

Silk fibroin safety in the eye: a review that highlights a concern

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ABSTRACT

The biomedical use of silk as a suture dates back to antiquity. Fibroin is the structural element that determines the strength of silk and here we consider the safety of fibroin in its role in ophthalmology. The high mechanical strength of silk meant sufficiently thin threads could be made for eye microsurgery, but such usage was all but superseded by synthetic polymer sutures, primarily because silk in its entirety was more inflammatory. Significant immunological response can normally be avoided by careful manufacturing to provide high purity fibroin, and it has been utilised in this form for tissue engineering an array of fibre and film substrata deployed in research with cells of the eye. Films of fibroin can also be made transparent, which is a required property in the visual pathway. Transparent layers of corneal epithelial, stromal and endothelial cells have all been demonstrated with maintenance of phenotype, as have constructs supporting retinal cells. Fibroin has a lack of demonstrable infectious agent transfer, an ability to be sterilised and prepared with minimal contamination, long-term predictable degradation and low direct cytotoxicity. However, there remains a known ability to be involved in amyloid formation and potential amyloidosis which, without further examination, is enough to currently question whether fibroin should be employed in the eye given its innervation into the brain.

INTRODUCTION

Silks are natural proteins. Most silk proteins are elongated molecules, belonging to the group of fibrous proteins that also includes collagens. Fibrous proteins have their polypeptide chains arranged in parallel along a single axis. Silks all contain highly repetitive amino acid sequences with ordered secondary-structure regions. This homogeneity leads to outstanding mechanical properties and remarkable functional performance.

Silk has been used for millennia as suture,¹ being accepted over the ages as a useful material that is generally safe to be used in the human body.² This allowed silk to gain regulatory approval, essentially on real-world evidence. While this follows a risk-assessment path, whereby a lack of known previous problems supports further use, a biomaterial should be reviewed again as scientific knowledge increases, potentially expanding the range of safety issues. Here we carry out such

a review on silk in ophthalmology to consider its safety in the eye.

THE ORIGIN AND APPLICATION OF SILK FIBROIN IN THE EYE

Silks are produced primarily by species of *Euarthropoda*, such as moths, spiders and centipedes. In general, the silk components are stored in the organism as liquids, which are then configured into fibres on extrusion. The number of natural silks is very large, as is the variation in their chemical composition, structure and properties. Human usage of silk has been contingent on the ease of its collection; spider silk fibres are stronger, but it is difficult to collect in large quantity and spiders cannot be domesticated. Some moths on the other hand, spin silk fibres to form a cocoon to protect the pupae and these cocoons can be collected in abundance. This type of silk has been valued for textile production for millennia and the wild silk moth was putatively domesticated to *Bombyx mori* to further ease supply.

As expected, the silk produced by the *B. mori* silkworm has been the most investigated. Each silk fibre consists of two core monofilaments made of the protein fibroin coated and glued together primarily by another protein, sericin. The strength lies in the fibroin monofilaments which have in this species a repeating primary sequence of Gly-Ala-Gly-Ala-Gly-Ser hexapeptide, with a three-step higher-order periodicity. This regularity determines the final strength, in conjunction with the proportions of the three molecular conformations dominating its macromolecular secondary structure of anti-parallel pleated sheets (β -sheets), α -helices or random coils.

Silk usage as suture arises from the strength of the silk fibre and the ease with which it can be spun into a thread. The silk suture was valued because of its lack of memory, good workability and knot tying security. As early as 1883, Kuhnt³ was advocating the use of silk for corneal sutures and silk also became the most prominent suture in general surgery.



Following the seminal work of Minoura *et al*,⁴ fibroin has now also been considered for many further biomedical applications,⁵ including those in ophthalmology.⁶ These range from the cornea, where differentiated epithelial cells,⁷ including those in a limbal construct,⁸ stromal keratocytes⁹ and endothelial cells¹⁰ have been grown on fibroin, through to a construct of film and sponge stacks of fibroin that incorporated three cell types of corneal nerve, epithelium and stromal cells.¹¹ In the retina, fibroin has been used to fabricate a substitute Bruch's membrane.¹²

As well as growing cells on fibroin, silk-derived proteins have been used for ocular wound healing to enhance corneal epithelial cell growth media.¹³ Furthermore, silk has been used for ocular drug delivery, with a hydrogel for slow-release of bevacizumab in a rabbit model for the treatment of age-related macular degeneration and for the introduction of cellular growth factors.¹⁴ Silk ocular prostheses are also advocated for corneal reshaping to restore visual acuity.¹⁵

THE SAFETY OF SILK

Sterilisation and decontamination

Biomaterials need to be sterile, not only to prevent the transmission of infectious agents from the source, but also to remove contaminant microbes gained during processing. Silk can be sterilised by all three of the standard methods of ethylene oxide, autoclaving or gamma irradiation, although with differing but predictable effects on chemical and physical structure. Increasing the irradiation dose can be used to speed the degradation rate, while steam sterilisation can make stronger, stiffer films that change cell attachment.¹⁶

By using water-based solvents to reconstitute fibroin,¹⁷ processing contamination risk can be limited to simple salts that can be reduced to very low levels by extensive dialysis against water.¹⁸ Only traces of degumming agents such as calcium carbonate and solvents such as lithium bromide should remain.⁷ Such an 'all green' chemistry¹⁷ approach has been used with silk fibroin wound-healing products.¹⁹

Infectious risk from organisms or viruses

The non-mammalian origin of silk confers a distinct advantage. Biomaterials such as collagen require their animal sources to be screened to reduce opportunity for transmission of mammalian diseases. In contrast, moths are not mammalian and they do not feed on mammalian blood, so they do not pose a zoonosis risk. A small group of insect retroviruses may be similar to mammalian retroviruses,²⁰ but there has been no documented disease in humans.

Silk is also a secretion that does not contain cellular elements that might sustain infection, indeed the European Economic Community does not classify silk to be an animal product. Taking all these aspects together, silk has a low infectious risk status, even before it is processed and sterilised. Processing can further reduce risk, as the

fibroin is dissolved, often purified by dialysis, and then reconstituted.¹⁸ Dissolving customarily involves lengthy boiling in a strongly alkaline chaotropic solution, such as lithium bromide, or the use of a strong acid or ionic liquid, often combined with filtration or centrifugation. Such treatments are effective decontamination processes in their own right.

Inflammatory response

Silk sutures have been used extensively in ophthalmology, first as so-called 'virgin silk', where the sericin coat is all but left intact and the fibres twisted together to the required thickness, then as 'black braided silk'. To produce black braided silk, the sericin coating is first removed by degumming, whereby the peptide bonds of the sericin are broken by acidic or alkaline hydrolytic or enzymatic treatment, usually along with heat,¹⁸ and the sericin washed off. The resulting fibrils of fibroin can then be spun into thread. Ophthalmic surgery is generally microsurgery, and monofilaments made of nylon or polypropylene were developed with enough strength for this purpose.

These synthetic alternatives displaced most of the use of silk, with adverse immunological reaction cited as the primary disadvantage of using virgin silk in the eye,²¹ although rabbit studies showed little difference between materials.²² Severe reaction from second cataract surgery suggested a possible role of prior sensitisation from a first encounter with virgin silk, with less dramatic reaction with black braided suture.²³ Silk suture usually elicits only a minimal acute inflammatory reaction which involves infiltrative migration of polymorphonuclear leucocytes, which is followed by gradual encapsulation of the suture by fibrous connective tissue. A fibroin film placed into a corneal stromal pocket in the rabbit was well tolerated, with after 6 months only a few lymphocytes and some activated keratocytes.²⁴ These data suggest that purified silk fibroin may produce a lesser response compared with the virgin silk suture. A murine model of dry eye suggested that a silk fibroin solution even has anti-inflammatory effects.²⁵

Recipient sensitisation

Sensitisation to virgin silk, and therefore sericin, has been repeatedly reported, including in ophthalmology.²³ However, despite the past widespread use of silk suture throughout the body, there have been few reports of delayed hypersensitivity.^{26 27} Now that silk usage is less common, generally only older patients will have prior sensitisation risk and there remains the option to carry out a preoperative allergenic skin test.

The conundrum of fibroin and sericin combined

Clinically, less corneal inflammation occurred when using black braided rather than virgin silk, which has a higher sericin content²³ and it was taken that sericin was inflammatory.⁵ However, sericin did not activate murine macrophages *in vitro* by itself, although it did have a

synergistic effect with bacterial lipopolysaccharide.²⁸ There was also low immunogenicity *in vivo* with mice, and Jiao *et al.*²⁹ reviewing previous studies, concluded they were mostly only suggestive of adverse reaction, and that subsequent improper referencinwas also low immunog gave a misperception regarding sericin's biosafety. The method of removing sericin from silk may change adsorption of exogenous proteins onto fibroin hampering cell adhesion.³⁰ Hence, we are left with an incomplete understanding of immunogenicity, but it appears that fibroin will benefit from reduced sericin concentration if cell adhesion is adequate. Quality control techniques to assess residual levels of sericin are now available³⁰ and overall, close attention to processing is required to ensure that sericin does not confound the biological response to fibroin.

Cytotoxicity

The widespread use of silk suture in ophthalmology is testament that it is not grossly cytotoxic to many cells. Cell death from direct contact does not normally occur, although necrosis has been reported, which is probably secondary to an immunological reaction.²³ With the ocular surface, epithelial cells rapidly grow down the apertures formed by silk suture, indicating that cells in close apposition retain viability.

The interaction of silk proteins *in vivo* throughout the body has been reviewed³¹; summarising that there are excellent bioresponses *in vivo* with low immunogenicity and an ability to be remodelled and replaced by native tissue. However, one group using fibroin nanoparticles, reported that they cause cellular and mitochondrial dysfunction in cultured fibroblasts, blood cells and umbilical vein cells.³²

Table 1 describes the major characteristics of the generic host response to silk and silk fibroin and relates these to the variables that could influence host response.

Neoplasia, carcinogenicity and teratogenicity and genotoxicity

Silk fibroin hydrogels have been shown to suppress tumour formation in chick chorioallantoic membrane.³³ *In vivo*, in mice with lung cancer, tumour growth was suppressed with fibroin.³⁴ These types of results, taken together with the extensive use of silk suture in the eye without the association of widespread resulting carcinoma, indicate that fibroin may be beneficial in this regard, rather than pathogenic.

In the early 1900s, silk suture was regularly used in human uterine surgery³⁵ without documented adverse effect. Studies of the effects of intrauterine silk thread on the fertility of female rats found no evidence of teratogenicity and no effect on the oestrous cycle, concluding it is safe for uterine use.³⁶ Yan *et al.*³⁷ performed a study for acute toxicity, genotoxicity and effects on the reproductive system after implantation of fibroin in nerve guides; no effect was found with any of the parameters measured.

Amyloidosis

Amyloid protein

Amyloid is a term describing insoluble protein aggregates of specific structure. Although no specific amyloidogenic peptide sequence pattern has been identified, there does appear to be a common core structure of polypeptide chains, generally known as the 'cross-β' structure.³⁸ Amyloids occur naturally, and in many roles do not result in disease, instead achieving a diverse range of advantageous biological functions,³⁹ for example in moths, where silk is produced as a protective case in larval development. In a similar way amyloid β (Aβ) peptide protects against fungal and bacterial infections in mammals.⁴⁰ It can promote recovery from injury, including repairing leaks in the blood–brain barrier⁴¹ and may act as a modulator of synaptic plasticity with implications in learning and memory.

Amyloid pathology

Conversely, it has been noted that Aβ may rapidly increase in response to a physiological challenge and often diminishes on recovery.⁴¹ It is also known that amyloids are involved in various human pathologies⁴² including Alzheimer's disease (AD) and Parkinson's disease where accumulation of abnormally misfolded proteins, a process known as amyloidosis, results in neurodegeneration. There is a balance between physiological and pathological effects of Aβ, and in disease the levels of Aβ are elevated from pM to nM or μM levels.⁴³ Amyloids can be toxic to cells in a variety of ways including: disruption of cytoskeleton, physical damage of cellular membranes, DNA damage, oxidative stress, mitochondrial dysfunction, apoptosis and adverse intracellular calcium signalling. Another dangerous feature of amyloids is that there is some form of pathogenic spread from region to region in the organ/body.⁴⁴

The cascade theory of Hardy and Higgins⁴⁵ proposed with AD that amyloid starts a cascade of events that then leads to amyloidosis and cellular damage. It is believed that neither the monomeric protein, nor the deposited fibrils, exert neurotoxicity *per se*. It is intermediate low molecular mass oligomers and protofibrils that are considered to be the likely neurotoxic species.⁴⁶ Although the cascade theory has been reconsidered and criticised,⁴⁷ there is still acknowledgement that a nucleus can initiate amyloid formation and a possible pathogenetic role of Aβ cannot be completely ruled out.⁴⁸ Regardless of any further mechanism, amyloid originating from nuclei is a concern and both fibroin and sericin have been shown to be seeds.⁴⁹ These proteins also bound to Aβ leading to aggregation, suggesting a potential role in the propagation of Aβ amyloidosis. Transmission of amyloid-β protein pathology from cadaveric pituitary growth hormone to intracerebrally inoculated mice has been demonstrated,⁵⁰ with the formation of a mutant, humanised amyloid precursor protein. The same report also documents iatrogenic transfer of Aβ pathology to humans.

**Table 1** Major characteristics of the generic host response to silk fibroin, based on the approach of Williams (2008)⁸⁹

Characteristics	Host response
Protein adsorption and desorption characteristics	<p>The method of silk processing affects protein adsorption:</p> <ul style="list-style-type: none"> ▶ Serum adsorption increases with fibroin fibre hydrophobicity. Some immunoproteins show different adsorption; Bb and C1q complement factors only attach well to fibroin fibres, not the film, whereas IgG and C3 fragments adsorb on both.⁹⁰ ▶ In contrast, when comparing different fibroin films, protein adsorption increased with hydrophilicity and the lowering of β-sheet content.⁹¹
Generalised cytotoxic effects	<p>Silk fibroin shows generally low cytotoxicity:</p> <ul style="list-style-type: none"> ▶ The widespread use of silk as suture has demonstrated real-world evidence of minimal cytotoxicity.³⁵ ▶ With silk films, no cytotoxicity was exhibited toward L929 cells or subcutaneously over 19 months in rats.⁹² ▶ Porous silk fibroin film placed into the rabbit cornea was gradually replaced by stromal tissue. Non-porous film had normal adjacent cells, even after 6 months.²⁴ ▶ Spider silk peptides have low cytotoxicity to neural cells.⁷¹
Neutrophil activation	<p>Neutrophil activation is generally low:</p> <ul style="list-style-type: none"> ▶ Rat mucosal epithelial cells attracted a significantly lower number of inflammatory neutrophils when used with silk fibroin compared with a control; there was no local or systemic immunological incompatibility of fibroin.⁹³ ▶ There was less neutrophil infiltration of wounds dressed with silk film than with a hydrocolloid dressing.⁹⁴
Macrophage activation, foreign body giant cell production, granulation tissue formation	<p>Macrophage activation occurs, but infiltration is generally low. Granulation tissue formation occurs:</p> <ul style="list-style-type: none"> ▶ With mouse macrophage cells, insoluble fibroin particles induced significant tumour necrosis factor (TNF) release and activation.²⁸ ▶ In mice, subcutaneous silk fibroin implantation produced macrophage migration inhibitory factor, but not other proinflammatory cytokines. There was no remarkable infiltration of macrophages or lymphocytes, although by day 180 some macrophages were present in the adjoining tissues.⁹⁵ ▶ Silk fibroin induced a cellular response in which proinflammatory macrophages and multinucleated giant cells were associated with vascularisation. The silk fibroin induced granulation tissue formation.⁹⁶
Fibroblast behaviour and fibrosis	<p>Fibroblasts can grow directly on fibroin:</p> <ul style="list-style-type: none"> ▶ Fibroin annealing treatment affects early-stage fibroblast adhesion. By adjusting fibroin surface characteristics, cell repellent areas and cell spreading direction can be controlled.⁹⁷
Microvascular changes	<p>Silk does not adversely affect microvasculature:</p> <ul style="list-style-type: none"> ▶ Silk fibroin fibres cocultured with human microcapillary endothelial and osteoblast cells produced a perfusable lumen containing red blood cells which anastomosed with host murine vasculature.⁹⁸
Tissue/organ-specific cell responses	<p>There are few tissue/organ-specific cell responses, however the response can depend on material preparation and biocompatibility is both host and location dependent:</p> <ul style="list-style-type: none"> ▶ Silk fibroin used as an electrospun scaffold for bladder reconstruction in the rabbit, resulted in a mild acute and chronic inflammatory reaction,⁹⁹ but inflammation did not occur when fibroin was used as a urethral sling in the rat.¹⁰⁰ ▶ There was considerable inflammatory cell infiltration when used as a mesh in the vesicouterine space, although the acute inflammation lasted no more than 4 weeks.¹⁰¹
Activation of clotting cascade	<p>The extent of activation of the clotting cascade depends on multiple factors in processing:</p> <ul style="list-style-type: none"> ▶ Coating polyester with solubilised fibroin reduces thrombogenicity. ▶ Fibroin thrombogenicity is reduced with methanol treatment. ▶ Fibroin films bound lower levels of fibrinogen than did two synthetic polymers while the same amounts of adsorbed human plasma complement fragment C3 and IgG were detected. ▶ Sulphation of silk fibroin can reduce the coagulant activity. ▶ Multiple factors in fibroin preparation such as treatment temperature and solvent influenced the biological response, but silk-based vascular grafts were considered viable.¹⁰²

Continued

Table 1 Continued

Characteristics	Host response
Platelet adhesion, activation, aggregation	<p>Platelet adhesion and activation can be controlled by silk preparation and blending:</p> <ul style="list-style-type: none"> ▶ Changing silk processing can affect platelet interaction,⁹¹ but multiple factors influence response.¹⁰² ▶ Chemicals or blending with other polymers can be used to decrease human platelet attachment. Heparin can be loaded as a drug into silk fibroin to reduce platelet attachment in vivo in pigs. ▶ Conversely, in areas where platelet adhesion may be desirable such as wound sites, von Willebrand factor can be attached to fibroin.¹⁰³
Complement activation	<p>Complement adsorption occurs with fibroin:</p> <ul style="list-style-type: none"> ▶ Fibroin preparation changes the extent of adsorption or activation with there being greater complement C3 fragment activation with films compared with fabrics.⁹⁰ Binding may be specific to hydrophobic (β sheet) areas.
Antibody production, immune cell responses	<p>Silk can produce an immune cell response, but removal of sericin may reduce this:</p> <ul style="list-style-type: none"> ▶ Silk can act as a potent allergen. Non-iatrogenic causes include type I allergic diseases, such as asthma, hypersensitivity pneumonitis and rhinitis. ▶ In the human eye, severe reactions to virgin silk sutures used in cataract surgery have occurred suggesting a possible role of prior sensitisation.²³ ▶ In the rabbit, porous fibroin is gradually replaced by stromal tissue without causing inflammatory reaction by CD4-positive and CD8-positive lymphocyte infiltration.²⁴
Acute hypersensitivity/anaphylaxis	<p>Silk can cause hypersensitivity:</p> <ul style="list-style-type: none"> ▶ There has been anaphylaxis after eating the silkworm in its entirety. ▶ In clothing, silk gives more positive reactions of eczema than any other protein.¹⁰⁴
Delayed hypersensitivity	<p>Delayed hypersensitivity can occur:</p> <ul style="list-style-type: none"> ▶ A reaction to silk fibroin was reported with recurrent granulomas where the patient may have developed the reaction from silk sutures used in a previous surgery.²⁷ ▶ Three paediatric neurosurgical patients had delayed inflammation 7 years following initial silk suture placement.²⁶
Mutagenic responses, genotoxicity	<p>There is no known evidence of a mutagenic or genotoxic response:</p> <ul style="list-style-type: none"> ▶ Silk fibroin genotoxicity has been tested in vitro using an Ames test employing a bacterial reverse mutation, and in vivo using a bone marrow micronucleus assay in the mouse. Genotoxicity was not evident.³⁷ ▶ Indeed, silk fibroin peptides actively chemoprotective and reduced DNA damage during challenge of a mutagen on mouse embryos. The size and the glycine plus alanine ratio of peptides affected activity and the action was due both to the protective interactions between cells and peptide molecules and also to the direct inactivation of the mutagen.¹⁰⁵
Reproductive toxicity	<p>There is no known evidence of a reproductive toxicity aspect of fibroin or sericin:</p> <ul style="list-style-type: none"> ▶ A teratozoospermia ratio assay and morphological observation of testis histology in the mouse showed that there was no difference between silk fibroin solution and control.³⁷ ▶ Indeed, in mice, silk fibroin amino acids improve male reproductive function by increasing testosterone and sperm count. ▶ Silk hydrogels into the cervixes of pregnant rats showed only an initial mild foreign body response and there was no upregulation of inflammatory markers with in vitro human cervical cells.¹⁰⁶ However, the longer-term impacts on pregnancy are unknown.
Tumour formation	<p>There is no known evidence of tumour formation from fibroin or sericin:</p> <ul style="list-style-type: none"> ▶ Indeed, silk has been shown to have a protective effect against tumour promotion.^{33 34}

In humans, 36 proteins/peptides are already known to generate amyloid deposits and disease.⁵¹ Each distinct form of amyloidosis is uniquely characterised by the chemical identity of the amyloid fibril protein that deposits in tissues to give rise to the disease. At least one, Enfuvirtide, is synthetic, and not a native human cellular protein, that binds with the HIV envelope protein gp41. Its presentation is also iatrogenic,⁵² highlighting how

amyloidosis risk may arise from medical treatment even with non-human peptides or proteins.

Amyloid and the eye

In primates, amyloid is associated with glaucoma; in a glaucoma model made by deliberate damage to the retina, AD-like pathologies are also established in the lateral geniculate nucleus.⁵³ Such an association is



concerning, as changes to the eye may therefore result in adverse change in the conduit to the brain. Studies to identify ocular markers as an Alzheimer's diagnostic have shown A β plaques in the eyes of those with the disease, indicating the close association between sites.⁵⁴ Indeed, in Alzheimer's-transgenic mice, retinal A β deposits preceded brain A β deposition.⁵⁵

In mice, subretinal injection of A β caused several adverse events leading to retinal degeneration. Understanding the role of A β may be important in diseases of retinal degeneration, such as age-related macular degeneration.⁵⁶

Prion characteristics

The prion diseases of Creutzfeldt-Jakob disease, Kuru, fatal familial insomnia and Gerstmann-Straussler-Scheinker syndrome diseases involve a conformation isoform of the normal cell surface glycoprotein PrP^C into the pathogenic protein PrP^{Sc}. The architecture of mammalian prions appears fundamental to their lethal pathogenicity, being self-propagating assemblies of misfolded host-encoded protein.⁵⁷ Infection can be transmitted not only by ingestion of tissues, but also by iatrogenic transfer.

There is evidence in mice that A β aggregates are prions by the demonstration of widespread cerebral β -amyloidosis following inoculation of purified A β aggregates derived from brain, or even synthetic A β aggregates,⁵⁸ such that a unifying process of prions has been proposed.⁵⁹ Furthermore, recent study in humans discovered similar prion-like propagation of A β aggregates.⁵⁰

Role of inflammation in amyloidosis

Many amyloidosis-associated processes are accompanied or triggered by inflammation.⁶⁰ In murine models with persistent inflammation, an amyloid-enhancing factor (AEF) protein has been identified that originates 1–2 days before amyloid A (AA).⁶¹ Specific fibrils from synthetic peptides with no amyloid relationship can induce AA amyloidosis during inflammation in an animal model.⁶² In addition, innocuous proteins can build up toxic amyloids under certain conditions.⁶³ An amyloid-related fibril can also act as an AEF where a characteristic property is their β -sheet organisation.⁶⁴ Taken together these show a relationship of β -sheet protein organisation and host inflammation in amyloidosis.

Association of fibroin β -sheet with amyloid and amyloidosis

Fibroin β -sheet formation of silk has similar structural characteristics to amyloid.⁶⁵ Dissolved fibroin has been reported to accelerate amyloid accumulation in mice⁶¹ with Lundmark *et al*⁶⁶ indicating that silk fibroin can act as a cross-seed nucleus and that there is, '*Transmissibility of systemic amyloidosis by a prion-like mechanism*'. The threat from a nucleation-polymerisation model⁶⁷ is that fibroin placed into humans might seed deleterious amyloid formation. Such risk may prevent devices that contain fibroin gaining regulatory approval for new usage,

particularly like those in ophthalmology where there is a close link between the eye via the optic nerve to the brain; the retina is effectively an extension of the central nervous system.⁶⁸

Silk fibroin gel with small fibrils and β -sheet content injected into mice resulted in a dose-dependent AA formation in the spleen, but only if the fibroin had first been sonicated and there was an inflammatory stimulus by use of silver nitrate. In the mouse, 1 μ g is sufficient to induce AEF.⁶¹ If animal studies are applicable to humans, using silk fibroin with a patient who has chronic inflammatory disease enhances the risk of amyloidosis.⁶⁹ Contrary to this view, not only is there no direct evidence of such occurrence in man, but there is a growing body of experimental evidence showing reduced risk. The cytotoxicity of amyloid peptides is related more to their nanoassembly, rather than the β -sheet content.⁷⁰ Furthermore, spider fibroin with the amino acid sequence of amyloid β -sheets had a marked cytotoxic effect on cultured human neural cells, but silk fibroin β -sheets did not.⁷¹ They attributed this to lack of surface charge on the silk peptides, even though they have the same β -sheet content as amyloid. Fibroin has also been shown to be a relatively poor seed in A β 40 and A β 42 aggregation.⁴⁹

A direct study of the risk of fibroin for amyloidosis by Tsukawak *et al*,⁷² concluded that silk occasionally promotes amyloidogenesis, but has a low potential for amyloidosis. Amyloid deposits were rarely observed in mice injected with silk fibroin in solution, but with certain methods of production, heavy amyloid deposit resulted from fibroin fabric placed in the abdomen; the production method is an important determinant of its biocompatibility.

BIODEGRADATION OF SILK

The routine use of silk as suture has resulted in it being recognised in regulation,⁷³ classifying it as non-absorbable because it is retained for more than 60 days. Despite this nomenclature, silk is eminently absorbable, as fibroin as a protein can be biodegraded by the major protein degradation pathways, via peptides to amino acids.⁷⁴ Fibroin is inherently slow to degrade; the long sequences inhibit substrate unfolding, decreasing the efficiency of the proteasomes and compact regions sterically delay degradation with the most crystalline regions degrading last.²

Proteases are ubiquitous in tears, anterior chamber and vitreous, but their concentration varies, not only with site, but also with age, race and disease state. The physical presentation also affects degradation rate with, for example, silk fibres being attacked at a slower rate than films.⁷⁴ Proteolytic action can also be delayed if the site encapsulates the silk with fibrous connective tissue. Thus, the degradation rate of fibroin fibres placed in an inflamed cornea may differ markedly from silk film embedded in retina.

Silk fibres during biodegradation show an increase of surface roughness and crystallinity before fragmentation occurs. Again, depending on site, traces of fibroin may

remain for years. The ability and the speed of degradation are not the only considerations for a successful biomaterial as it must continue to function as it degrades. Cracking, void formation⁷⁴ and fragmentation can also be important, as this can dislodge cells or allow the movement of the biomaterial to unsuitable areas. For example, in the eye, fragments of silk might lodge in the iris angle. Although models of degradation have been used in vitro, it is only through in vivo modelling that more accurate simulation can be demonstrated. Nevertheless, animal models may differ from the human eye, and clinical trials are the ultimate test of suitability.

BIOCOMPATIBILITY OF SILK

The aim of suture is purely to provide mechanical strength; ideally it is chemically and biologically inert and does not interact with the host. Silk fibroin for tissue engineering on the other hand has a need for specific and direct interactions with tissue components.⁷⁵ Silk in tissue engineering is subject to sterile inflammation⁷⁶ that can present different conditions to a conventional pathogenic response. As reviewed by Altman *et al.*,⁵ there are many reports stating silk fibroin is biocompatible which are routinely based on the long history of use without direct pathology and often supported by the short-term growth of a single cell type from the planned site of use. Short-term growth of a monoculture in the laboratory only demonstrates the material is not cytotoxic to those cells. Biocompatibility requires us to show that the material does not induce a negative biological response when implanted into a tissue composed of many cell types with a complex arrangement of blood supply and nerves.

Biocompatibility varies with the application.⁷⁶ It is not a property of the material alone, but it is the biomaterial in a host system. For this reason biocompatibility keeps getting redefined.⁷⁷ Thus, the biocompatibility of a fibroin film placed on the surface of an avascular cornea may markedly differ from the same product placed in an inflamed, vascularised cornea, even though it is the same tissue. Numerous differences may be involved: different enzymes may be present, immune reaction may be primed, mechanobiology may change cell growth, and so on.

NON-*B. MORI* SILK

There is a vast range of fibroin types, with individual species producing fibroin with very different characteristics from different types of gland.⁷⁸ Thus, knowledge of the safety of fibroin from *B. mori* alone is insufficient to assess all fibroins. Importantly, where the fibroin is not a direct moth secretion, then there can be potential additional hazards carried over from the source and the manufacturing process. Here fibroin has been divided into those arising from moth and non-moth sources.

Moth fibroin

The fibroin taken directly from moths other than *B. mori* has been investigated, particularly those of the *Antheraea*

genus such as *mylitta*⁷⁹ and *pernyi*,⁸⁰ which, unlike *B. mori*, have an RGD sequence that binds to integrin receptors in cell membranes to improve cell adhesion. The processing techniques used are similar to those used for *B. mori*, hence only the resultant moth-protein characteristics may cause an additional hazard. However, to increase cellular attachment and growth, or to select for other requirements, blends of fibroins from different species, coatings¹⁰ or fibroin mixed with cell attachment or growth factors have been used.¹⁴ In these cases the safety may differ from the individual components, and must be assessed on a case-by-case basis.

Non-moth fibroin

The use of non-moth fibroin has been limited by the quantity of supply. Sources such as ants have been little studied, but the unsurpassed strength of spider silk has meant that alternative methods of manufacture have been justified to increase supply. Gene insertion into *B. mori* to code chimeric spider fibroin has been one method.⁸¹ In this case, the lack of cell transfer from moth to fibroin will again limit the resultant hazard to the protein characteristics, although a poorly designed gene insertion vector may also propagate its own protein. Other types of manufacture can further increase risk. Methods already used involve gene insertion into bacteria, yeast, tobacco or potatoes, which could produce foreign gene proteins from those organisms, as may the use of transgenic animal sources, such as goats, where methods have been established to harvest the protein from the milk.⁸² However, the highest risk arises from a cultured mammalian cell source, where cell proteins and disease agents have a more direct route to potentially contaminate any silk product. Additional risk from non-*B. mori* fibroin can be reduced by preventing all but the desired protein to be coded. This can be achieved by following these procedures: prior screening of animals, cell lines and organisms for disease; careful selection of the method of gene insertion; and precise control of polymerisation by the use of non-regenerable flanking restriction enzyme sites during gene control.⁸³ The resultant protein can then be purified by methods such as immobilised metal affinity chromatography.⁸³ In these ways the outcome can be a defined product.

REGULATORY APPROVAL

Not only is silk suture regulated and approved by the United States Pharmacopeia,⁷³ but a fibroin scaffold⁸⁴ (SERI surgical scaffold, Allergan, USA) has been used for abdominal wall repair. There have also been clinical trials of fibroin for tympanic membrane⁸⁵ and breast reconstruction⁸⁶ with at least three devices already with approval for clinical use.¹⁹

Most authorities have harmonised their regulation to the ISO 10993 standards.⁸⁷ If there is similarity to a predicate, clinical trials may not even be required for device approval and a less onerous route to approval permitted, for example 510(k) in the USA. Thus, levels of evidence

of silk fibroin biocompatibility may relate primarily to the specific function of the cell therapy in order to gain regulatory approval because it will be assumed that fibroin has been proven safe because of real-world results over many years.

A safety review according to ISO 10993 for acute toxicity, genotoxicity, toxicological effects on the reproductive system and local effects after implantation of fibroin as nerve guides showed no effect on any of the parameters measured.³⁷ Similar studies are required for silk fibroin deployed in the eye. The issue remains however, that although such a study with the eye may also suggest absence of an adverse effect, it may not address, for example, if there is a risk from fibroin amyloid in a human host, over long periods, with associated inflammation. If a material has been approved in a medical device such as suture, this does not mean that the material is safe to use in a tissue engineering role, where there is different biological activity. ISO 10993 may currently be a poor mechanism to assess this.⁸⁸

CONCLUSIONS

Silk is a well-established biomaterial; as a suture it has been taken to cause little harm. It can have an inflammatory response, but this can be minimised by precise manufacture to produce pure fibroin. Fibroin meets many of the criteria for a competent biomaterial, such as lack of direct cytotoxicity, and its long usage in the human body has supported regulatory approval. However, lack of direct cytotoxicity does not guarantee biocompatibility. Williams,⁸⁹ in his definition of biocompatibility, importantly includes that a biomaterial should perform its desired function, ‘without eliciting any undesirable local or systemic effects in the recipient’.

β-amyloid is a molecule with unclear nature, behaviour and pathological roles that is an important participant in the pathogenesis of many neurodegenerative diseases. Currently, despite centuries of use, there appears to be little evidence that human amyloidosis has been caused by silk, but conversely there has also been insufficient specific long-term investigation. A low potential for amyloidosis⁷² needs to be risk-assessed with long-term studies for specific sites involving simultaneous inflammation.⁶⁹ First, to do no harm, remains a basic tenet of medical treatment and potential harm needs to be fully assessed before fibroin is used as a biomaterial in the eye.

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