Journal of Materials Chemistry B

ROYAL SOCIETY OF CHEMISTRY

REVIEW

View Article Online
View Journal

Cite this: DOI: 10.1039/d4tb01231c

Immune response profiles induced by silk-based biomaterials: a journey from 'immunogenicity' towards 'immuno-compatibility

Nilotpal Majumder,[a] Maumita Bhattacharjee,[a] Giulio C. Spagnoli[b] and Sourabh Ghosh *[a]

Received 6th June 2024,
Accepted 20th August 2024

DOI: 10.1039/d4tb01231c

rsc.li/materials-b

Silk is a widely accepted biomaterial for tissue regeneration owing to its tunable biomechanical properties and ease of chemical modification. However, a number of aspects associated with its clinical use are still debated. Indeed, to achieve clinical success, a biomaterial must favorably interact with host tissues without evoking local or systemic immuno-inflammatory responses. The analysis of immune responses associated with silk under *in vitro* and *in vivo* conditions provides useful insights, improving the understanding of the functional characteristics of silk biomaterials and further promoting their clinical application. Silk evokes moderate immune responses upon implantation *in vivo*, depending on the material structure, fabrication method, degradation time, and implantation in soft or hard tissue sites, which rapidly subside within a few days/weeks. *In vitro* studies indicate that its immune-stimulatory properties are largely due to inherent protein conformation and differential processing parameters. Strategically controlled levels of immune responses *in vivo* with marginal immunogenicity of silk-based biomaterials may contribute to matrix remodeling and replacement by native tissue matrix around the implanted site. Therefore, immunomodulatory strategies should be developed to promote the use of silk-based biomaterials as promising candidates for numerous clinical applications.

1. Introduction

Silk materials have accompanied the development of human civilizations for at least three thousand years. Their applications have ranged from use in luxury garments and curtains in palaces to contributions to healthcare domains in manufacturing surgical sutures and synthetic grafts for organ regeneration (Fig. 1).

[a] *Regenerative Engineering Laboratory, Department of Textile and Fibre Engineering, Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110016, India. E-mail: sourabh.ghosh@textile.iitd.ac.in*
[b] *National Research Council Institute of Translational Pharmacology, Rome, Italy*

Nilotpal Majumder

Nilotpal Majumder is a PhD student at the Department of Textile and Fibre Engineering, Indian Institute of Technology Delhi, India. His research mainly focuses on devising advanced 3D bioprinting strategies while modulating cell signaling pathways to study articular cartilage development and regeneration. He has completed his B. Tech-M. Tech dual degree in Biotechnology from Kalinga Institute of Industrial Technology Deemed to be University (KIIT-DU), India.

Giulio C. Spagnoli

Giulio Spagnoli is a medical professional with extensive experience in immunobiology and immunotherapy of human cancers. Within this context, he has developed a specific interest in studying immune responses to tumor spheroids and biomaterials.

Fig. 1 Silk has traveled across continents and centuries. Traditionally, silk has represented a basic material for expensive garments. However, silk fibers have long been used upon further processing to manufacture different products, such as surgical sutures and rods. More recently, silk proteins, chemically decorated with different biochemical moieties, have also been used to form scaffolds or 3D bioprinted constructs to be used as cytocompatible and immuno-compatible biomaterials for regenerative therapy applications.

Clinical applications of silk materials have garnered favorable attention in recent years owing to their unique amalgamation of tunable mechanical properties, tailored biodegradability, flexible transformation in different forms, such as films, fibers, scaffolds, or sponges, ease of biomedical modification,[1] and biocompatible nature, as also supported by FDA approval.[2–4]

As a protein-based amphiphilic polymeric material mainly composed of amino acids, silk has inherent advantages due to its controlled rheology[5] and mechanical stability, external pH tuning, ionic strength and protein secondary conformation, allowing the fabrication of structurally stable tissue constructs for clinical uses.[6–8] Although sufficient information regarding silk's physiological and biochemical characterization has been gathered to support its tissue engineering potential, a detailed immunogenic profile is required to fully evaluate its prospective impact.

Indeed, the clinical success of any biomaterial-based implant/graft critically depends on its biocompatibility. The degree of response induced in the hosts' immune system ultimately decides the fate of the graft, be it complete degradation or remodeling of the surrounding niche to promote tissue regeneration.[9] In particular, biomaterials should be characterized by a controlled capacity to activate the immune system, promoting wound healing and regeneration processes while avoiding chronic inflammation and limiting "foreign body" responses.

Immediately after the implantation of a biomaterial into the host, a foreign body reaction is usually generated.[10–12] Proteins from blood and interstitial fluids adhere to the biomaterial surface and initiate a cascade of events marking the onset of an inflammatory response.[13,14] Initially, the intrinsic and extrinsic coagulation cascade is activated by Factor XII and tissue factor (TF), followed by fibrinogen adhesion onto the biomaterial surface, initiating clot formation with blood platelet recruitment.[15–17] This process is immediately followed by activating classical and alternative complement pathways, ultimately converging on the C3 convertase step, generating C3a and C5a proteins at the implant site.[18,19] These anaphylatoxins

Sourabh Ghosh

Sourabh Ghosh is a Professor at the Department of Textile and Fibre Engineering, Indian Institute of Technology Delhi, India. He is the head of the Regenerative Engineering laboratory. His research focuses on the interface between fundamental and applied research by combining the principles of Textile Technology and Tissue Engineering, using silk and collagen proteins, 3D bioprinting and establishment of in vitro disease models.

promote inflammatory responses with degranulation of mast cells, enhanced permeability of blood vessels, release of proteolytic enzymes and reactive oxygen species (ROS) production, resulting in the generation of a chemoattractant gradient recruiting and sequestering inflammatory cells, like monocytes, and polymorphonuclear (PMN) granulocytes.[20–22] In particular, the interaction between pattern recognition receptors and integrins expressed on their surfaces and the protein-adsorbed biomaterials promotes PMN degranulation and release of proteolytic enzymes and ROS.[21,22] While PMNs are generally rapidly cleared from the wound site within a day or two, their prolonged persistence may lead to implant failure and promote infiltration by macrophages, dendritic cells and lymphocytes due to the secretion of several chemokines, including, among others, CXCL8, CCL3 and CCL4.[23]

The chronic inflammation stage is marked by the recruitment of circulating monocytes into the implant site and their subsequent polarization into pro-inflammatory M1 or anti-inflammatory M2 macrophages, respectively.[12] While M2 macrophages around the implant is desirable since it will promote healing by initiating tissue repair,[24,25] a predominantly M1 phenotype might lead to tissue damage due to the release of pro-inflammatory molecules.[26]

Within this context, foreign body giant cells (FBGC), generated by a fusion of macrophage membranes during phagocytosis and stimulated by T lymphocytes and mast cells, might play a major role.[27] Inflammation may evolve towards a chronic state with additional recruitment of immune cells and proteins onto implant surfaces, and subsequent FBGC formations generally result in implant degradation and graft rejection. However, if inflammation can be arrested in the acute phase, M2 macrophages and anti-inflammatory, immunomodulatory mediators might favor the remodeling of surrounding tissues and promote neo-tissue regeneration. Importantly, T-lymphocyte activation also represents a major hallmark of chronic inflammation and regulates macrophage polarization.

Therefore, deciphering the overall immunocompatibility of silk biomaterials requires a detailed analysis of the immune system's role in its innate and adaptive components, critically impacting the functionality of silk constructs post-implantation and their translational potential for biomedical purposes. Indeed, although silk has been used in its native form for surgical sutures for centuries, it was eventually replaced by synthetic sutures due to undesired pro-inflammatory effects.[28] For instance, in early studies, virgin silk suture implantation in the eyes of patients undergoing cataract surgery resulted in acute and chronic inflammation and infiltration by multinucleated giant cells.[29]

Silk sutures are usually produced from *B. mori* silk. However, most silk sutures also contain waxy materials or coatings, thus complicating the assessment of *in vivo* bio-responses and degradation.[29] Soluble factors produced upon sonication of native silk have also been shown to induce the production of pro-inflammatory cytokines and increase phagocytosis. Earlier studies suggested that sericin, the outer waxy layer of native silk fibers, was causing Type-I allergic responses[30–32] even at very low concentrations (0.003 units per mg). Instead, while silk sutures also generated chronic inflammatory responses with cellular infiltration and fibrosis when used for peripheral nerve ligation or tracheal anastomosis in a rabbit model, sericin-free fibroin, obtained upon degumming only, caused minimal immune activation.[33,34] However, more recent studies have classified sericin as a biologically safe material that promotes wound healing and osteogenesis and is suitable for other biomedical applications.[35]

Various additional factors, including surface chemistry, topography, protein secondary conformation, and direct interactions between immune cells and proteins adsorbed on silk implants,[36] have been proposed to play important roles in the modulation of immune response to silk. Thus, several issues must be addressed to reliably assess the immune compatibility of silk-based implants. In particular:

1. Which factors, including amino acid sequences, associated secondary conformation, surface topography, or 3D structure of silk biomaterials, are more likely to induce undesired immune response?[37]

2. Does the association of the sericin component of silk protein with the fibroin part favor the induction of an immunological response?

3. Do processing procedures modifying silk properties or degradation of silk fibroin trigger immune responses?

This review aims at stimulating the discussion on fundamental aspects of the innate and adaptive immune response to silk-based biomaterials *in vitro* and *in vivo*. Furthermore, to favor innovative clinical applications of silk-based biomaterials, we propose clarifying factors potentially contributing to regulating immune responses and devise immune-modulating strategies.

2. Immune response to silk sericin

Silk sericin (mol wt. 10–300 kDa) is a globular glue protein covering fibrous fibroin proteins. It comprises 18 amino acids with strong polar side groups, such as hydroxyl, carboxyl, and amino groups.[38] Sericin has been shown to possess several beneficial properties, including antioxidant and anti-inflammatory potential.[39–41] Being hydrophilic with good adhesive properties, sericin supports the adhesion and proliferation of several mammalian cell lines, such as mouse fibroblasts, human skin fibroblasts, and human and mouse hybridomas. Furthermore, it has been shown to promote collagen production, enhancing wound healing.[42,43]

However, despite these reports, its use in biomedical applications is still limited, mainly due to its potential role in the induction of allergy and hypersensitivity reactions[29,30,44,45]

2.1. Inflammatory response

2.1.1. *In vitro* response. The ability of sericin to promote the activation of macrophages and the production of inflammatory cytokines has been repeatedly investigated *in vitro*. Sericin was shown to dose-dependently induce the production of TNF-α and IL-1β by human (NR8383) and murine (J774.2)

macrophage-like cell lines at 0.2–1.0 mg ml^{-1} concentrations. However, maximal levels of TNF-α and IL-1β released upon sericin stimulation were relatively low, *e.g.*, 500 pg ml^{-1} and 350 pg ml^{-1}, respectively, and did not induce overt inflammatory responses or prevent cell proliferation.[46] Instead, the RAW 264.7 macrophage cell line failed to produce TNF-α in the presence of sericin-recoated fibers or sericin-coated wells.[47] Accordingly, negligible levels of TNF-α were produced by RAW 264.7 cells cultured for 7 days on sericin films directly isolated from the silk gland.[48] These reports indicate that soluble sericin does not induce significant innate immune responses, even at relatively high concentrations.

However, sericin might undergo conformational changes upon binding to silk fibers, potentially triggering macrophage stimulation, and sericin-coated fibers were found to serve as templates for macrophage adhesion. These results were recently corroborated by a report showing that increased sericin concentrations in silk-sericin electrospun scaffolds (7 : 3) promote the expression of CCR7, an M1 macrophage marker, inflammatory cytokine secretion, and vascularization.[49]

2.1.2. *In vivo* response. Earlier studies showed that activation and phagocytic activities of resident and thioglycolate-elicited rat peritoneal macrophages could be elicited upon exposure to silk suture-primed media[50] without direct silk-macrophage interaction. More recently, silk sericin has been reported to promote mild innate and adaptive immune response *in vivo* to an extent significantly higher than silk fibroin.[51] Immune responses included antibody secretion, the activation of $CD4^+$ T cells and cellular apoptosis. In this context, remarkably, earlier *in vivo* investigations have shown that hypersensitivity reactions to silk, particularly sericin, may be associated with type I allergic responses and upregulated levels of specific IgE, with respiratory symptoms, including asthma.[30,50,52] Accordingly, subcutaneous implantation of sericin in combination with collagen in Wistar rats has been shown to induce acute inflammation, regressing to moderate degradation within 2 weeks, followed by fibro-encapsulation.[53] The implantation site was marked by abundant macrophage infiltration, with multinucleated giant cells completely degrading the biomaterial and disappearing after complete resorption.

These investigations suggested that while sericin evoked a negligible to very mild immune response *in vitro*, it could trigger a strong immune activation *in vivo*.

2.2. Anti-inflammatory response

2.2.1. *In vitro* response. Anti-inflammatory effects of sericin have also been reported. In addition, sericin was also shown to inhibit the expression of pro-inflammatory factors, TNF-α, and COX-2, which mediate skin inflammation, cell proliferation, and skin tumor promotion.[54] Anti-inflammatory properties elicited by repressing the activities of IL-18, TLR-4 and NF-kB pathways associated with various inflammatory diseases have also been reported.[55]

A low molecular weight sericin (< 10 kDa) could successfully differentiate the U937 macrophages towards an anti-inflammatory M2 phenotype with an enhanced expression of Arginase 1 (Arg-1), making it a suitable biomaterial for wound healing applications. This anti-inflammatory property was further validated by the detection of high expression levels of genes encoding anti-inflammatory cytokines and chemokines, including CXCL9, IL12a, and IL-10, at a minimum concentration of 0.1 mg ml^{-1}.[56]

Silk sericin, in combination with other polymers like poly lactic-*co*-glycolic acid (PLGA), might provide an excellent composite for dental tissue engineering applications. When cultured with LPS-induced RAW 264.7 macrophages, it induced decreased levels of pro-inflammatory markers like MMP-3/13, IL-6, *etc.*, at the relatively low concentration of 0.5 mg ml^{-1} while promoting cellular viability of human gingival fibroblast cells.[57]

2.2.2. *In vivo* response. *In vitro* studies are further supported by *in vivo* investigations. The topical application of a sericin solution in a carrageenan-induced rat edema model induced downregulation of COX-2 and iNOS genes, consistent with an anti-inflammatory effect.[58] In addition, in sericin-treated tissues, a decrease in inflammatory response regarding polymorphonuclear cells and macrophage infiltration is comparable to that induced by indomethacin. In addition, sericin was shown to play a favorable role in treating full-thickness wounds in rats based on its ability to promote collagen synthesis.[59]

Moreover, implantation of a pure sericin hydrogel in BALB/c mice was accompanied by low-level infiltration of inflammatory immune cells and vascular endothelial cell migration within 3 days, resulting in anti-inflammatory tissue regeneration without triggering allergic reactions.[60] Consistently, Sapru *et al.* reported that sericin-containing matrices elicited minimal inflammatory immune response, evaluated by TNF-α, IL-1β and CD68 expression, and accelerated wound healing with enhanced angiogenesis when implanted in Wistar rats.[61] Accordingly, sericin appeared to reduce lymphocyte and mast cell migration in full-thickness rodent wounds, as compared to Bactigras®, a commercially available woven gauze dressing containing chlorhexidine acetate, without irritating throughout the implantation period, and therefore qualifying as a potential candidate for wound healing applications.[62]

Thus, published data on immune responses to sericin appear to be, by and large, contradictory. However, most *in vivo* studies support the biocompatibility of sericin and attribute its immunogenic potential to its physical association with fibroin filaments. It is also possible that, based on the extent of degumming, sericin may be denatured and cleaved to small amino acid fragments, potentially promoting regeneration rather than eliciting immune responses. It is tempting to speculate that a more accurate investigation of the type of immune responses generated, including the extent of macrophage polarization and inflammation time course, coupled with detailed conformational studies of fibroin and sericin core silk proteins in mono- or copolymer states, may provide important information, for future applications. Within this context, as with purified fibroin or sericin, combined fibroin–sericin structures have been suggested to elicit immune responses and allergy.

3. Immune response to silk fibroin

Native silk fibroin consists of three protein components: a heavy chain of approximately 350 kDa, a light chain of approximately 26 kDa, and a P-25 glycoprotein of approximately (30 kDa),[63,64] with a unique organization of hydrophobic and hydrophilic blocks in its structure. This organization provides silk fibroin with many beneficial characteristics, including the ability to modulate cell signaling pathways and favor tissue regeneration, cytocompatibility, hemocompatibility, and biodegradability.[65,66]

3.1. Inflammatory immune response

3.1.1. *In vitro* response. Several studies support better biocompatibility of silk fibroin over other commonly used biomaterials such as sericin, collagen, polystyrene or poly(2-hydroxyethyl methacrylate).[67–69] However, it is highly regulated by the adhesion of cells and the associated protein onto materials' surfaces and the activation of PMN and macrophages.[70] Santin *et al.*[68] conducted a detailed study of the inflammatory response of complement fragment C3 to fibroin films. Once the C3 protein adheres to biomaterial surfaces, a disulfide bond in C3 is cleaved, causing the adhesion of leukocytes and macrophages and activating inflammatory responses.[71] Importantly, leukocytes and other inflammatory cells can adhere directly to biomaterials only in the presence of defined proteins such as fibrinogen or IgG.[68] Compared to polystyrene and poly-hydroxyethyl methacrylate, fibroin is characterized by lower fibrinogen absorption, whereas a similar absorption of plasma complements fragments C3 and IgG was observed. Accordingly, silk fibroin films showed significantly lower macrophage adhesion than these materials, consistent with a decreased inflammatory response.

In another study comparing the responsiveness of bone marrow stromal cells, a significantly lower expression of IL-1β, COX-2, and PGE2 in cells cultured on silk fibroin over collagen films was observed, further decreasing with time (96 hours). Moreover, upon *in vivo* intramuscular implantation, silk films showed a relative abundance of bound fibroblasts and collagen fibers and lower numbers of macrophages and multinucleated giant cells compared to polylactic acid and collagen films, suggesting improved biocompatibility. In another study, fibroin and collagen comparably stimulated TNF-α release by murine macrophage cell line RAW 264.7, but to significantly lower extents than LPS, and minimal immune responses were evoked.[67]

However, in a study[47] on the activation of RAW 264.7 macrophages, while macroscopic silk fibers failed to activate macrophages under both short (18 hours) and long-term culture (7 days), silk fibroin microparticles (10–200 mm) significantly stimulated macrophages, as indicated by increased TNF-α release.

Investigations by our research group also support an *in vitro* immune-stimulating potential of silk fibroin biomaterial, dependent on silk source and chemical processing. We observed that ethanol-treated silk films and *A. mylitta* silk braids induced the secretion of TNF-α, IL-6 and IL-8 pro-inflammatory cytokines associated with M1 phenotype polarization. However, water-annealed silk films and B. Mori silk braids promoted the polarization of THP-1 cells towards an anti-inflammatory M2 phenotype with decreased secretion of pro-inflammatory cytokines.[72] Accordingly, in previous studies, we demonstrated that 3D silk fibroin matrix induced higher IL-1β and IL-6 gene expression and protein secretion compared to 2D fibroin matrices but lower than sericin film.[37] This data indicates that various physio-chemical characteristics of silk-based biomaterials govern their ability to induce immune responses.

3.1.2. *In vivo* response. Although the previous studies suggest that silk fibroin is superior to other commonly used biomaterials, most silk fibroin-based biomaterials have also been shown to activate the complement system *in vivo*. These reports suggest that processing, including using chemicals such as HFIP/formic acid and fabrication methods for 3D structures of films, fibers, sponges, or foam silk-based biomaterials used for specific biomedical applications rather than intrinsic chemical composition, might be responsible for these effects. Indeed, Meinel *et al.* showed that silk fibroin films prepared by hexafluoro-2-propanol (HFIP) attracted and activated macrophages at the implant-tissue interface upon intramuscular implantation.[67] Silk fibroin tubes used for vascular grafts in the abdominal aorta of rats showed substantial infiltration of macrophages with phagocytosis of fibroin remnants.[73] Similarly, infiltration of multinucleated giant cells and macrophages was also observed in porous silk fibroin scaffolds prepared from solutions by formic acid evaporation upon implantation in a bone defect model.[74]

Other studies have also shown that silk fibroin can activate macrophages and the complement system, but this activation is short-lived and regresses within a few days.[75] Indeed, subcutaneous implantation of lyophilized silk fibroin sponges induced immune cell infiltration at 2 weeks, followed by fibrosis at 4 weeks. Silk gels also induced mild complement system activation at days 7 and 14 after subcutaneous implantation with macrophage and neutrophil infiltration.[76] Another study also confirmed the short-lived activation of immune cells, reporting that subcutaneous implantation of silk fibroin gels initially induced inflammation with eosinophil, neutrophil, and macrophage infiltration, markedly reduced after 1 month. Furthermore, no inflammatory cells were detectable after 3 months.[76] Long-lasting biocompatibility of fibroin was also demonstrated by *in vivo* subcutaneous implantation in C57BL6 mice for as long as 180 days, resulting in neo-tissue formation similar to vascularized reticular connective tissue with mild foreign body response and no fibrosis.[77]

Importantly, studies with fibroin-coated polyester sutures have demonstrated that regenerated silk fibroin does not induce significant thrombocytosis.[78] Notably, a higher macrophage infiltration has been observed upon subcutaneous implantation of small rather than larger and medium microfiber-reinforced scaffolds, with the latter displaying lower numbers of giant cells, 4–5 cell sheets thick fibroblast layers, and increased vascularization. Further studies have confirmed the minimal incidence of inflammation when electro-spun silk

matrices were implanted in rats for 8 weeks.[79] Similar responsiveness was detected in 3D silk scaffolds after subcutaneous implantation in Lewis rats, with a mild immune response observed after a year, with negligible expression of TNF-α, IFN-γ, IL-4, IL-6, and IL-13 genes.[80] Accordingly, MSCs cultured on a knitted silk mesh, showing enhanced proliferation potential and differentiation into fibroblast-like cells, have successfully been used for anterior cruciate ligament (ACL) reconstruction in a swine model.[81]

On the other hand, when non-mulberry silk fibroin *A. mylitta* and mulberry silk *B. mori* acellular scaffolds were implanted in critical-sized defects of rat calvariae, suboptimal bone formation was observed within 6 months of implantation of silk scaffolds of *B. mori* origin. Indeed, the *B. mori* scaffold was completely degraded due to extensive infiltration by multinucleated giant cells, phagocytes, macrophages, and other immune cells, as detected by CD11b staining. Instead, nominal numbers of inflammatory cells were observed in *A. mylitta* scaffolds, resulting in complete ossification and perfect healing of the defect.[82]

Therefore, the inflammatory response of the fibroin component of the silk protein under both *in vitro* and *in vivo* conditions is highly dependent on different factors, including time of exposure, protein size and conformation, and source of silk fibroin (Section 4).

3.2. Anti-inflammatory response

3.2.1. *In vitro* response. Interestingly, silk fibroin has also been suggested to efficiently treat inflammation and stabilize tear films in dry eye disease.[83] Indeed, silk fibroin was found to inhibit the secretion of inflammatory factors by lacrimal glands in the dry eye model, consistent with an anti-inflammatory effect.[84] Furthermore, silk fibroin matrices have also been shown to induce a lower expression of MCP-1, CCR7 and VEGF pro-inflammatory M1 markers and an enhanced expression of CCL8, CD206 and PDGF-BB anti-inflammatory M2 marker genes, as compared to fibroin–sericin composites.[49] Moreover, although MSCs cultured on silk films produced inflammatory cytokines and prostaglandins, they generally declined following 96 hours of culture.[67]

3.2.2. *In vivo* response. Silk fibroin films have been investigated in rabbit and porcine full-thickness skin defect models, and these studies confirmed their long-term safety and effectiveness with no cytotoxicity or delayed-type hypersensitization. Furthermore, no acute systemic toxicity, hemolysis, subchronic systemic toxicity, intracutaneous reactivity, or genotoxicity were observed in these conditions.[4] Their biocompatibility was further validated by negligible macrophage invasion and giant cell infiltration following intramuscular implantation in rat models.[67] Clinical experimentation has shown that silk fibroin films significantly reduce the time to wound healing and show no adverse effects compared to commercial dressing.[4] Remarkably, a silk fibroin material is used as an anti-inflammatory carrier for the Baicalein flavonoid drug. This combination has demonstrated an enhanced free radical scavenging rate (95%) compared with the Baicalein drug in solution, resulting in a 77.6% inhibition of abdominal edema.[85] Furthermore, in a colitis rat model, intrarectally administered RGD-functionalized silk fibroin nanoparticles ameliorated colon damage with reduced neutrophil infiltration, nitric oxide production and expression levels of IL-1β and IL-6/12 pro-inflammatory cytokines.[86]

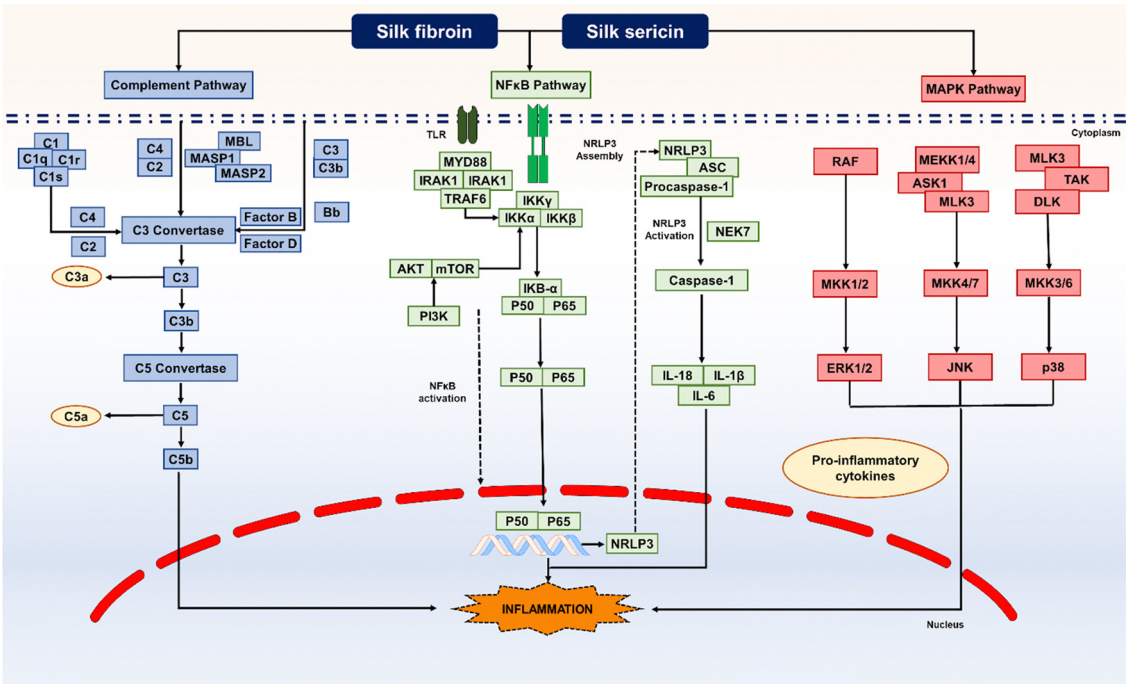

Fig. 2 Major signaling pathways regulating the inflammatory immune response to silk-based biomaterials.

4. Factors governing immunity of silk-based biomaterials

Biomaterial immunogenicity critically depends on multiple factors: size, shape, morphology, surface chemistry, surface topography, surface stiffness, physical and chemical properties, overall architecture, protein conformation, implantation site, and processing-induced degradation products.[87–90] Accordingly, the duration and intensity of the immune responses may vary significantly (Fig. 3). For instance, polyvinyl pyrrolidone (PVP) with a 10 000 Da MW is a very weak immunogen,[91] whereas 360 000 Da MW PVP is strongly immunogenic. The biomaterial's surface area to volume ratio is also a critical factor. Particles <20 mm in diameter are markedly more immunogenic than particles >50 mm. Moreover, Bartneck *et al.*[87] reported on the role of biomaterial surface morphology in 3D *vs.* 2D nanofibers with variable matrix porosities and surface chemistries. They observed anti-inflammatory and proinflammatory effects of 2D and 3D systems, respectively. Therefore, different factors determining the biocompatibility of silk-based biomaterials should be carefully analyzed.

The interaction between immune cells and proteins adsorbed on silk fibroin implants regulates immune responses (see above). Soon after implantation, biomaterials contact extracellular fluids and blood proteins. The absorption of plasma proteins on material surfaces results in the formation of a "provisional matrix" rich in several growth factors, chemo-attractants, and cytokines, capable of recruiting and activating immune cells, thereby mediating inflammatory responses and ultimately dictating the fate of the implanted material.[14,22] The degree and composition of the provisional matrix depend on the surface chemistry, morphology, and structure of the implant. Therefore, surface morphology, chemistry, and structure play critical roles in activating immune response.

Several studies have shown that soluble silk fibroin and silk-collagen composites promote rapid blood coagulation.[92,93] Furthermore, silk fibers bind serum proteins such as IgG and α-1-microglobulin to higher extents than methanol-treated films and promote fibrin polymerization.[94] Protein conformation also plays a significant role in serum protein adsorption since regenerated films with reduced β-sheet content obtained by casting from water solution and stabilized by methanol treatment do not bind serum proteins. Further studies on silk films have confirmed that several other factors, such as treatment temperature, sterilization method, relative humidity, and solvent used in processing, influence the biocompatibility of fibroin-based biomaterials.[95–97]

4.1. Protein size

The nature of the degradation products of silk proteins critically impacts the immune response profile induced by silk scaffolds.[98]

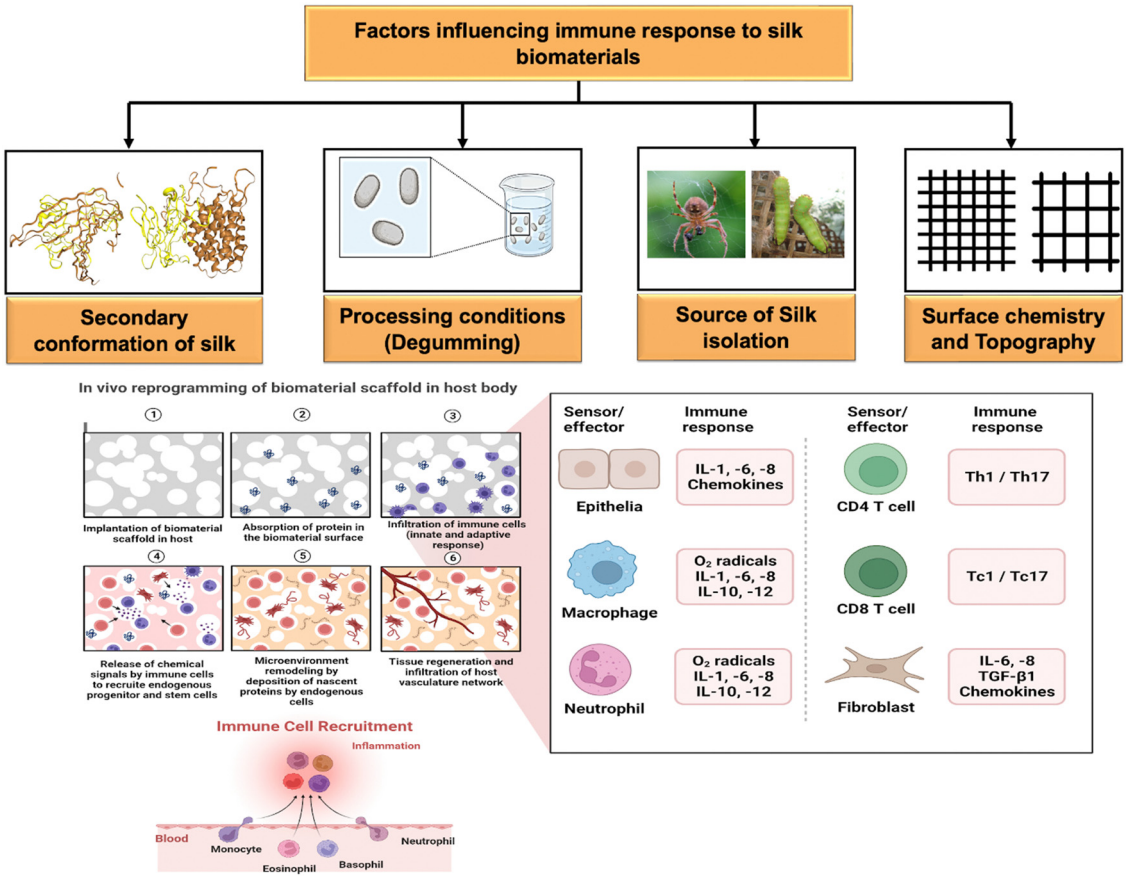

Fig. 3 Factors contributing to the differential immune response profile of silk-based biomaterial in its innate and adaptive mechanisms.

In an experimental study in mice, focusing on silk microfibers of different sizes (10–20, 150–200, and 400–500 μm) used for bone regeneration, small microfibers (10–20 μm silk fibers) were reported to induce a higher adhesion with effective phagocytosis by surrounding macrophages, as compared to larger particles.[99]

4.2. Surface chemistry/topography

The surface chemistry and topography of silk-based biomaterials also play important roles in platelet and THP-1 myeloid cell adhesion and activation. Moreover, amino acid composition influences film crystallinity, hydrophobicity, surface roughness, and biological interactions. The adsorption of proteins and, particularly, of the chemotactic factors (C3a, C5a, and C3b) was lower in samples with higher crystallinity and hydrophobicity.[95]

The role of protein conformational change on thrombogenicity was studied by treating silk fibroin films with 50% aqueous methanol for 15, 30, 60, 120, and 240 minutes. This treatment resulted in the transition of random coils to β-sheets, increasing surface tension, polarity, and crystallinity. Platelet adhesion and anti-thrombogenic effect were enhanced by increasing methanol treatment time and crystallinity, consistent with a possible influence of surface free energy, especially its polar component. It was also observed that thick fiber, highly porous, silk fibroin scaffolds attracted more bone marrow mononuclear cells displaying M1 and Th1 pro-inflammatory phenotypes than thin fiber from low porous scaffolds that, in contrast, appeared to suppress the immune response.[100] This data suggests that the nature of the silk fibroin surface could control initial protein adsorption. In contrast, the adsorbed protein layer would critically influence platelet adherence, providing an important basis for the blood coagulation process.[101]

4.3. Protein secondary structures

The role of secondary conformation in the immune response to silk proteins was investigated in a study where silk films with different degrees of β-sheet content were prepared using water annealing and organic solvents. Dried films were processed using (1) 100% methanol treatment for 6 hours, (2) 70% ethanol treatment for 6 hours, (3) water vapor annealing at 25 °C for 6 hours, (4) incubation at 37 °C for 12 hours, and (5) 20 min steam autoclaving at 121 °C before exposure to human whole blood.[102] The β-sheet content was found to increase (35%, 42%, and 56%) with increasing processing temperature from 25 °C to 37 °C and 121 °C. Silk samples treated with ethanol or methanol showed β-sheet contents ranging between 36% and 54%. Most importantly, C3b, responsible for leukocyte adhesion, C5a and CD11b were expressed at the highest levels in cells exposed to films treated with methanol. Consistently, the inflammatory response to silk treated with methanol was higher than the other materials. Instead, silk films water annealed at 25 °C for 6 hours showed high biocompatibility with blood components, as evaluated based on hemostasis and inflammatory parameters, strongly suggesting that processing parameters, including treatment temperature and solvent, may considerably affect protein conformation, and play important roles in steering the biological response.[102] Interestingly, residual solvent molecules within silk scaffolds have been shown to influence cytokine production in rat models.[80]

Our studies strongly suggest that the architecture of silk biomaterials (2D *vs.* 3D) impacts immune activation.[37] The production of IL-1β and IL-6 by human peripheral blood monocytes was relatively higher upon stimulation with 3D fibroin, compared to 2D film, with reduced cytokine release after 6 days in all experimental groups. The immune response may also be evoked by differential protein conformation. Indeed, IR spectra indicated the predominance of silk I conformations (27.1% α-helices, 8.7% β-sheet (weak) or aggregated β-strands, 12.2% random coils, and 7.9% β-turns) in the 2D silk fibroin film. The silk II conformational state (51.6% strong β-sheet crystal and 28.9% aggregated strands) in 3D silk fibroin rendered the structure more hydrophobic. Accordingly, 2D fibroin film with silk I conformation and reduced β-sheet content was a poorer immune stimulator than 3D fibroin (Fig. 4). However, 2D and 3D silk fibroin did not elicit an adaptive immune response with $CD4^+$ and $CD8^+$ T cells (Fig. 6). Also, the study by Roy *et al.* showed how *B. mori* braids with the highest β-sheet content (73.51%) evoked mild immune response compared to *A. mylitta* braids (72.60%), indicating the role of secondary conformation in triggering immune reactions.[72] However, ethanol treatment, resulting in higher β-sheet content (67.19%), induced a higher pro-inflammatory response than water-annealed silk films (63.49% β-sheet content) (Fig. 4). Thus, regarding fabrication parameters, not only the secondary conformation but also the internal microstructure of the protein in terms of hydrogen bonding plays a role in the immune response.

This data largely agrees with earlier studies indicating that peptides, irrespective of their size, are usually poor immunogens, whereas protein aggregates or supramolecular assembled peptides and proteins are strongly immunogenic.[103,104] Assembled peptides mediated immunogenicity and antibody response was first reported by Rudra *et al.*[104] to evoke T cell-dependent B cell responses. In the cited study, purified $CD4^+$ and $CD8^+$ T cells from healthy donors were also cultured in different forms in the presence of silk biomaterials to analyze adaptive immune responses. T lymphocyte activation in the presence of autologous $CD14^+$ cells was monitored by testing the expression of CD69 and CD25 markers and IFN-γ gene expression. No evidence of pre-existing sensitization was observed by using cells from healthy donors. However, other studies have shown that fibroin contains antigenic sites within the nanocrystalline region, which may be generated by chymotryptic digestion. Furthermore, it was observed that glycine, serine, alanine, and tyrosine residues might represent important components of fibroin-derived antigenic determinants.[105]

4.4. Source of silk

Silk sources also play important roles in governing the immune response to derived biomaterials. For instance, extensive biological characterization *in vitro* using bone marrow-derived macrophages and *in vivo* indicates that *A. assamensis* silk is

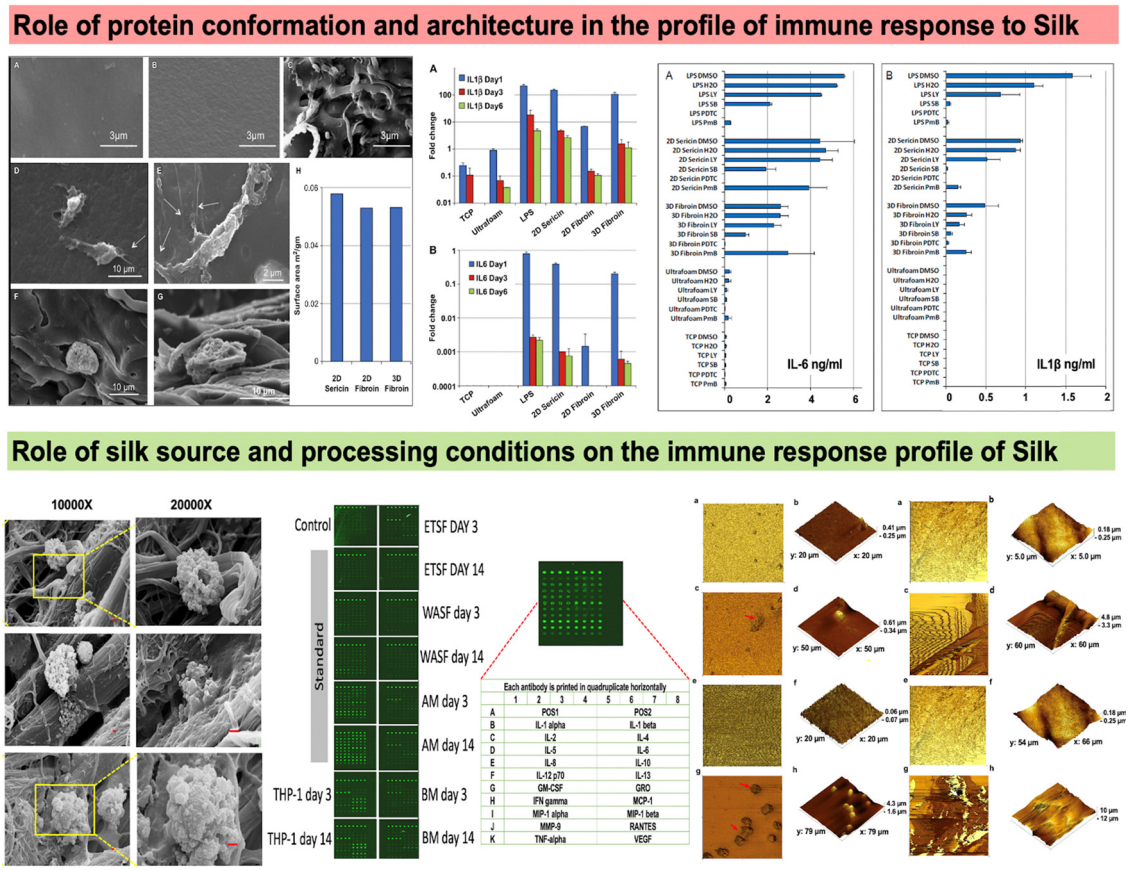

Fig. 4 (a) The role of protein architecture and conformation on immune response profiles induced by silk-based biomaterials. The scanning electron microscopy (SEM) image denotes the different morphologies of sericin in 2D and 3D forms. The bar graph depicts IL-1β and IL-6 inflammatory cytokines gene expression by $CD14^+$ monocytes cultured on silk scaffolds. Activation of different inflammatory pathways by different silk protein conformations and the role of specific inhibitors is also shown. Reproduced with permission[37] Copyright 2013, Elsevier. (b) Role of silk source and processing conditions in the immune response profile induced by silk biomaterials. Silk from two sources (*A. mylitta* and *B. mori*) and scaffolds prepared by water annealing or ethanol treatment were considered. The SEM image shows the morphology of the cultured THP-1 monocytes on the *B. mori* silk braids for 3, 7 and 14 days. Microarray analysis of cytokine production unravels the anti-inflammatory role of *B. mori* silk scaffold in native and water-annealed form, whereas *A. mylitta* silk film and ethanol-treated silk scaffolds display an inflammatory immune response profile. The AFM image illustrates the surface roughness of the respective silk scaffolds with poor cell attachment on *B. mori* silk film and increased adhesion on *A. mylitta* silk films. Reproduced with permission[72] Copyright 2022, American Chemical Society.

more effective than *B. mori* silk in inhibiting multinuclear giant cell formation while stimulating pro-remodeling macrophage activation.[106] On a similar line, Roy *et al.* have shown that at difference with *B. mori* silk braids, *A. mylitta* braids induce a pro-inflammatory M1 phenotype with the secretion of inflammatory cytokines by THP-1 cells.[72] On the other hand, in a study where *B. mori* and *A. mylitta* scaffolds were implanted into a calvarial bone defect without any bone-precursor cells (Fig. 5), the non-mulberry silk scaffold demonstrated enhanced bone regeneration with a slower degradation rate. However, extensive infiltration by immune cells, including lymphocytes, multinucleated giant cells and pro-inflammatory macrophages, was observed in the *B. mori* scaffold 3 months after implantation, leading to rapid implant degradation. The presence of macrophages was further corroborated by CD11b staining, identifying more $CD11b^+$ cells over the *B. mori* than over *A. mylitta* scaffolds.[82]

Our research group conducted a clinical trial with the implantation of *B. mori* and *A. mylitta* silk braids into the socket resulting from the extraction of maxillary anterior gum. An initial inflammatory phase for 1 week was observed for both silk implants. Still, the inflammation subsided earlier for *B. mori* braids, contributing to the preservation of the alveolar ridge and augmentation of the respective socket, thereby promoting healthy tissue regeneration.[107] In contrast, *mylitta* braids' implantation was associated with prolonged inflammation (Fig. 7). Therefore, variations in silk sources contribute to variable amino acid sequences and physiochemical properties responsible for differential host immune responses. Notably, *in vitro* and *in vivo* assays have also yielded contradictory findings. For instance, dragline silk isolated from *Nephila edulis* spider showed no cytotoxicity and inflammatory potential when cultured with neuronal cells *in vitro*. Still, it generated a chronic inflammatory immune response

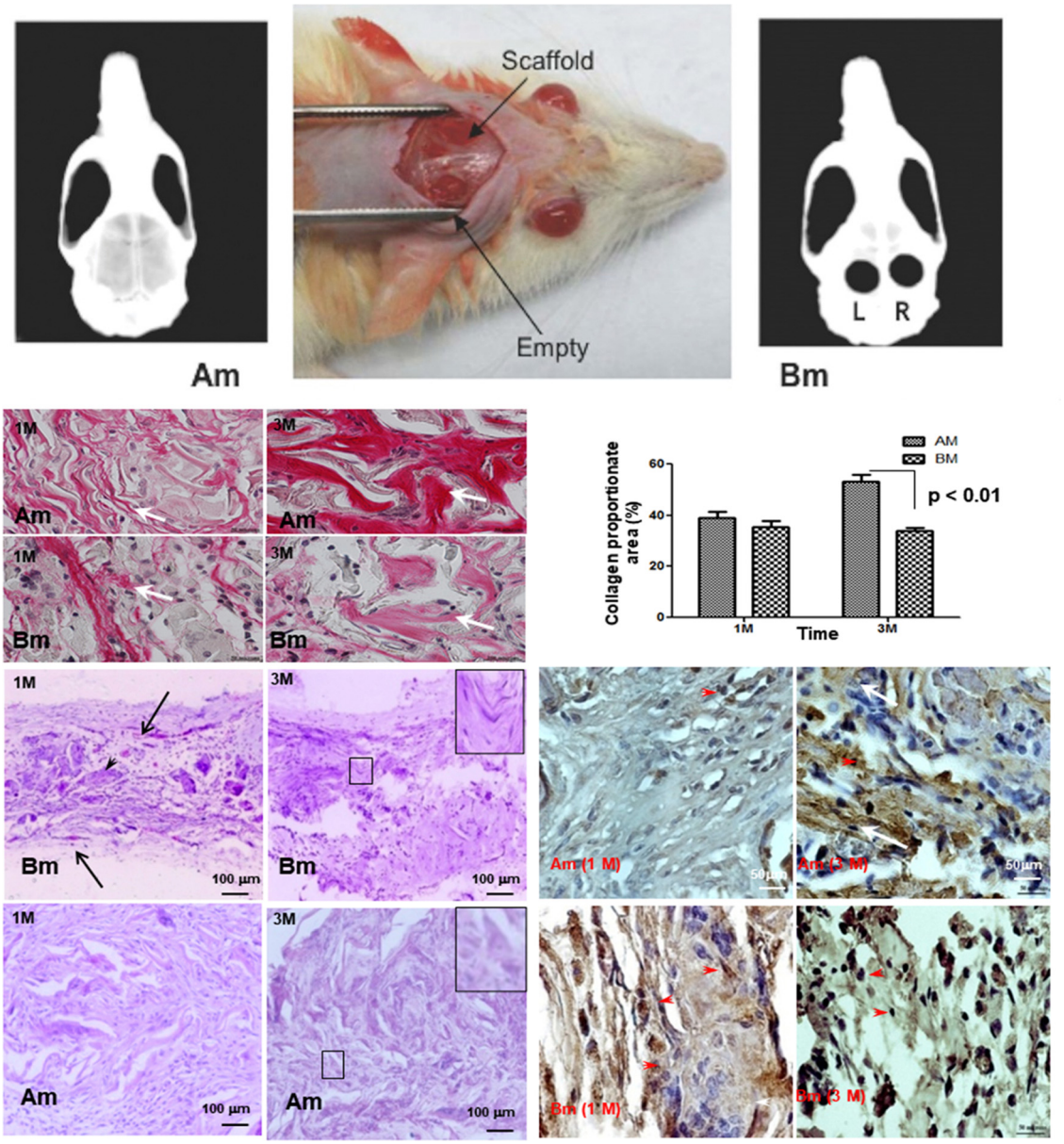

Fig. 5 Comparative analysis of immune response profiles induced by *A. mylitta* and *B. mori* scaffolds *in vivo* upon implantation in a calvarial defect rat model. The degradation rate of *B. mori* scaffolds was higher than that of *A. mylitta* scaffolds 12 months after implantation. The degradation could be attributed to increased infiltration by inflammatory immune cells like lymphocytes, macrophages and mononuclear giant cells into the defect site, already detectable after 3 months in the *B. mori* scaffold implantation defect with a formation of soft tissue. This was further validated by CD11b staining and counting the $CD11b^+$ cells, with a higher number in *the B. mori* group over *A. mylitta*, consistent with increased inflammatory immune response. At the same time, increased collagen intensity in the *A. mylitta* scaffold was observed, as compared to the *B. mori* group, denoting more effective bone regeneration *in vivo*. Reproduced with permission[82] Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

with granuloma tissue formation when implanted in a rat model.[108]

4.5. Processing conditions

Silk processing conditions also impact the biocompatibility of derived materials. For instance, the degumming process leads to the degradation of silk protein into low-molecular-weight products by disrupting inner intermolecular hydrogen and van der Waals bonds. Remarkably, a higher release of IL-1β and IL-6 cytokines by $CD14^+$ monocytes has been observed upon stimulation by fibroin films degummed for 15 min compared to fibroin film degummed for 60 min. These effects could most likely be due to fibroin films of higher molecular weight fragments due to lower protein degradation in samples with shorter degumming. However, no significant differences in TNF-α release were observed.[109]

Other studies have shown that sericin processing methods such as heat, acid, alkaline, and urea extraction producing fractions with different molecular weights may significantly impact biological responses to silk-based materials. Indeed,

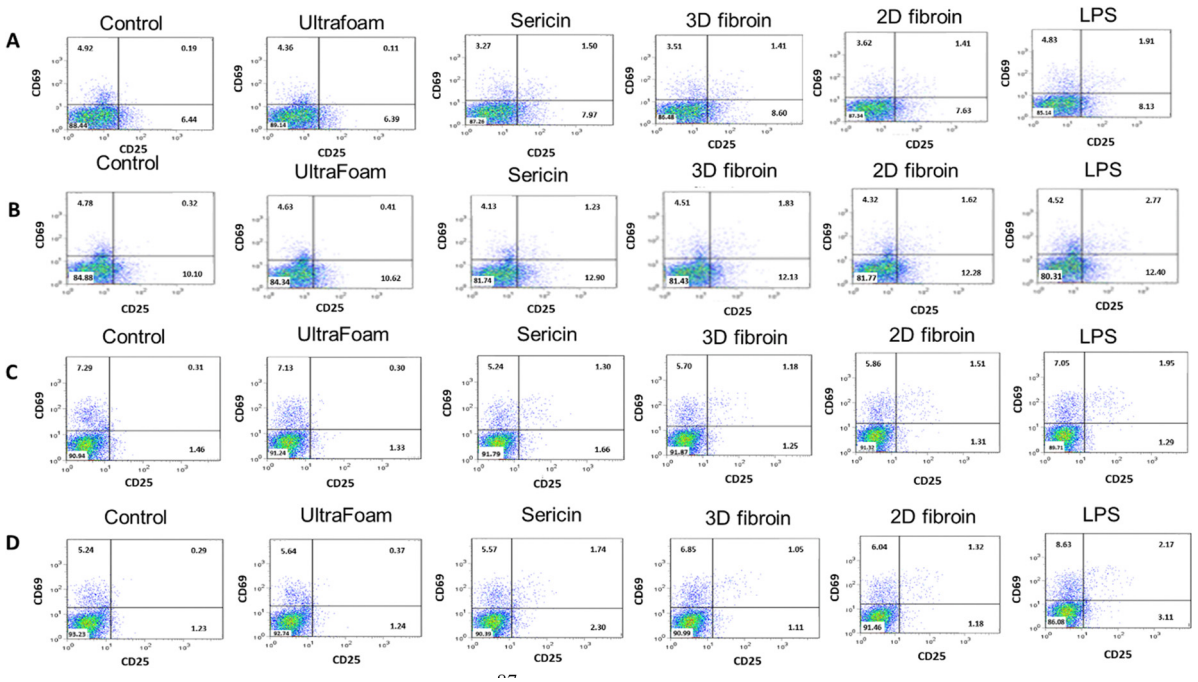

Fig. 6 The adaptive immune system response to silk-based biomaterials was investigated by culturing purified $CD4^+$ and $CD8^+$ T cells together with silk-based biomaterials in the presence of autologous $CD14^+$ cells as antigen-presenting cells. T-Cell activation was monitored by testing the expression of CD69 and CD25 lymphocyte activation markers. None of the biomaterials under investigation induced a significant upregulation of the phenotypic markers (CD25 and CD69).

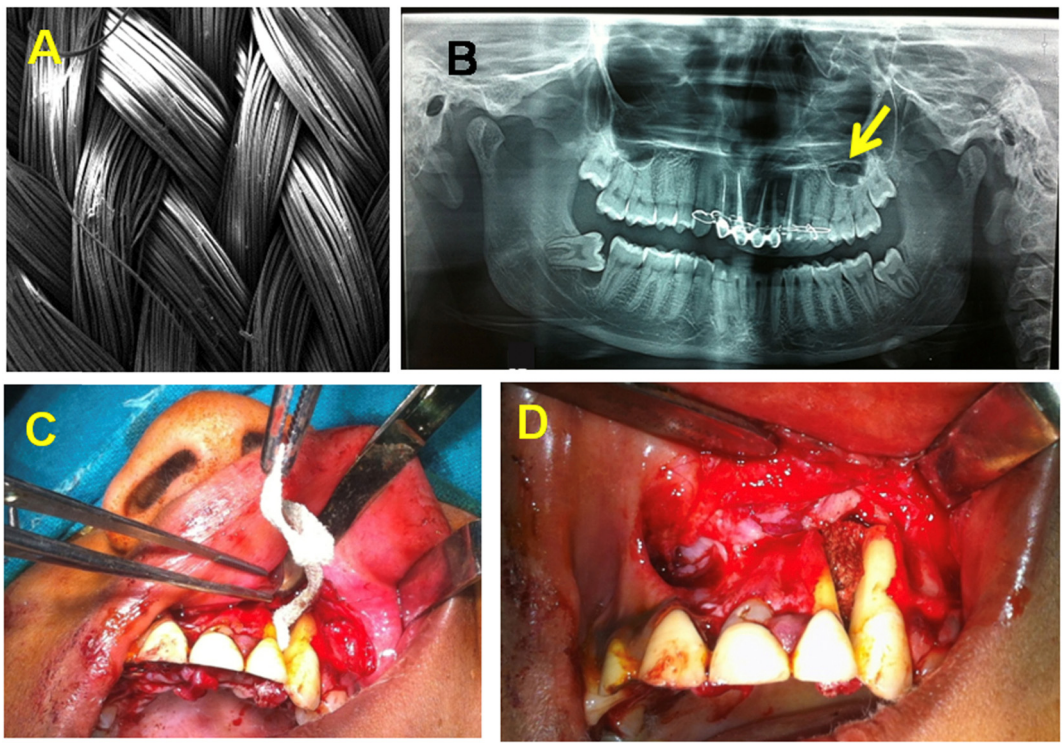

Fig. 7 *In vivo* implantation of silk braids (A) into the maxillary anterior tooth socket post-extraction (B)–(D). After implantation, both *B. mori* and *A. mylitta* braids cause inflammation for 1 week. However, *A. mylitta* implantation resulted in prolonged inflammation compared to *B. mori*. Reproduced with permission[107] Copyright 2017, Springer Nature.

the water-soluble fraction of sericin with a molecular mass ranging between 10 and 70 kDa and mostly containing polar amino acids, with hydroxyl and amino groups, including serine and lysine accounting for 72% of its MW, is devoid of immunogenicity. This water-soluble fraction of sericin was used to increase the affinity of L-asparaginase.[110] However, silk sericin peptides with higher molecular mass (approximately 100–300 kDa) were only soluble in hot water. In contrast, lower molecular mass peptides (<20 kDa) were readily soluble in cold water. Importantly, water-soluble silk sericin within a 15–60 kDa molecular weight range is not immunogenic and is as biocompatible as fibroin.[111]

Sericin processing by urea produces a 22.5–23 kDa MW polypeptide reportedly endowed with antimicrobial properties.[112] Heat-degraded and alkali-degraded sericin have been reported to be the most suitable forms for cell culture at an optimal concentration of 100 μg ml^{-1}. In addition, heat-treated sericin contains sulfur-rich amino acids (methionine and cysteine), inducing high levels of collagen production have high effectiveness in wound healing.[113]

5. Effect of silk material on the inflammatory/anti-inflammatory signaling pathways

As discussed above, sericin and fibroin's inflammatory and anti-inflammatory immune response profiles are closely associated with the up-and downregulation of several cell signaling pathways, including complement, NF-κβ, MAP kinase and TLR pathway.

A previous study from our research group demonstrated the predominant role of the 3D silk fibroin matrix in triggering the activation of NF-κβ and p38 MAP kinase pathways for the production of IL-6 pro-inflammatory cytokine.[37] Interrelation between NF-κβ and TLR activation was also observed, as also validated by Park *et al.*, who demonstrated the activation of the NF-κβ pathway by silk fibroin with expressions of IKKα, IKKβ, and p65 NF-κβ downstream targets.[114]

However, inhibiting the NF-κβ pathway by silk fibroin hydrolysates, hindering its activation ligand RANKL and MAPK cascade and inducing osteoclast apoptosis has also been reported.[115] In sharp contrast, sericin at a concentration of 1 mg ml^{-1} was sufficient to induce the pro-inflammatory NF-κβ pathway with enhanced expression levels of its downstream targets, IKKα and IKKβ.[116] The anti-inflammatory effects of sericin decrease the keratinocyte proliferation in psoriasis and control the mTOR and IL-17 signaling pathways.[117] Conversely, contradictory data have been reported regarding Wnt/β-catenin pathway activation by silk fibroin protein as both inflammatory and anti-inflammatory.[118,119]

Therefore, silk fibroin and silk sericin may activate or downregulate specific cell signaling pathways, playing crucial roles in the overall immunogenicity of silk materials (Fig. 2).

6. Strategies to modulate immunogenicity of silk-based biomaterials

6.1. Inflammation inhibitors

The use of small anti-inflammatory molecules could help improve biomaterials' tissue compatibility. Within this context, the possibility of inhibiting signal transduction induced in monocytes/macrophages in response to 3D silk-fibroin scaffolds has been investigated using polymyxin B (PMB), Pyrrolidine dithiocarbamate (PDTC), LY294002 (LY)-PI3 Kinase Inhibitor, and SB202190 (SB)-MAP Kinase Inhibitor. Polymyxin B is known to block lipopolysaccharide (LPS)-induced activation by specific lipid A binding, while PDTC blocks NF-κβ activation and SB202190 (SB) and LY294002 are p38 MAP and phosphatidylinositol 3-kinase (PI3K) inhibitors, respectively.

However, while Polymyxin B blocks IL-6 production induced by LPS stimulation, it fails to inhibit IL-6 production induced by sericin and 3D fibroin scaffolds.[120] Moreover, silk-based biomaterials stimulate the activation of pro-inflammatory NF-κβ and a sizeable production of IL-6 by $CD14^{+}$ cells in the presence of PDTC.

NF-κβ and p38 MAP kinase play crucial roles in monocyte activation by silk materials as indicated by the effects of specific inhibitors. However, SB202190 is more active than LY294002 in inhibiting IL-6 production. Moreover, LY294002 did not affect IL-1β production.[37] Since NF-κβ and p38 MAP kinase play significant roles in monocyte activation driven by Toll-like receptor (TLR) triggering, future research is warranted to clarify whether the immunostimulatory ability of 3D silk-based biomaterials also resides in their capacity to trigger selected TLR. Nevertheless, these reports suggest that inhibitors of specific inflammatory transduction pathways can be conjugated or immobilized into silk material scaffolds to limit or abolish short-term immune responses that may be induced post-implantation *in vivo.*

6.2. Chemical modification

Biocompatibility may be increased by surface modification or coating of implants. This has become a major area of research.[121,122] Hemocompatibility, a decisive factor in implant fates, is also being addressed to reduce protein adsorption on biomaterial surfaces.

Sulfation by chloro-sulfonic acid/pyridine has been used to increase the anticoagulant activity of silk fibroin. Sulfate groups were added to the hydroxyl moieties of Ser and Tyr residues in fibroin, and the anticoagulant activity of sulfated fibroin depended on the number of sulfate groups.[92,123] Sulfation increases surface wettability, reducing protein adsorption and platelet adhesion. This could be particularly interesting for sericin fractions with high molecular weight, which are known to be characterized by higher anticoagulant activity.[124]

In another study, the inflammatory response to silk fibroin materials was inhibited by tropoelastin incorporation, reducing IL-1β, IL-6, MMP-9, and MMP-2 release and acute and chronic

inflammatory response, thereby improving silk fibroin biocompatibility *in vivo*.[125] Other methods include the incorporation of anti-inflammatory biochemical mediators into the silk biomaterial through a wide range of covalent and non-covalent conjugation strategies. For instance, glucocorticoids suppress the production of several cytokines and chemokines.[126] Indeed, the incorporation of dexamethasone into the biomaterials has been reported to suppress the immune response by reducing the number of infiltrating macrophages and lymphocytes and the subsequent fibrous capsule formation.[127] The chemically modified silk biomaterial can then be processed with different biopolymers and cells for various tissue engineering applications.

6.3. Macrophage polarization as a marker for immunogenicity

Macrophages dictate biomaterial function upon implantation. However, macrophages may switch their polarization depending on local microenvironmental conditions.[25] This process can unravel mechanisms that might be exploited to minimize immunogenic responses.[128] For instance, tailoring the surface chemistry of biomaterials may restrict macrophage adhesion, activation, and fusion to FBGCs. Notably, the attachment of macrophages to hydrophilic and anionic biomaterial surfaces may provide fewer integrin-binding sites, thus promoting macrophage apoptosis.[129]

Altering surface chemistry, topography, and protein conformation of silk-based materials may help improve their biocompatibility. With an increasing understanding of the host responses to implants and the wound healing process, researchers have developed biomaterials with ''immunomodulating'' capacities favoring implant integration and healing without systemically affecting normal immune cell functions.

Macrophage plasticity originates from their diverse identities and variable activation states. The heterogenicity and versatility of macrophages in a particular tissue are due to their dynamic shift from the bone marrow progenitor cells. Renewed interest in recent years has identified the involvement of monocytes/macrophages besides the T- and B-lymphocytes in the implantation site in any graft rejection. Although macrophage activation, polarization and cytokine secretion are recognized as key determinants of inflammatory or non-inflammatory immune response to biomaterials, several aspects still need clarification.

Different forms of native and regenerative silk matrices have been used to identify specific adhesion/activation paradigms associated with immune response during longer periods. Surface roughness, β sheet content, crystallinity, and arginine have emerged as crucial players in silk biomaterial degradation and immune response. Moreover, prolonged (14 days) cytokine microarray analysis has revealed the intermediate plasticity of healthy monocytes on different native and regenerative biomaterial surface.[72] Capitalizing on this background, macrophage polarization towards a stable anti-inflammatory phenotype has been obtained using silk-elastin and silk-sericin-like protein through recombinant DNA technology.[130,131]

On the other hand, the role of tissue-resident macrophage, derived from embryonic hematopoietic progenitor cells, and their specific plasticity under healthy and pathological conditions are still unclear. Indeed, many reports suggest that tissue-resident and recruited macrophages have distinct origins. Moreover, whether biomaterial-mediated immune response is due to macrophage-mediated hemangiogenesis or lymphangiogenesis is still debated.

7. Conclusions

Silk has made a long way from the material of choice for emperors' garments to an integral component of innovative biomaterials for biomedical applications. However, for its use in clinical applications, biomaterials must either be immune-inert or able to generate anti-inflammatory responses without possessing any immuno-toxicity. In the case of silk proteins, both the sericin and fibroin components elicit ambivalent, mild inflammatory and anti-inflammatory immune responses. Such differential immune response profiles can be attributed to surface chemistry, protein conformation, source of origin, and association with another protein polymer. The existing literature reports provide decisive advances in identifying immune cells, factors, and signal transduction pathways involved in the response to silk-based implants. However, analyzing immune responsiveness to silk-based implants is still considered a 'nascent' research field, particularly compared to millennia-old textile technology.

Contradictory results should be interpreted within this context. As an example, it is somewhat difficult to compare ''*in vitro*'' studies with cells derived from ''differentiated'' established, transformed cell lines with studies performed with freshly derived cells. Moreover, the potential significance of different myeloid cell subsets and their ''*in vivo*'' location has rarely been addressed. In addition, *in vivo* models include, among others, topical, subcutaneous and rectal administration involving different types of potentially responding resident cells. Our analysis urges the development of standardized assays, allowing a more reliable assessment of the biocompatibility of silk-derived materials obtained by established procedures, with defined steps ranging from *in vitro* analysis to experimentation in clinically relevant *in vivo* models.

Within this context, tissue engineers, material scientists, and immunologists are working together to devise cutting-edge strategies to develop immunomodulatory silk-based biomaterials through a wide array of chemical modifications and macrophage profiling studies to steer the immunological fate of the biomaterial. Such advancements in the future will stand as yardsticks for silk-based biomedical implants to progress from mildly immunogenic materials to the safest immune-compatible devices.

Data availability

The data will be available with the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts to declare.

References

1 N. Majumder, S. Seit, N. S. Bhabesh and S. Ghosh, *Adv. Healthcare Mater.*, 2024, **13**, 2303513.
2 S. Midha, S. Murab and S. Ghosh, *Biomaterials*, 2016, **97**, 133–153.
3 J. Melke, S. Midha, S. Ghosh, K. Ito and S. Hofmann, *Acta Biomater.*, 2016, **31**, 1–16.
4 W. Zhang, L. Chen, J. Chen, L. Wang, X. Gui, J. Ran, G. Xu, H. Zhao, M. Zeng, J. Ji, L. Qian, J. Zhou, H. Ouyang and X. Zou, *Adv. Healthcare Mater.*, 2017, **6**, 1700121.
5 N. Majumder, A. Mishra and S. Ghosh, *Bioprinting*, 2022, **28**, e00241.
6 N. Majumder, C. Roy, L. Doenges, I. Martin, A. Barbero and S. Ghosh, *ACS Appl. Mater. Interfaces*, 2024, **16**, 9925–9943.
7 P. Admane, A. C. Gupta, P. Jois, S. Roy, C. C. Lakshmanan, G. Kalsi, B. Bandyopadhyay and S. Ghosh, *Bioprinting*, 2019, **15**, e00051.
8 A. Sharma, P. Rawal, D. M. Tripathi, D. Alodiya, S. K. Sarin, S. Kaur and S. Ghosh, *ACS Biomater. Sci. Eng.*, 2021, **7**, 3861–3873.
9 A. Remes and D. F. Williams, *Biomaterials*, 1992, **13**, 731–743.
10 R. A. Villarreal-Leal, G. D. Healey and B. Corradetti, *Adv. Drug Delivery Rev.*, 2021, **179**, 113913.
11 E. Mariani, G. Lisignoli, R. M. Borzì and L. Pulsatelli, *Int. J. Mol. Sci.*, 2019, **20**, 636.
12 A. Vishwakarma, N. S. Bhise, M. B. Evangelista, J. Rouwkema, M. R. Dokmeci, A. M. Ghaemmaghami, N. E. Vrana and A. Khademhosseini, *Trends Biotechnol.*, 2016, **34**, 470–482.
13 M. B. Gorbet and M. V. Sefton, *Biomaterials*, 2004, **25**, 219–241.
14 C. J. Wilson, R. E. Clegg, D. I. Leavesley and M. J. Pearcy, *Tissue Eng.*, 2005, **11**, 1–18.
15 R. Zhuo, C. A. Siedlecki and E. A. Vogler, *Biomaterials*, 2006, **27**, 4325–4332.
16 E. A. Vogler and C. A. Siedlecki, *Biomaterials*, 2009, **30**, 1857–1869.
17 L. Tang, *J. Biomater. Sci., Polym. Ed.*, 1998, **0**, 1257–1266.
18 D. Perala, R. Chapman and J. Gelfand, *Int. J. Oral. Maxillofac Implants*, 1991, **6**, 136–141.
19 Y. Mödinger, G. Q. Teixeira, C. Neidlinger-Wilke and A. Ignatius, *Int. J. Mol. Sci.*, 2018, **19**, 3367.
20 J. Andersson, K. N. Ekdahl, R. Larsson, U. R. Nilsson and B. Nilsson, *J. Immunol.*, 2002, **168**, 5786–5791.
21 G. Nimeri, L. Öhman, H. Elwing, J. Wetterö and T. Bengtsson, *Biomaterials*, 2002, **23**, 1785–1795.
22 G. Nimeri, M. Majeed, H. Elwing, L. Öhman, J. Wetterö and T. Bengtsson, *J. Biomed. Mater. Res., Part A*, 2003, **67**, 439–447.
23 P. Krzyszczyk, R. Schloss, A. Palmer and F. Berthiaume, *Front. Physiol.*, 2018, **9**, 419.
24 H. Zhang, Z. Li and W. Li, *Front. Immunol.*, 2021, **12**, 648539.
25 R. Sridharan, A. R. Cameron, D. J. Kelly, C. J. Kearney and F. J. O'Brien, *Mater. Today*, 2015, **18**, 313–325.
26 Y. T. Konttinen, J. Pajarinen, Y. Takakubo, J. Gallo, C. Nich, M. Takagi and S. B. Goodman, *J. Long Term Eff. Med. Implants*, 2014, **24**, 259–265.
27 Z. Sheikh, P. J. Brooks, O. Barzilay, N. Fine and M. Glogauer, *Materials*, 2015, **8**, 5671–5701, 28793529.
28 C. Vepari and D. L. Kaplan, *Progress Polym. Sci.*, 2007, **32**, 991–1007.
29 H. K. Soong and K. R. Kenyon, *Ophthalmology*, 1984, **91**, 479–483.
30 C. Wen, S. Ye, L. Zhou and Y. Yu, *Ann. Allergy*, 1990, **65**, 375–378.
31 S. Kurosaki, H. Otsuka, M. Kunitomo, M. Koyama, R. Pawankar and K. Matumoto, *Nippon Ika Daigaku Zasshi*, 1999, **66**, 41–44.
32 M. Dewair, X. Baur and K. Ziegler, *J. Allergy Clin. Immunol.*, 1985, **76**, 537–542.
33 Y. K. Seo, H. H. Yoon, K. Y. Song, S. Y. Kwon, H. S. Lee, Y. S. Park and J. K. Park, *J. Orthop. Res.*, 2009, **27**, 495–503.
34 C. Cassinelli, G. Cascardo, M. Morra, L. Draghi, A. Motta and G. Catapano, *Int. J. Artif. Organs*, 2006, **29**, 881–892.
35 L. Lamboni, M. Gauthier, G. Yang and Q. Wang, *Biotechnol. Adv.*, 2015, **33**, 1855–1867.
36 J. M. Anderson, A. Rodriguez and D. T. Chang, *Semin. Immunol.*, 2008, **20**, 86–100.
37 M. Bhattacharjee, E. Schultz-Thater, E. Trella, S. Miot, S. Das, M. Loparic, A. R. Ray, I. Martin, G. C. Spagnoli and S. Ghosh, *Biomaterials*, 2013, **34**, 8161–8171.
38 Y. Q. Zhang, *Biotechnol. Adv.*, 2002, **20**, 91–100.
39 R. Dash, C. Acharya, P. C. Bindu and S. C. Kundu, *J. Biochem. Mol. Biol.*, 2008, **41**, 236–241.
40 S. Zhaorigetu, N. Yanaka, M. Sasaki, H. Watanabe and N. Kato, *Oncol. Rep.*, 2003, **10**, 537–543.
41 S. Zhaorigetu, N. Yanaka, M. Sasaki, H. Watanabe and N. Kato, *J. Photochem. Photobiol., B*, 2003, **71**, 11–17.
42 P. Aramwit, S. Kanokpanont, T. Nakpheng and T. Srichana, *Int. J. Mol. Sci.*, 2010, **11**, 2200–2211.
43 P. Aramwit and A. Sangcakul, *Biosci., Biotechnol., Biochem.*, 2007, **71**, 2473–2477.
44 W. Zaoming, R. Codina, E. Fernández-Caldas and R. F. Lockey, *J. Invest. Allergol. Clin. Immunol.*, 1996, **6**, 237–241.
45 J. C. Celedón, L. J. Palmer, X. Xu, B. Wang, Z. Fang and S. T. Weiss, *Pediatrics*, 2001, **107**, E50.
46 P. Aramwit, S. Kanokpanont, W. De-Eknamkul and T. Srichana, *J. Biosci. Bioeng.*, 2009, **107**, 556–561.
47 B. Panilaitis, G. H. Altman, J. Chen, H. J. Jin, V. Karageorgiou and D. L. Kaplan, *Biomaterials*, 2003, **24**, 3079–3085.
48 B. C. Dash, B. B. Mandal and S. C. Kundu, *J. Biotechnol.*, 2009, **144**, 321–329.
49 Y. Wang, D. Yao, L. Li, L. Li, Z. Qian, W. He, R. Ding, H. Liu, H. Liu and Y. Fan, *ACS Biomater. Sci. Eng.*, 2020, **6**, 3502–3512.

50 C. R. Uff, A. D. Scott, A. G. Pockley and R. K. S. Phillips, *Biomaterials*, 1995, **16**, 355–360.
51 Y. Zhang, N. Shi, L. He, S. Wang, X. Li, S. Lu, Q. Zhang and H. Niu, *J. Biomed. Nanotechnol.*, 2021, **17**, 2433–2443.
52 E. Rossitch, D. E. Bullard and W. J. Oakes, *Child's Nervous System*, 1987, **3**, 375–378.
53 O. Akturk, A. Tezcaner, H. Bilgili, M. S. Deveci, M. R. Gecit and D. Keskin, *J. Biosci. Bioeng.*, 2011, **112**, 279–288.
54 P. Aramwit, S. Kanokpanont, W. De-Eknamkul, K. Kamei and T. Srichana, *J. Biomater. Sci., Polym. Ed.*, 2009, **20**, 1295–1306.
55 Y. Sun, W. Shi, Q. Zhang, H. Guo, Z. Dong, P. Zhao and Q. Xia, *Int. J. Mol. Sci.*, 2022, **24**, 259.
56 J. H. Cherng, S. J. Chang, Y. K. Chiu, Y. H. Chiu, T. J. Fang and H. C. Chen, *Front. Bioeng. Biotechnol.*, 2022, **10**, 925197.
57 K. Chachlioutaki, C. Karavasili, E. Adamoudi, N. Bouropoulos, D. Tzetzis, A. Bakopoulou and D. G. Fatouros, *Biomater. Adv.*, 2022, **133**, 112723.
58 P. Aramwit, P. Towiwat and T. Srichana, *Nat. Prod. Commun.*, 2013, **8**, 501–504.
59 F. Munir, H. M. Tahir, S. Ali, A. Ali, A. Tehreem, S. D. E. S. Zaidi, M. Adnan and F. Ijaz, *ACS Omega*, 2023, **8**, 32090–32098.
60 Z. Jiao, Y. Song, Y. Jin, C. Zhang, D. Peng, Z. Chen, P. Chang, S. C. Kundu, G. Wang, Z. Wang and L. Wang, *Macromol. Biosci.*, 2017, **17**, 1700229.
61 S. Sapru, S. Das, M. Mandal, A. K. Ghosh and S. C. Kundu, *Acta Biomater.*, 2018, **78**, 137–150.
62 T. Siritientong, A. Angspatt, J. Ratanavaraporn and P. Aramwit, *Pharm. Res.*, 2014, **31**, 104–116.
63 K. Tanaka, S. Inoue and S. Mizuno, *Insect Biochem. Mol. Biol.*, 1999, **29**, 269–276.
64 P. Dubey, S. Murab, S. Karmakar, P. K. Chowdhury and S. Ghosh, *Biomacromolecules*, 2015, **16**, 3936–3944.
65 R. L. Horan, K. Antle, A. L. Collette, Y. Wang, J. Huang, J. E. Moreau, V. Volloch, D. L. Kaplan and G. H. Altman, *Biomaterials*, 2005, **26**, 3385–3393.
66 R. E. Unger, K. Peters, M. Wolf, A. Motta, C. Migliaresi and C. J. Kirkpatrick, *Biomaterials*, 2004, **25**, 5137–5146.
67 L. Meinel, S. Hofmann, V. Karageorgiou, C. Kirker-Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. Vunjak-Novakovic and D. L. Kaplan, *Biomaterials*, 2005, **26**, 147–155.
68 M. Santin, A. Motta, G. Freddi and M. Cannas, *J. Biomed. Mater. Res.*, 1999, **46**, 382–389.
69 A. E. Thurber, F. G. Omenetto and D. L. Kaplan, *Biomaterials*, 2015, **71**, 145–157.
70 J. M. Anderson, *ASAIO Trans.*, 1988, **34**, 101–107.
71 C. H. Gemmell, *J. Biomed. Mater. Res.*, 1997, **37**, 474–480.
72 S. Roy, A. Sharma and S. Ghosh, *ACS Biomater. Sci. Eng.*, 2022, **8**, 659–671.
73 S. Enomoto, M. Sumi, K. Kajimoto, Y. Nakazawa, R. Takahashi, C. Takabayashi, T. Asakura and M. Sata, *J. Vasc. Surg.*, 2010, **51**, 155–164.
74 S. Ghanaati, R. E. Unger, M. J. Webber, M. Barbeck, C. Orth, J. A. Kirkpatrick, P. Booms, A. Motta, C. Migliaresi, R. A. Sader and C. J. Kirkpatrick, *Biomaterials*, 2011, **32**, 8150–8160.
75 J. Rnjak-Kovacina, L. S. Wray, J. M. Golinski and D. L. Kaplan, *Adv. Funct. Mater.*, 2014, **24**, 2188–2196.
76 O. Etienne, A. Schneider, J. A. Kluge, C. Bellemin-Laponnaz, C. Polidori, G. G. Leisk, D. L. Kaplan, J. A. Garlick and C. Egles, *J. Periodontol.*, 2009, **80**, 1852–1858.
77 I. Dal Pra, G. Freddi, J. Minic, A. Chiarini and U. Armato, *Biomaterials*, 2005, **26**, 1987–1999.
78 P. W. Madden, I. Klyubin and M. J. Ahearne, *BMJ Open Ophthalmol.*, 2020, **5**, e000510.
79 J. Zhou, C. Cao, X. Ma, L. Hu, L. Chen and C. Wang, *Polym. Degrad. Stab.*, 2010, **95**, 1679–1685.
80 Y. Wang, D. D. Rudym, A. Walsh, L. Abrahamsen, H. J. Kim, H. S. Kim, C. Kirker-Head and D. L. Kaplan, *Biomaterials*, 2008, **29**, 3415–3428.
81 H. Fan, H. Liu, S. L. Toh and J. C. H. Goh, *Biomaterials*, 2009, **30**, 4967–4977.
82 N. Sahu, P. Baligar, S. Midha, B. Kundu, M. Bhattacharjee, S. Mukherjee, S. Mukherjee, F. Maushart, S. Das, M. Loparic, S. C. Kundu, S. Ghosh and A. Mukhopadhyay, *Adv. Healthcare Mater.*, 2015, **4**, 1709–1721.
83 S. H. Tran, C. G. Wilson and F. P. Seib, *Pharm. Res.*, 2018, **35**, 248.
84 C. E. Kim, J. H. Lee, Y. K. Yeon, C. H. Park and J. Yang, *Sci. Rep.*, 2017, **7**, 44364.
85 F. H. Holanda, A. N. Ribeiro, B. L. Sánchez-Ortiz, G. C. de Souza, S. F. Borges, A. M. Ferreira, A. C. Florentino, S. A. Yoshioka, L. S. Moraes, J. C. T. Carvalho and I. M. Ferreira, *Biotechnol. Lett.*, 2023, **45**, 235–253.
86 A. Rodriguez-Nogales, F. Algieri, L. De Matteis, A. A. Lozano-Perez, J. Garrido-Mesa, T. Vezza, J. M. de la Fuente, J. L. Cenis, J. Gálvez and M. E. Rodriguez-Cabezas, *Int. J. Nanomed.*, 2016, **11**, 5945–5958.
87 M. Bartneck, K. H. Heffels, Y. Pan, M. Bovi, G. Zwadlo-Klarwasser and J. Groll, *Biomaterials*, 2012, **33**, 4136–4146.
88 J. K. Yun, K. DeFife, E. Colton, S. Stack, A. Azeez, L. Cahalan, M. Verhoeven, P. Cahalan and J. M. Anderson, *J. Biomed. Mater. Res.*, 1995, **29**, 257–268.
89 H. Gelb, H. Ralph Schumacher, J. Cuckler and D. G. Baker, *J. Orthop. Res.*, 1994, **12**, 598.
90 P. H. Wooley, R. Morren, J. Andary, S. Sud, S. Y. Yang, L. Mayton, D. Markel, A. Sieving and S. Nasser, *Biomaterials*, 2002, **23**, 517–526.
91 M. Zimecki and D. R. Webb, *Clin. Immunol. Immunopathol.*, 1978, **9**, 75–79, 336252.
92 Y. Tamada, *Biomaterials*, 2004, **25**, 377–383.
93 Q. Lu, S. Zhang, K. Hu, Q. Feng, C. Cao and F. Cui, *Biomaterials*, 2007, **28**, 2306–2313.
94 A. Motta, C. Migliaresi, A. W. Lloyd, S. P. Denyer and M. Santin, *J. Bioact. Compat. Polym.*, 2002, **17**, 23–35.
95 A. Motta, D. Maniglio, C. Migliaresi, H. J. Kim, X. Wan, X. Hu and D. L. Kaplan, *J. Biomater. Sci., Polym. Ed.*, 2009, **20**, 1875–1897.

96 B. Kundu, C. J. Schlimp, S. Nürnberger, H. Redl and S. C. Kundu, *Sci. Rep.*, 2014, **4**, 4945.
97 F. P. Seib, M. F. Maitz, X. Hu, C. Werner and D. L. Kaplan, *Biomaterials*, 2012, **33**, 1017–1023.
98 J. Sapudom, M. Kongsema, A. Methachittipan, S. Damrongsakkul, S. Kanokpanont, J. C. M. Teo, M. Khongkow, K. Tonsomboon and P. Thongnuek, *J. Mater. Chem. B*, 2023, **11**, 3607–3616.
99 B. B. Mandal, A. Grinberg, E. S. Gil, B. Panilaitis and D. L. Kaplan, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 7699–7704.
100 N. Yang, M. J. Moore, P. L. Michael, M. Santos, Y. T. Lam, S. Bao, M. K. C. Ng, J. Rnjak-Kovacina, R. P. Tan and S. G. Wise, *Adv. Healthcare Mater.*, 2021, **10**, e2100615.
101 W. H. Park, W. S. Ha, H. Ito, T. Miyamoto, H. Inagaki and Y. Noishiki, *Fibers Polym.*, 2001, **2**, 58–63.
102 F. P. Seib, M. F. Maitz, X. Hu, C. Werner and D. L. Kaplan, *Biomaterials*, 2012, **33**, 1017–1023.
103 C. Maas, S. Hermeling, B. Bouma, W. Jiskoot and M. F. B. G. Gebbink, *J. Biol. Chem.*, 2007, **282**, 2229–2236.
104 J. S. Rudra, Y. F. Tian, J. P. Jung and J. H. Collier, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 622–627.
105 F. Oyama, S. Mizuno and K. Shimura, *J. Biochem.*, 1984, **96**, 1689–1694.
106 G. Janani, L. Zhang, S. F. Badylak and B. B. Mandal, *Biomater. Adv.*, 2023, **145**, 213223.
107 B. Basu and S. Ghosh, *Biomaterials for musculoskeletal regeneration*, Springer, 2017.
108 F. Koop, S. Strauß, C. T. Peck, T. Aper, M. Wilhelmi, C. Hartmann, J. Hegermann, J. Schipke, P. M. Vogt and V. Bucan, *PLoS One*, 2022, **17**, e0264486.
109 L. S. Wray, X. Hu, J. Gallego, I. Georgakoudi, F. G. Omenetto, D. Schmidt and D. L. Kaplan, *J. Biomed. Mater. Res., Part B*, 2011, **99**, 89–101.
110 Y. Q. Zhang, M. L. Tao, W. De Shen, J. P. Mao and Y. H. Chen, *J. Chem. Technol. Biotechnol.*, 2006, **81**, 136–145.
111 Y. Q. Zhang, Y. Ma, Y. Y. Xia, W. De Shen, J. P. Mao and R. Y. Xue, *J. Controlled Release*, 2006, **115**, 307–315.
112 M. Žurovec, C. Yang, D. Kodrík and F. Sehnal, *J. Biol. Chem.*, 1998, **273**, 15423–15428.
113 P. Aramwit, S. Kanokpanont, P. Punyarit and T. Srichana, *Wounds*, 2009, **21**, 198–206.
114 Y. R. Park, M. T. Sultan, H. J. Park, J. M. Lee, H. W. Ju, O. J. Lee, D. J. Lee, D. L. Kaplan and C. H. Park, *Acta Biomater.*, 2018, **67**, 183–195.
115 J. W. Chon, H. Kim, H. N. Jeon, K. Park, K. G. Lee, J. H. Yeo, H. Kweon, H. S. Lee, Y. Y. Jo and Y. K. Park, *Int. J. Mol. Med.*, 2012, **30**, 1203–1210.
116 H. L. Jeong, E. Bin Kang, S. G. Yun, D. B. Park, J. O. Lim and J. S. Suh, *Appl. Sci.*, 2022, **13**, 288.
117 P. Aramwit, K. Fongsodsri, K. Tuentam, O. Reamtong, T. Thiangtrongjit, T. Kanjanapruthipong, V. K. Yadavalli and S. Ampawong, *Sci. Rep.*, 2023, **13**, 12133.
118 S. Chawla, G. Desando, E. Gabusi, A. Sharma, D. Trucco, J. Chakraborty, C. Manferdini, M. Petretta, G. Lisignoli and S. Ghosh, *J. Mater. Res.*, 2021, 1–17.
119 B. Ma and M. O. Hottiger, *Front. Immunol.*, 2016, **7**, 378.
120 Y. Cheng, J. Du, J. Han, W. Sun, F. Gao, P. Zhang, H. Zhao, M. Chen, J. Wang, M. Wang, S. Dong, D. Sun, Y. Zhang, J. Cui, J. Cai and C. Liu, *Cell. Physiol. Biochem.*, 2017, **42**, 1120–1126.
121 E. M. Hetrick and M. H. Schoenfisch, *Chem. Soc. Rev.*, 2006, **35**, 780–789.
122 J. S. Lewis, T. D. Zaveri, C. P. Crooks and B. G. Keselowsky, *Biomaterials*, 2012, **33**, 7221–7232.
123 Y. Tamada, *J. Appl. Polym. Sci.*, 2003, **87**, 2377–2382.
124 Y. Tamada, M. Sano, K. Niwa, T. Imai and G. Yoshino, *J. Biomater. Sci., Polym. Ed.*, 2004, **15**, 971–980.
125 H. Liu, S. G. Wise, J. Rnjak-Kovacina, D. L. Kaplan, M. M. M. Bilek, A. S. Weiss, J. Fei and S. Bao, *Biomaterials*, 2014, **35**, 5138–5147.
126 D. Franchimont, T. Kino, J. Galon, G. U. Meduri and G. Chrousos, *Neuroimmunomodulation*, 2002, **10**, 247–260.
127 S. D. Patil, F. Papadmitrakopoulos and D. J. Burgess, *J. Controlled Release*, 2007, **117**, 68–79.
128 J. Chakraborty, S. Roy, S. Murab, R. Ravani, K. Kaur, S. Devi, D. Singh, S. Sharma, S. Mohanty, A. K. Dinda, R. Tandon and S. Ghosh, *ACS Biomater. Sci. Eng.*, 2019, **5**, 165–179.
129 W. G. Brodbeck, J. Patel, G. Voskerician, E. Christenson, M. S. Shive, Y. Nakayama, T. Matsuda, N. P. Ziats and J. M. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 10287–10292.
130 T. Unzai, T. Washisaka and Y. Tabata, *ACS Appl. Bio Mater.*, 2022, **5**, 5657–5664.
131 S. Gou, W. Meng, A. C. Panayi, R. Wang, R. Zhang, P. Gao, T. He, W. Geng, S. Hu, Y. Yu, Q. Feng and K. Cai, *Adv. Funct. Mater.*, 2023, **33**, 2213867.

