PERSPECTIVE

Silkworm silk biomaterials for spinal cord repair: promise for combinatorial therapies

Background: Traumatic injury to the adult mammalian spinal cord results in minimal axonal regrowth, cystic cavity formation at the injury site, poor functional recovery and there is no cure available. Due to the complex nature of spinal cord injury (SCI), a combination of therapeutic strategies may offer the most promise for successful regeneration (Ahuja et al., 2017). A key element considered for a combination strategy is a biomaterial scaffold to fill the cavity and to deliver growth promoting factors and transplanted cells. In the last few decades many synthetic and natural biomaterials have been explored for their suitability to repair damaged spinal cord, including hydrogels, guidance conduits and nanoparticles, but none has led to successful clinical translation (Siebert et al., 2015), likely due to failure in optimization of biomaterial characteristics required. The aim of this perspective is to first briefly outline the key characteristics of a biomaterial suited to spinal cord repair and then discuss the potential of using silkworm silk biomaterials such as degummed *Antheraea pernyi* filaments (DAPF) in a combinatorial context.

Key biomaterial properties for spinal cord repair: In our recently published work we demonstrated that DAPF meet the biomaterial properties essential for aiding spinal cord repair (Varone et al., 2017), which are outlined as follows.

Biomaterial stiffness and cell alignment cues: Growth of injured central nervous system (CNS) axons is highly sensitive to the mechanical stiffness of an implanted biomaterial scaffold, so the biomaterial substrate must have a suitable stiffness. There is conflicting data on the optimal mechanical properties required to support CNS axonal regrowth; the effects of mechanical mismatch vary significantly depending on cell type, cell density and the biomaterial type (Moshayedi et al., 2014). Therefore, when developing a biomaterial-based combination strategy it is important to first identify the optimal biomaterial substrate stiffness that supports outgrowth of CNS neuronal and non-neuronal cells, including glial or neural stem cells. Ideally, this would be studied *in vitro* for proof of concept, and then followed up *in vivo* using an appropriate SCI model. The *in vivo* experiments are essential because the material's mechanical properties are likely to change after implantation (especially biodegradable materials) and because spinal cord stiffness changes after injury and glial scar formation (Moeendarbary et al., 2017).

Axons in the mature, intact spinal cord are highly aligned and this architecture plays an important role in cell behaviour and tissue function. After SCI, neuronal processes grow in a disorganized manner, failing to extend past the lesion cavity. In this context, alignment cues from biomaterials could direct and encourage injured axons to extend to targets distal to the injury site. Physical alignment cues can be provided by patterned grooves on the surface of biomaterials (Rajnicek et al., 1997) or by inherently fibre-like biomaterials such as DAPF (**Figure 1**).

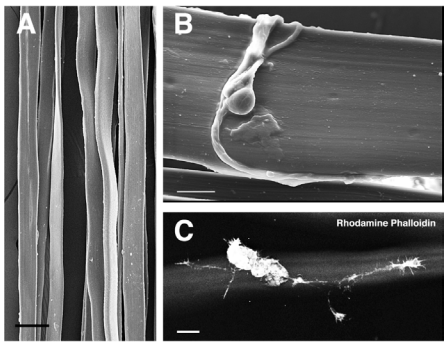
Cell adhesion: Cell adhesion is a critical property of biomaterials for spinal cord repair because neurons must attach to them first to initiate axonal outgrowth. The biomaterial may be modified to change its charge (*e.g.*, with polylysine) or roughness. Alternatively, it can incorporate specific adhesion proteins (*e.g.*, laminin or fibronectin) or short amino acid sequences (*e.g.*, RGD (arginine- glicine-aspartic acid) or IKVAV (isoleucine-lysine-valine-alanine-valine) peptides) that interact with extracellular matrix binding sites (*e.g.*, integrins), thus mimicking the natural extracellular matrix environment present *in vivo*. Extracellular matrix peptides are preferable over proteins because they can be conjugated precisely within the structure of the biomaterial, thus hindering their rapid degradation and impeding an undesirable immune response (Hersel et al., 2003). For these reasons, amino acid sequences of extracellular matrix peptides are of great interest in biomaterial-based combination strategies. A non-synthetic biomaterial, like *Antheraea pernyi* silk, which naturally contains integrin-binding RGD peptides and supports nerve growth and attachment is also attractive in a commercial context because it is cost effective and can be prepared in a degummed, purified form (Varone et al., 2017). Other types of silkworm silk biomaterials, including *Bombyx mori* (BM), can also be functionalized to include relevant peptides (Sun et al., 2017).

Biocompatibility and biodegradation: Biomaterial compatibility and degradation properties are of considerable importance because implantation of a foreign body in the spinal cord triggers a temporal inflammatory response, rapidly activating microglia and attracting neutrophils. The response varies depending on the type of biomaterial, so initial *in vitro* screening should be performed on materials, already proven to support cell growth, to indicate whether the candidate material needs modification to minimise the acute immune response (Moshayedi et al., 2014). Following this modification, the optimal cell growth should be iteratively retested. Ideally, a biomaterial to be implanted or injected should also degrade gradually, leaving only inert, naturally cleared or biodegradable residue. After serving its original role in support of pioneer nerve regrowth across the lesion site; gradual degradation of the biomaterial is desirable because it prevents chronic immune responses, avoids the necessity for further surgery to remove it and it does not obstruct repair processes, such as remyelination.

Considerations for future biomaterials developments in SCI: We recently reported on the potential of DAPF for the regrowth of injured nervous systems (**Figure 1A**). Nerve conduits containing DAPF were implanted into a rat sciatic nerve injury model *in vivo* and promoted extensive and rapid axonal regeneration in gaps of 8–13 mm (Huang et al., 2012). We subsequently explored the potential of DAPF for spinal cord repair by investigating its key biomaterial properties and its ability to support nerve growth. In the context of CNS axonal regrowth DAPF offered numerous advantages, compared to other fibre-like biomaterials, being mechanical suitability, axonal growth alignment, cell adhesion, biocompatibility, and biodegradation (Varone et al., 2017). This sets the stage for future development of DAPF, which will be tailored to the type of SCI. A contusion or compression of the cord commonly leads to large lesions with irregularly shaped cavities (**Figure 2A**), but laceration or transection of the cord tends to lead to small lesions, with a well-defined cavity (**Figure 2B**). An ideal material for spinal cord repair would be applicable in both circumstances. If DAPF or BM silk could be developed into an injectable hydrogel for use in contusion cavities or an implantable hydrogel scaffold for insertion into transection injuries, it would fill this need. DAPF contains repeated RGD peptide sequences but BM silk based hydrogels would need to be functionalized with RGD or IKVAV peptides to aid cell attachment.

Tuneable physical properties: DAPF can be made into a self-assembling hydrogel material with many beneficial properties for *in vivo* applications. We showed recently that the stiffness of the cervical region of the spinal cord is lower than that of the lumbar region (Varone et al., 2017) so it may be advantageous to tune the stiffness of the hydrogel to match the region into which it is injected/implanted. Silk hydrogels have adaptable properties that make it highly attractive in this regard (Floren et al., 2016). The DAPF hydrogel stiffness can be tuned easily by adjusting the concentration of silk fibroin protein in the mixture, adapting to the mechanical requirements of different spinal cord levels (cervical, thoracic or lumbar) and for traumatic brain injuries. Therefore, the ability to modulate the stiffness of DAPF or other self-assembling silkworm silk hydrogels is a major advantage.

A source of growth promoting molecules: An ideal biomaterial would also provide active growth support to damaged axons by delivering growth promoting molecules. A distinctive property of self-assembling hydrogels from DAPF or other silkworm silk is that it can be used as a "depot" to hold biomolecules and to deliver them locally and gradually when transplanted into the lesion cavity. Conventional systemic delivery methods are not optimal for spinal cord repair strategies because the low permeability of the blood-brain barrier and blood-spinal cord barrier may limit diffusion. This means large molecules may not cross and in other cases high systemic doses may be necessary to achieve the required therapeutic concentration at the injury site. Therefore, encapsulation of growth promoting molecules in the biomaterial scaffold is considered a better approach. The notion of using a silkworm silk



(A) Scanning electron microscopy (SEM) image of a longitudinal view of a bundle of DAPF. (B) SEM image of a postnatal rat cortical neuron extending its longest neurite along DAPF after two days in culture. (C) Fluorescence image of an embryonic *Xenopus laevis* spinal cord neurons extending neurites along a DAPF after 5 hours in culture. Scale bars: 100 µm in A, 10 µm in B, C.

Figure 1 Degummed *Antheraea pernyi* filaments (DAPF) and neuronal growth alignment.

hydrogel *e.g.*, from either DAPF or BM silk, is highly promising since it is capable of slow, sustained local release of bioactive substances including neurotrophic factors (Hopkins et al., 2013).

Injectable and self-assembling in vivo: Contusive/compressive SCI therapies may require an injectable biomaterial because the lesion cavities are often irregular, large and situated proximal to the central canal (**Figure 2A**). Hydrogels from DAPF or other silkworm silk can be developed into an injectable self-assembling format. The silk fibroin solution molecules could be assembled into a gel with elongated nano fibrils using a simple one-step sonication process just before injection. The hydrogel could be injected in a semi-liquid form, permitting it to conform exactly to the amorphous lesion cavity *in vivo*. Furthermore, it is possible to adapt the length of the syringe needle to reach the exact area of the gap, causing minimal disruption to the surrounding tissue (**Figure 2A**). Injecting hydrogels incorporating therapeutic drugs is considered a minimally invasive technique and can be easily applied by neurosurgeons.

Implantable hydrogel scaffold: In spinal cord laceration/transection SCI the lesion gap is usually short and with a defined geometry. For this type of injury DAPF embedded in a 3-dimensional hydrogel (made from DAPF or other silkworm silk) containing growth promoting molecules may be more suitable for supporting axonal regrowth (**Figure 2B**). The hydrogel scaffold can be shaped to fit the precise dimensions of the lesion following measurements with CT or MRI scans. The implantation of the readily assembled hydrogel scaffold benefits precise filling of every area of the lesion gap and it may also prevent potential complications of the gel not setting *in vivo* with altered physical complications (*e.g.*, pH or temperature changes). Furthermore, DAPF embedded in the hydrogel will provide a linear array of guidance cues, which may encourage aligned axonal regrowth across the lesion and towards their appropriate targets as described above.

Conclusions: Biomaterials intended for spinal cord repair therapies have so far failed to translate effectively to the clinic, perhaps due to a lack of complete and systematic studies of biomaterial design and subsequent characterization. We propose that in biomaterial design for spinal cord repair the key biomaterial properties to assess are: mechanical stiffness, alignment cues for axonal growth, cell adhesion, biocompatibility and degradation. In addition, it may be essential to develop two types of biomaterial scaffolds: a self-assembling injectable hydrogel for contusive/compressive SCI and a precisely shaped 3-dimensional hydrogel scaffold that can be implanted directly in a laceration/transection SCI. Hydrogels technology incorporating DAPF may be ideal for spinal cord repair because of the ease of synthesis, chemical adaptability and easily tuneable properties. Hydrogels also permit active growth support because of the unique ability to carry and deliver growth promoting molecules within a 3-dimensional, resorbable, textured environment, thus delivering a combinatorial therapy that is more likely to be effective than a monotherapy. Moreover, this technology could be adapted for other types of CNS injuries, such as brain trauma and stroke, which share similar pathophysiologies to SCI.

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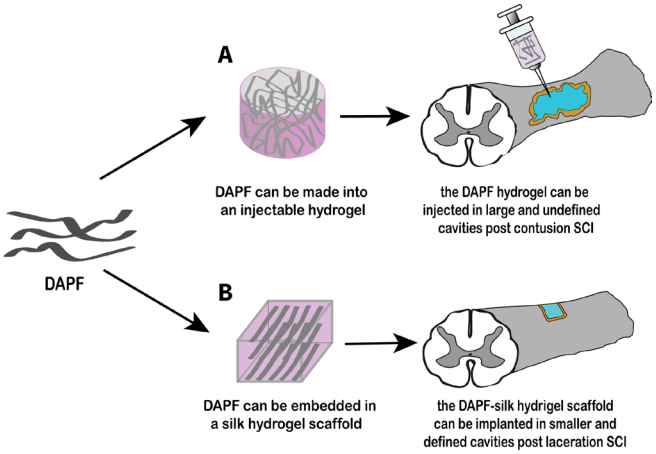

Figure 2 Development of degummed *Antheraea pernyi* filaments (DAPF) for *in vivo* spinal cord injury (SCI) models.
(A) DAPF can be dissolved and made into a hydrogel form which can be injected directly in a large and undefined cavity typical of contusive SCI. (B) DAPF can also be embedded in a silk hydrogel and implanted in a transection SCI in which the lesion gap is small and with a defined geometry.

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