EGF. The functionalized bioactive silk mat demonstrated complete epithelialization of the wound in comparison to the non-functionalized silk mat; images reproduced with permission from [109] ©2009 Acta Materialia Inc. Published by Elsevier Ltd. (II) Functionalized 4RepCT spider silk containing fluorophore (4RepCT^{3Aha}) and efficacy of antibiotic-Levofloxacin (Lev)-loaded spider silk fiber against microbial culture; images reproduced with permission from [203] ©WILEY-VCH Verlag

GmbH & Co. KGaA, Weinheim. (III) The schematic design depicts a fabrication strategy of cationized BmSF scaffold with PEI/pDNA complexes and grafting location for gene delivery and wound healing applications; images reproduced with permission from [204].

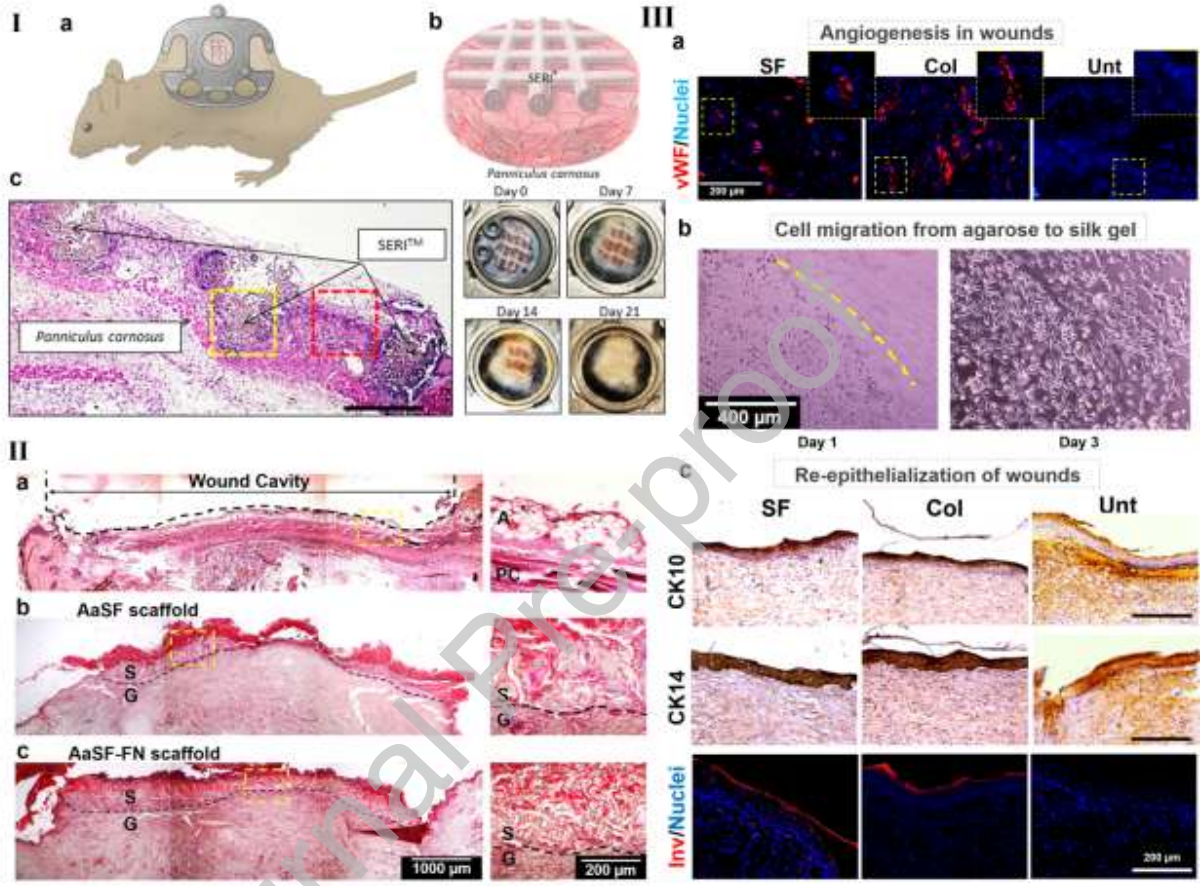

Figure 9: Wound healing efficacy of silk-based matrices under *in vivo* studies: (I) Animal study of SERI scaffold using dorsal skinfold chamber (DSC) mouse model to examine the integration of SERI surgical scaffold and healing efficiency. The haematoxylin and eosin (HE)-stained tissue section and gross wound images revealed scaffold integration post-implantation; images reproduced with permission from [14]© 2016 British Association of Plastic, Reconstructive and Aesthetic Surgeons. Published by Elsevier Ltd. (II) Histology images of *A. assama* silk fibroin (AaSF scaffolds) implanted on the wounds in rat showing integration of scaffolds with the host tissue day 7 post implantation. The magnified images represent the infiltration of cells in the microporous structures of the scaffolds (S) showing an early recruitment of cells and development of granulation tissue (G) at the wounded site; images reproduced with permission

from [68] ©2018 American Chemical Society. (III) Wound healing efficacy of silk-based hydrogel (SF) comparable to collagen (Col) gel showing (a) vascularization potential, (b) migration of cells using agarose drop assay as depicted by the cells migrating from agarose gel towards the gel and (c) re-epithelialization potential of the silk-based hydrogel as shown by the suprabasal expression of cytokeratin 10 (CK10) and basal expression of cytokeratin 14 (CK14); images reproduced with permission from [22] ©2018 John Wiley and Sons.

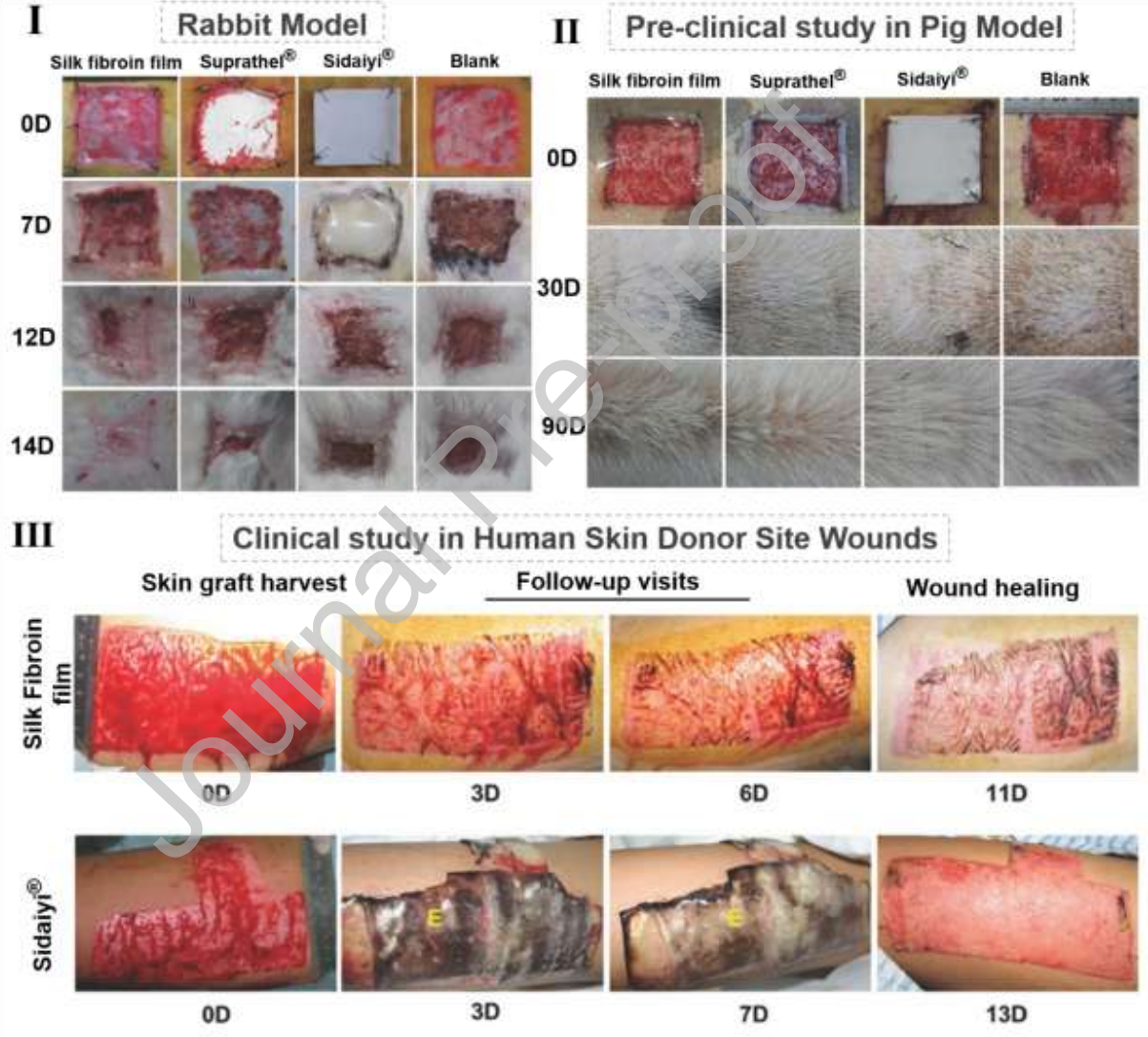

Figure 10: Pre-clinical and clinical studies using *B. mori* silk fibroin thin film representing better healing efficacy of silk film in comparison to polyurethane wound dressing - Suprathel, silk-silicone composite dressing - Sidaiyi, and blank control under (I) rabbit model, (II) porcine

model and (III) clinical study performed on human skin donor site wounds as shown by the macroscopic wound images; reproduced with permission from [8] ©WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Table 1: List of silk-based composite matrices and design of the constructs.

Material format	**Composition**	**References**
3D Porous Scaffolds	Silk Fibroin + Keratin	[123]
	Silk Fibroin + Chondroitin Sulfate + Hyaluronic acid	[161]
	Silk Fibroin + Elastin	[134]
	Silk Fibroin + Alginate	[133]
	Silk Fibroin + Citrus Pectin	[162]
	Silk Fibroin + poly(ethylene glycol) (PEG)	[163]
	Silk Fibroin + Recombinant Spider Silk Fusion Proteins	[26]
	Silk Fibroin + Hyaluronic acid + Sodium Alginate	[164]
	Silk Sericin + Gelatin	[87]
	Silk Sericin + Chitosan	[155, 157]
	Silk Sericin + PVA	[165]
Silk-based Hydrogels	Silk Fibroin + β-cyclodextrin + polyethyleneimine	[166]
	Silk Fibroin + Calcium Alginate + Carboxymethyl Cellulose	[167]

	Silk Fibroin + Collagen	[131]
	Silk Fibroin + Platelet Gel	[168]
	B. mori SF + *A. assama* SF	[22]
	Silk Sericin + PVA	[169, 170]
	Silk Sericin + Carboxymethyl Chitosan + Gelatin	[158]
	Silk Sericin + Agarose	[171]
	Silk Sericin + Polyacrylamide	[159]
	Silk Sericin + Carboxymethyl Cellulose	[172]
Silk-based Films / Thin Membrane	Silk Fibroin + Chitosan	[173]
	Silk Fibroin + Aloe vera gel	[174]
	Silk Sericin + Collagen	[154]
	Silk Sericin + Agar	[175]
Nanofibrous matrices	Silk Fibroin + poly(caprolactone) (PCL) + Hyaluronic Acid	[176]
	Silk Fibroin + Chitosan	[135]
	Silk Fibroin + Collagen	[177]
	Silk Fibroin + PCL	[178]
	Silk Fibroin + PVA	[30]
	Silk Fibroin + Aloe vera	[179]
	Silk Sericin + PVA	[44, 180]
	Silk Sericin + PLCL	[156]

	Silk Sericin + Hyaluronan + Chondroitin Sulfate + Cationic Gelatin	[181]
Silk - decellularized ECM Composites	Silk Fibroin + Human amniotic membrane	[182]
	Silk Fibroin + Goat's Dermal Matrix	[183]
	Silk Fibroin + Duck's Feet Collagen	[184]
	Silk Fibroin + Human Placental derived ECM	[185]
3D Printed Constructs	Gelatin-sulfonated Silk	[186]
	Silk Fibroin + Gelatin bioink	[118]
	Silk Sericin + GelMA bioink	[47]

Table 2: List of silk-based functionalized wound dressings loaded with bioactive molecules/antibiotics/antimicrobial peptides/growth factors.

Material format	**Additional Component**	**References**
SF-based nanofibers	Astragaloside IV	[213]
	Fenugreek	[214]
	Thyme essential oil and Doxycycline monohydrate	[215]
	Grape Seed Extract	[216]
	Human Platelet Lysate	[217]
	Vitamin E + Curcumin	[218]

	Vitamin E	[219]
	Pantothenic acid (Vitamin B_5)	[220]
	Riboflavin (Vitamin B_2)	[221]
	Vitamin C	[222]
	Manuka Honey	[223]
	Antimicrobials - Silver Oxide Nanoparticles, Cathelicidin Peptide (LL37), Silver Sulfadiazine	[110, 224, 225]
	EGF, bFGF	[30, 109]
	Type I Collagen Peptides and Nitric Oxide Donor	[226]
	Quinone-based Chromenopyrazole Antioxidant	[227]
Porous silk-based scaffold	Curcumin	[228]
	Silver nanoparticles (AgNPs)	[229]
	Antibiotic loaded Gelatin Microsphere	[230]
	VEGF165–Ang-1 coexpression plasmid DNA	[204]
	Nuerotensin	[231]
SF-based Hydrogel	Platelet-Rich Plasma Exosomes	[232]
	Curcumin	[233]
	Liposomes with bFGF	[234]
	Polarized Hydroxyapatite	[235]
	GMSC-Derived Exosomes	[236]

SF-based thin films	Antibacterial $MoSe_2$ nanosheet	[237]
	Strontium	[238]
	EGF and Silver sulfadizine	[110]
Transgenic Silkworm-based Genetically Engineered Silk	FGF-, EGF-, KGF-, PDGF- and VEGF	[239]
	VEGF and RGD	[240]
	Platelet-Derived Growth Factor (PDGF-BB) in Silk Cocoons	[241]
	FGF2 and TGF-β1	[242]
	EGF	[243]
	acidic fibroblast growth factor	[244]
	HGF	[245]

Table 3: List of the published patents that highlight the application of silk-based materials and uses thereof. Source: Data retrieved from Google patents (accessed 23.06.19).

S. No.	Title	Assignee Or Applicant	Patent number/Reference
1	Silk fibroin materials and use thereof	Trustees of Tufts College and Massachusetts Institute of Technology	US20130158131A1 [259]
2	Methods, compositions and systems for production of recombinant spider silk polypeptides	Entogenetics, Inc., Charlotte	WO2009097540A1 [260]
3	A 3D bioprinted scar tissue model	Indian Institute of Technology, Delhi, New Delhi	WO2019106695 A1 [261]

4	A personal care composition comprising sericin	Unilever N. V. / Unilever PLC / Conopco, Inc., D/B/A Unilever	WO2019101524A1 [262]
5	Topical silk compositions and methods of using	The regents of the University of Colorado and University of Central Florida	WO2019040850Al [263]
6	Silk fibroin-based personal care compositions	Trustees of Tufts College	US20150079012A1 [264]
7	Chimeric spider silk and uses thereof	KRAIG BIOCRAFT LABORATORIES Inc.	US20130212718A1 [265]
8	Silk - based capsules	Patheon Softgels Inc., High Point, NC	US20160045443A1 [266]
9	Stable silk protein fragment compositions	Silk Therapeutics Inc., Nature Inc	US9187538B2 [267]
10	Implantable biomedical devices on bioresorbable substrates	University of Illinois, Northwestern University, University of Pennsylvania and Tufts University	US8666471B2 [268]
11	Silk fibroin systems for antibiotic delivery	Trustees of Tufts College	US20120052124A1 [269]
12	Silk fibroin-based microneedles and methods of making the same	Trustees of Tufts College	US20130338632A1 [270]
13	Methods of producing and using silk microfibers	Trustees of Tufts College	US9925301B2 [271]
14	Compositions comprising low molecular weight silk	Sofregen Medical, Inc., Medford	US20180272030A1 [272]

	fibroin fragments and plasticizers		
15	Cross linked silk - hyaluronic acid composition	Allergan, Inc., Irvine, CA	US20140315828A1 [273]
16	Thin-layered, endovascular silk-covered stent device and method of manufacture thereof	Lifeshield Sciences LLC	US20010053931A1 [274]
17	Silk fibroin hydrogels and uses thereof	Allergan, Inc., Irvine, CA	US8420077B2 [275]
18	Method for making a knitted mesh	Allergan, Inc., Irvine, CA	US9078731B2 [276]
19	Tissue-engineered silk organs	Trustees of Tufts College	US9102916B2 [277]
20	Method for using a silk derived bioresorbable scaffold in breast reconstruction	Allergan, Inc., Irvine, CA	US20140088700A1 [278]
21	Implantable silk prosthetic device and uses thereof	Allergan, Inc., Irvine, CA	US20140277000A1 [279]
22	Method of forming an implantable knitted fabric comprising silk fibroin fibers	Allergan, Inc., Irvine, CA	US8628791B2 [280]
23	Electrospun silk material systems for wound healing	Trustees of Tufts College	US8728498B2 [281]
24	Silk based implantable medical devices and methods for determining suitability for use in humans	Allergan, Inc., Irvine, CA	US20130253646A1 [282]
25	Silk fibroin and polyethylene glycol-based biomaterials	Trustees of Tufts College	US20130287742A1 [283]

26	Methods for stepwise deposition of silk fibroin coatings	Trustees of Tufts College	US8354501B2 [284]
27	Silk-based drug delivery system	Trustees of Tufts College and Eidgenossisches Technische Hochschule (The Swiss Federal Institute of Technology)	US8530625B2 [285]
28	Bioengineered silk protein-based nucleic acid delivery systems	Trustees of Tufts College	US20120171770A1 [286]
29	Dermal fillers comprising silk fibroin hydrogels and uses thereof	Allergan, Inc., Irvine, CA	US8288347B2 [287]
30	Drug delivery platforms comprising silk fibroin hydrogels and uses thereof	Allergan, Inc., Irvine, CA	US20110052695A1 [288]