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Emerging peptide nanomedicine to regenerate tissues and organs

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Abstract. Peptide nanostructures containing bioactive signals offer exciting novel therapies of broad potential impact in regenerative medicine. These nanostructures can be designed through self-assembly strategies and supramolecular chemistry, and have the potential to combine bioactivity for multiple targets with biocompatibility. It is also possible to multiplex their functions by using them to deliver proteins, nucleic acids, drugs and cells. In this review, we illustrate progress made in this new field by our group and others using peptide-based nanotechnology. Specifically, we highlight the use of self-assembling peptide amphiphiles towards applications in the regeneration of the central nervous system, vasculature and hard tissue along with the transplant of islets and the controlled release of nitric oxide to prevent neointimal hyperplasia. Also, we discuss other self-assembling oligopeptide technology and the progress made with these materials towards the development of potential therapies.

Keywords: bioactive nanostructures, peptide amphiphiles, regenerative medicine, self-assembly.

Introduction

The expectation of humans to live long lives free from morbidity and pain demands continuous development of new therapeutic strategies that can promote the regeneration and optimal healing of tissues and organs damaged by trauma, disease, or congenital defects. The field of regenerative medicine aims to meet these demands, focusing on restoring lost, damaged, aged, or dysfunctional cells and their extracellular matrices to return function to tissues. There are many targets where new therapies could greatly improve both the span and quality of life; of special importance amongst these are conditions where normal physiological regeneration is limited or nonexistent. For example, the ability to regenerate the central nervous system would provide a higher quality of life to individuals paralysed by spinal cord injury, suffering serious dysfunction from stroke, and living with degenerative diseases such as Parkinson's, Alzheimer's and multiple sclerosis. Regenerative strategies are also needed to combat heart disease and heart failure, which remain some of the leading causes of mortality, and to prompt the development of new vasculature to deliver blood to ischaemic tissues and organs. Regeneration of pancreatic β cells would bring a higher quality of life to the many young people suffering from diabetes, who for now are reliant on regular insulin injections. The regeneration of teeth would eliminate the need for dentures and other dental implants. Musculoskeletal ailments such as damage to bones, tendons, and ligaments and osteoarthritis resulting from irreversible cartilage damage all are an enormous source of pain and truly compromise one's ability to carry on an active lifestyle. These are but a

few of the many targets that are in need of new regenerative strategies. Human ailments are truly complex, and innovative therapies necessitate the merging of many diverse fields including stem cell science, developmental biology, molecular biology, genetics, materials science, chemistry, bioengineering and tissue engineering [1]. These collaborative efforts are the only foreseeable means by which to make progress in the treatment of disease and ultimately improve the human condition. This review demonstrates how we, and others, have initiated efforts towards regenerative therapies using peptide-based strategies for self-assembly, supramolecular chemistry and nanotechnology.

The need for new regenerative strategies has coincided with, and likely promoted, the emergence of the field of bionanotechnology. Over the past decade, the focus of nanoscience has shifted from the synthesis, development and characterization of novel nanostructures to the exploration of potential applications for this technology to assist in crucial problems as diverse as energy and medicine. The organization of human tissue begins on the nanoscale, with complex biological molecules providing the structural and functional infrastructure for life's processes. Thus, therapies designed on this scale with specific structure and function have the potential to similarly serve as matrix for cells, participate in biological signalling, and to efficiently deliver proteins and drugs, all of this through minimally invasive therapies. To date, a broad range of nanostructures including peptide nanofibres [2], carbon nanotubes [3, 4], inorganic nanoparticles such as quantum dots [5], and dendrimers [6, 7] have been explored in disease diagnostics, drug delivery and regenerative strategies.

Molecular self-assembly is a very attractive strategy to construct nanoscale materials for applications in regenerative medicine due to both its simplicity in application and its unique capacity to produce a variety of diverse nanostructures [8–10]. This spontaneous process organizes small molecules into structures that are ordered on multiple length scales. The structural features of the final supramolecular assemblies can be controlled through molecular design and by finely tuning assembling conditions and kinetics. For applications of these self-assembled materials, the ultimate goal is for the material to achieve its desired function. For example, assemblies used for cell scaffolds would ideally incorporate both the necessary structural support as well as the ability to signal transplanted or native cells, thus serving to mimic the complex signalling machinery of native extracellular matrix. Furthermore, molecular design can be used to create structures that biodegrade over an appropriate time scale. To achieve maximal function in a minimally invasive way, these networks can be designed to start as small molecules in a liquid that can be injected easily into tissues and self-assembly would then transform them *in situ* into solid scaffolds or nanostructures, specifically designed with application-specific control over degradation to further limit invasiveness of the therapy.

Peptides are a unique platform for the design of self-assembled materials with controllable structural features at the nanoscale. The chemical design versatility afforded by amino acid sequences leads to a variety of possible secondary, tertiary and quaternary structures through folding and hydrogen bonding. β-Sheet forming peptides, in particular, have demonstrated the extraordinary ability to use intermolecular hydrogen bonding for the assembly of one-dimensional nanostructures [11]. For applications in regenerative medicine, peptides have additional appeal because of their inherent biocompatibility and biodegradability. Entanglement of peptide-based nanostructures can lead to the formation of three-dimensional networks, allowing these nanofibres to mimic the structure of native extracellular matrix. Further, the ability to incorporate biological peptide-based signalling sequences affords control over the function of these nanofibre networks. Though the large-scale synthesis of proteins comprising hundreds of residues whilst ensuring appropriate folding and post-translational modifications remains a formidable challenge, oligopeptides can be produced rather easily using standard solution or solid-phase synthetic methods. Futhermore, the design of self-assembly peptides for targeted functions can also include their modification with other biomolecular units such as sugars, lipid components and nucleic

acid monomers. These oligopeptides, whilst designed for supramolecular self-assembly, could also serve to functionally mimic large proteins. For these reasons, self-assembling, nonimmunogenic peptides project as promising new therapeutics for human disease.

Peptide amphiphiles: technology

Design of peptide amphiphiles

Within the past decade, the Stupp laboratory has synthesized several amphiphilic molecules designed to self-assemble into biomaterials [2, 12–15]. A broad class, known as peptide amphiphiles (PAs), incorporates a short hydrophobic domain on one end of a hydrophilic oligopeptide sequence [2, 8, 9, 11, 15]. Figure 1 shows the chemical structure of a representative PA molecule, which is composed of four key structural domains [2, 16]. Domain 1, the hydrophobic domain, typically consists of an alkyl chain analogous to lipid tails and can be tuned by using different chain lengths or different hydrophobic components [15]. Domain 2, immediately adjacent to the hydrophobic segment, consists of a short peptide sequence designed to promote intermolecular hydrogen bonding, typically β-sheet formation, which induces the assembly of the molecules into high aspect-ratio cylindrical or twisted nanofibres, and in

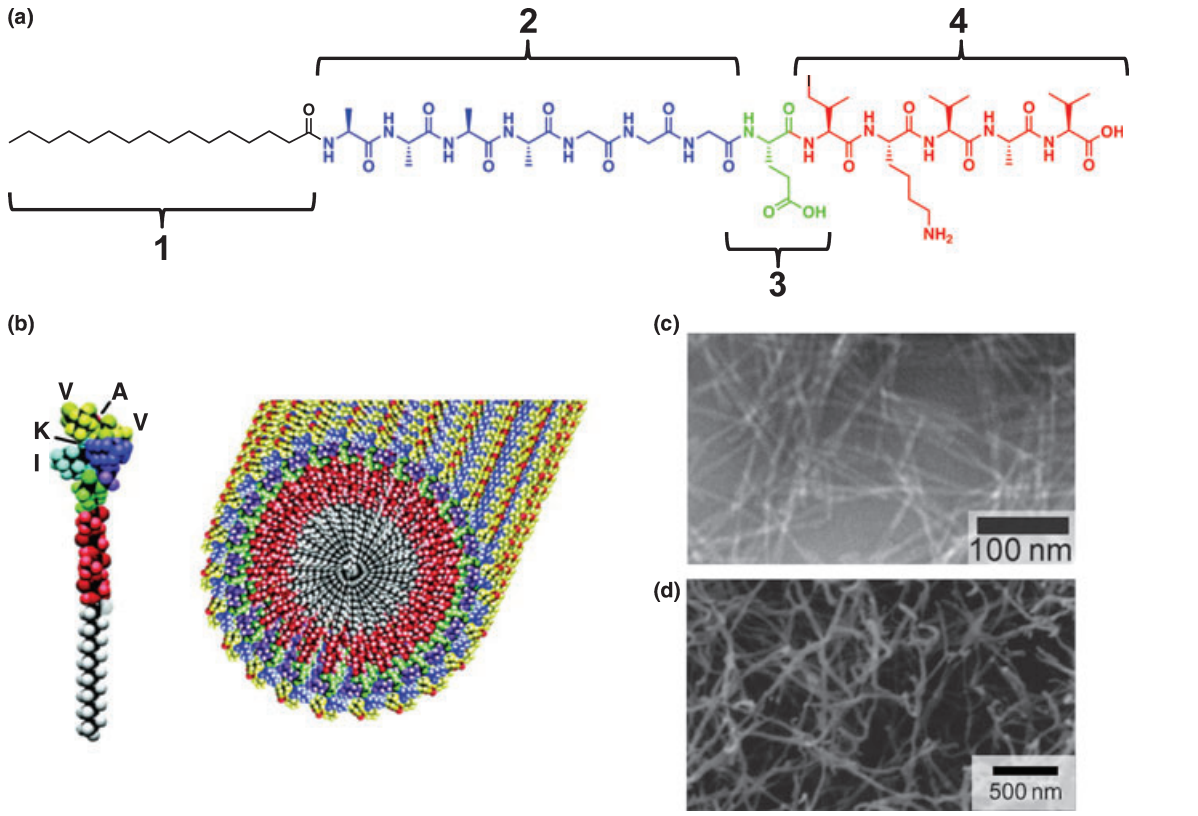

Fig. 1 (a) Molecular structure of a representative peptide amphiphile with four rationally designed chemical entities. (b) Molecular graphics illustration of an IKVAV-containing peptide amphiphile molecule and its self-assembly into nanofibres; (c) transmission electron microscopy (TEM) micrograph of IKVAV nanofibres; (d) SEM micrograph of IKVAV nanofibre gel network. Parts (b) and (d) from Silva *et al.* [16]. Reprinted with permission from Niece et al. [Self-assembly combining two bioactive peptide amphiphile molecules into nanofibres by electrostatic attraction. J Am Chem Soc 2003; **125**: 7146–47]. Copyright © 2003 American Chemical Society.

some cases nanobelts [17, 18]. Domain 3 incorporates charged amino acids to enhance solubility in water and to enable the design of pH or salt-induced self-assembly of the molecules. Domain 4 can be used for the display on the nanostructure surface of bioactive signals in the form of oligopeptide sequences designed to interact with cells, proteins, or biomolecules. If domain 4 has a sufficient number of charged amino acids to provide solubility and charge screening possibilities, then domain 3 is not necessary in the design of the PA. This classical PA molecule assembles into high aspect-ratio nanofibres with dimensions similar to native extracellular matrix fibrils. The driving force for this assembly is based on both hydrophobic collapse of domain 1 away from the aqueous environment and propensity for intermolecular hydrogen bond formation. In physiological media, the charged amino acids become screened by electrolytes, diminishing the electrostatic repulsion. Hydrophobic collapse drives the alkyl tails to aggregate away from the aqueous media, and intermolecular hydrogen bonds form parallel to the long axis of the fibre [18]. This assembly mechanism allows the bioactive region of PA molecule to be presented on the surface of the nanofibre, where it can interact with cells, proteins, or biopolymers. These 1D nanostructures can further entangle into networks, and form self-supporting gels at relatively low concentrations, on the order of 1% by weight [19, 20].

The salt- or pH-responsive design element is a critical feature that makes PAs ideal candidates for minimally invasive therapies. This feature allows unassembled PA molecules to be combined with bioactive entities, such as growth factors, DNA or glycosaminoglycans, or with cells to form a liquid cocktail with very low viscosity. Upon injection into the tissue, electrolytes in the physiological environment can immediately induce the self-assembly of PAs into nanofibres and subsequent formation of gelled networks encapsulating the desired payload. From the standpoint of applications for regenerative medicine, we are mainly interested in using individual PA nanofibres to signal cells through the display of bioactive epitopes, whilst employing three-dimensional networks of these high aspect-ratio nanofibres as bioactive scaffolds to simultaneously support, deliver and signal cells.

Cell scaffolds and substrates

Hydrogels have the potential, because of their fibrillar interconnected structure, to serve as a three-dimensional support to study the behaviour of cells *in vitro* or deliver and support therapeutic cells *in vivo*. One-dimensional PA nanofibres can bundle and entangle into such a self-supporting three-dimensional gel network. This transition typically requires screening by multivalent ions in physiological media, and allows cells to be encapsulated in the resulting nanofibre matrix [19]. These cells, once entrapped within PA nanofibre networks, are viable for several weeks and continue to proliferate, indicating no cell cycle arrest. Transmission electron microscopy (TEM) suggests internalization of PA by these encapsulated cells, and biochemical assays demonstrate evidence of PA metabolism. PA nanofibres are not only capable of displaying signals to cells, but also provide structural support to the encapsulated cells and could be eventually metabolized into nutrients. This supports the idea that three-dimensional networks of PA nanofibres can serve as an artificial extracellular matrix, and the architectural similarities between PA nanofibres and the filamentous structures found in natural extracellular matrix could allow these PA networks to function as a highly biomimetic artificial matrix. The gelation kinetics of PA nanofibre networks are also tunable by changing the internal peptide sequence whilst keeping the bioactive domain constant, producing nanofibre gels with a range of gelation times [21]. Changes in the region promoting β-sheet formation to include more bulky and hydrophilic residues, such as SLSLGGG instead of AAAAGGG, significantly increases the time for gelation. This gives design flexibility to tune the gelation time of these injectable PA nanofibre gels for a desired application.

Peptide amphiphiles as scaffolds for cells can be further tuned by the addition of a bioactive sequence to signal the encapsulated cells. For example, in order to enhance matrix–cell interaction, the Arg-Gly-Asp-Ser (RGDS) peptide epitope has been incorporated into

PA molecular design. Using orthogonal protecting group chemistry, the covalent architecture in which this epitope is presented on the nanofibre surface can be varied, producing PAs that present RGDS in linear, branched, double-branched or cyclic geometries [22]. PAs designed with a branched architecture show improved cell attachment and migration, likely due to decreased molecular packing altering the density of epitopes on the nanofibre surfaces [23]. The role of nanostructure shape on the bioactivity of the displayed epitope was recently evaluated, comparing the bioactivity of RGDS presented on nanospheres with that of the epitope presented on nanofibres [24]. It was found that epitopes presented on the surface of nanofibres are significantly more bioactive than those presented at equal density on the surface of nanospheres. Co-assembly of bioactive and nonbioactive PA molecules can enable control over the epitope densities presented to cells. Using this approach, a PA system presenting RGDS was prepared and optimized for biological adhesion as a scaffold for the therapeutic delivery of bone marrow mononuclear cells [25]. In a preliminary *in vivo* study, this material contributed significant support to the therapeutic cells following percutaneous co-injection (Fig. 2). In addition to being the main component of a scaffold, PAs can also be used as coatings to lend bioactivity to traditional tissue engineering materials. For example, coating RGDS PAs onto poly(glycolic acid) significantly enhances the adhesion of primary human bladder cells to the scaffold *in vitro* [26].

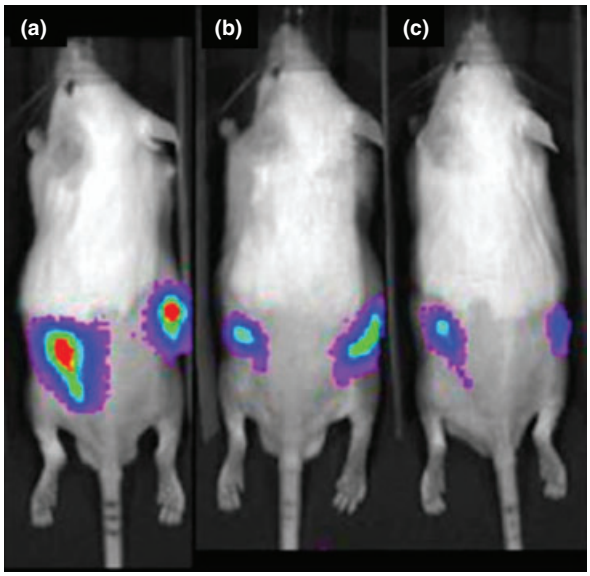

Fig. 2 (a) Therapeutic bone marrow mononuclear cells derived from a luciferase transgene mouse and transplanted within an RGDS-presenting PA network into either flank of a wild-type mouse, imaged at 4 days following transplant; (b) the same cells when transplanted within a PA network that did not contain the RGDS epitope; (c) The same cells, transplanted with only a saline vehicle. Reprinted with permission from Webber *et al.* [25].

Not only have PAs been used as scaffolds that provide structural support and bioactive signals, but these materials have also been molded to allow for the study of the effects of matrix geometry on the behaviour of cells. We have used microfabrication and lithography techniques to develop well-defined topographical patterns of PAs with microscale and nanoscale resolution [27–29]. Self-assembly of PAs containing both a photo-polymerizable moiety and the RGDS cell adhesion epitope were designed for use within microfabrication molds. This resulted in networks of nanofibres with well-defined topographical patterns such as holes, posts, or channels produced from networks of either randomly oriented PA nanofibres or prealigned PA nanofibres [27]. These surfaces were used as substrates to culture human mesenchymal stem cells (MSCs), examining the effects of the topographical and nanoscale features on the migration, alignment and differentiation of these cells. Topographical patterns produced from aligned PA nanofibres were able to promote the alignment of MSCs, indicating cell sensing of both micro- and nanoscale substrate features. Specifically, MSCs underwent osteogenic differentiation when cultured on substrates of randomly oriented nanofibres that had been patterned into hole microtextures.

Peptide amphiphile–polymer hybrids

Recently, a new assembly strategy has been explored to produce hybrid structures by mixing PA molecules with oppositely charged biopolymers. Mixing a solution of high molecular weight hyaluronic acid (HA) with a solution containing an oppositely charged PA

results in immediate formation of a solid membrane at the interface (Fig. 3) [30]. When the dense HA solution is placed on top of the PA solution, the HA fluid sinks into the PA solution, leading to continuous growth of the membrane at the progressively renewed liquid–liquid interface. This process eventually results in the formation of a liquid-filled sac. These sacs can be made instantly by injecting HA solution directly into the PA solution, and the resulting robust macroscopic structures can encapsulate human MSCs, which remain viable for up to 4 weeks in culture. These cells, when stimulated by soluble factors in the media, undergo chondrogenic differentiation whilst encapsulated in the sac. This synergistic assembly also allows us to produce membranes of arbitrary shape and highly ordered strings. The strategy of utilizing the electrostatic attractions between a large charged molecule and oppositely charged self-assembling small molecules has great potential for the development of highly functional materials organized

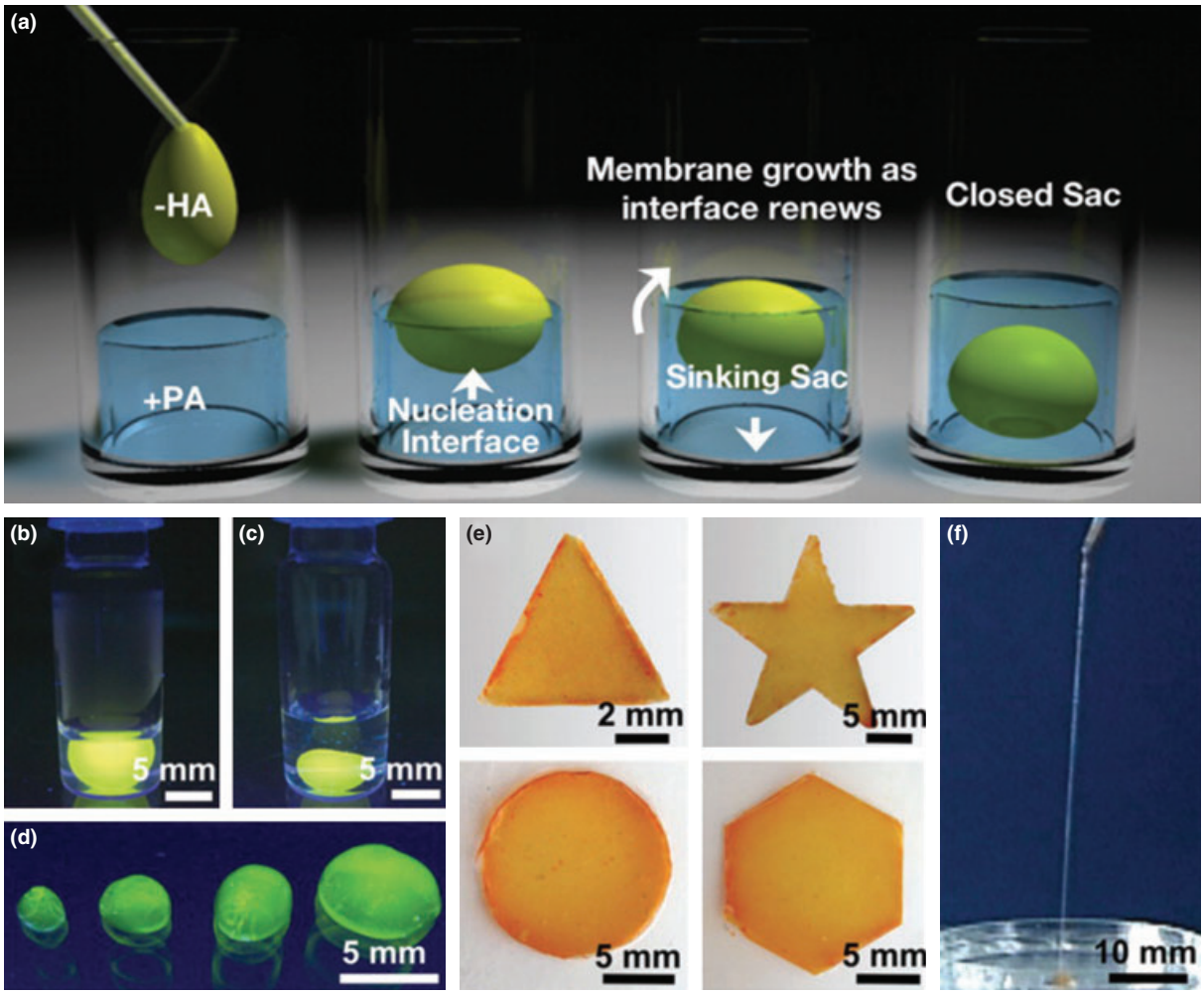

Fig. 3 (a) Schematic representation of one method to form a self-sealing closed sac. A sample of the denser negatively charged biopolymer solution is dropped onto a positively charged peptide amphiphile (PA) solution. (b) Open and (c) closed sac formed by injection of a fluorescently tagged hyaluronic acid (HA) solution into a PA solution. (d) Self-assembled sacs of varying sizes. (e) PA-HA membranes of different shapes created by interfacing the large- and small molecule solutions in a very shallow template (~1 mm thick). (f) Continuous strings pulled from the interface between the PA and HA solutions. From Capito *et al.* [30]. Reprinted with permission from AAAS.

across multiple length scales that could be used to deliver cells within an isolated and controlled environment for regenerative medicine.

Peptide amphiphiles: medical applications

Neural regeneration

Regeneration of the central nervous system presents a formidable challenge within regenerative medicine, as neurons in the brain and spinal cord have very limited potential for healing and reorganization. The Ile-Lys-Val-Ala-Val (IKVAV) peptide sequence, derived from laminin, has been incorporated into PAs for applications in neural regeneration to enhance neural attachment, migration and neurite outgrowth. Our previous work with PAs presenting this epitope found that neural progenitor cells (NPCs) cultured *in vitro* within networks of IKVAV PA quickly undergo selective and rapid differentiation into neurons with the formation of astrocytes being largely suppressed [16]. This selective differentiation was even greater for NPCs cultured in our PA networks than for cells cultured with laminin, the natural protein bearing the IKVAV sequence on which the synthetic peptide is based. To establish cell lineage the proteins β-tubulin III and glial fibrillary acidic protein (GFAP) were used as markers for neurons and astrocytes, respectively. NPCs cultured within our PA gel overwhelmingly express β-tubulin III compared with GFAP as they differentiate. This observation is presumably due to the high density at which the epitope is presented on the nanofibre surface. Control experiments using a mixture of soluble IKVAV peptide and PA nanofibres without the IKVAV epitope did not reveal this same response.

The *in vitro* results showing selective differentiation of NPCs suggested that the IKVAV PA may be a useful material in the treatment of spinal cord injury, where the formation of a glial scar, comprised primarily of astrocytes, prevents axonal regeneration after injury [31]. Indeed, when IKVAV PA nanofibres were applied to a mouse spinal cord injury model, the response in treated animals was quite promising [32]. The animal model used mice treated with an injection of IKVAV PA solution 24 h following spinal cord injury; at the site of injection this solution should form nanofibres by self-assembly through electrolyte screening of the molecules. The material reduced cell death at the injury site and decreased the astrogliosis involving a hyperplastic state of astrocytes. The injected nanofibre gel also increased the number of oligodendroglia, the cells responsible for the formation of the myelin sheath around neurons in the central nervous system, at the injury site. Histological evidence was also obtained for the regeneration of descending motor axons as well as ascending sensory axons across the site of spinal cord injury in animals treated with the IKVAV PA (Fig. 4). This was accompanied by behavioural improvement as well, with treated animals demonstrating enhanced hind limb functionality [32]. The results of this animal study offers great promise for the potential of PAs to restore function to those paralysed by spinal cord injury, and this target remains an area of focus for our group.

Angiogenesis

The enhancement of angiogenesis, the development of new blood vessels from existing vasculature, holds promise for the treatment of ischaemic diseases of the heart, peripheral vasculature, and chronic wounds [33, 34]. A PA, termed the heparin-binding peptide amphiphile (HBPA), was designed with a Cardin-Weintraub heparin-binding domain to specifically bind heparan sulphate-like gylcosaminoglycans (HSGAG). This glycosaminoglycan screens charges on the HBPA molecules, triggering PA self-assembly into nanofibres that display heparin on their surface [35, 36]. The heparin-binding character of HBPA was verified using isothermal titration calorimetry (ITC), determining an association constant of 1.1×10^7. The presentation of heparin enables these nanofibres to capture many potent signalling proteins through their heparin-binding domains; such proteins include fibroblast growth factor 2 (FGF-2), bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF). The heparin-presenting HBPA showed a prolonged release of FGF-2 compared with an HBPA network prepared using divalent phosphate ions to screen charges and promote self-assembly. FGF-2 and

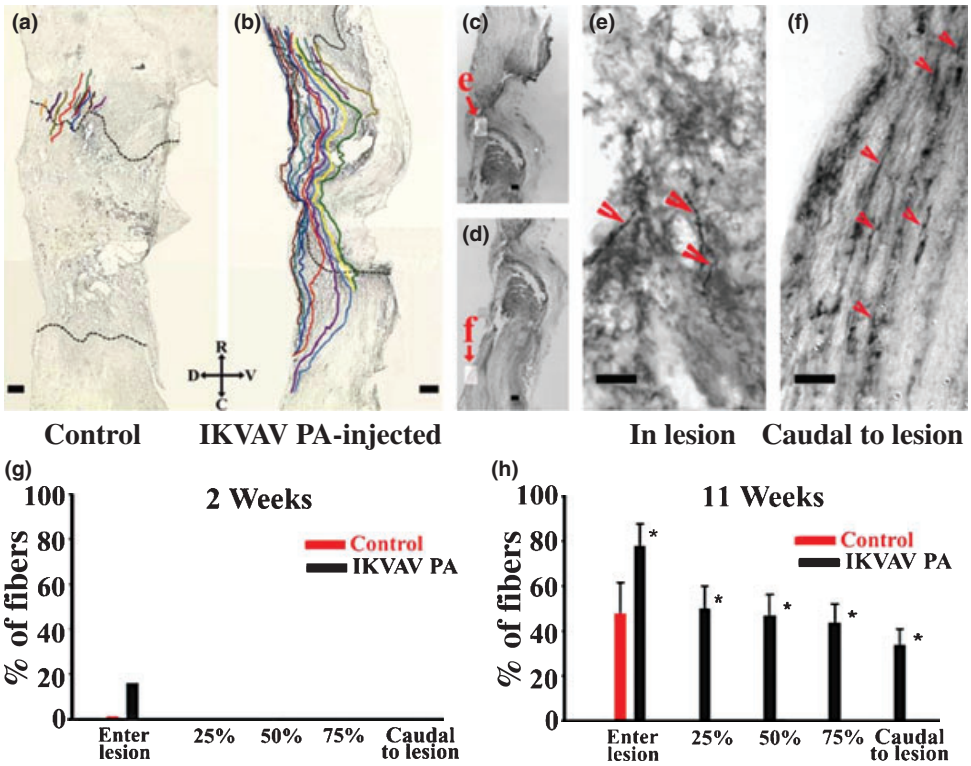

Fig. 4 IKVAV PA promotes regeneration of descending corticospinal motor axons after spinal cord injury. (a, b) Representative neurolucida tracings of BDA-labelled descending motor fibres within a distance of 500 μm rostral of the lesion in vehicle-injected (a) and IKVAV PA-injected (b) animals. The dotted lines demarcate the borders of the lesion. (c–f) Bright-field images of BDA-labelled tracts in lesion (c, e) and caudal to lesion (d, f) used for Neurolucida tracings in an IKVAV PA-injected spinal cord (a, b). (g, h) Bar graphs show the extent to which labelled corticospinal axons penetrated the lesion. *The groups representing three control and three IKVAV PA mice and the tracing of 130 individual axons differ from each other at $P < 0.03$ by the Wilcoxon rank test. (R, Rostral; C, caudal; D, dorsal; V, ventral. Scale bars: a–d: 100 μm and e, f: 25 μm.) Reprinted with permission from Tysseling-Mattiace *et al.* [32].

VEGF are well known to participate in angiogenesis, so our laboratory first explored the HBPA-heparin system for the promotion of new blood vessels. When nanogram quantities of the bioactive factors were combined with the HBPA-heparin and implanted into a surgical pocket of a rat cornea, the material induced significant vascularization compared to growth factors alone, HBPA-heparin without growth factors, and similar materials with and without growth factors (Fig. 5) [37]. Scrambling of the heparin-binding sequence presented on the PA diminished the observed angiogenic effect in a tube formation assay using endothelial cells [36]. Since the scrambled version of the PA and HBPA had similar association constants with heparin, the difference in bioactivity was attributed to a slower off-rate for the interaction between heparin and HBPA, stabilizing the protein from enzymatic degradation and leading to more efficient growth factor signalling.

Assessing the tissue reaction to these HBPA-heparan sulphate nanofibre gels using a mouse subcutaneous implantation model and percutaneous injection revealed excellent biocompatibility and also demonstrated retention of the material for at least 30 days *in vivo* [38]. The more exciting finding from this study was the discovery that, as the material was biodegraded, it was quickly remodelled into a well-vascularized connective tissue without the addition of any exogenous growth factors. Dynamic analysis

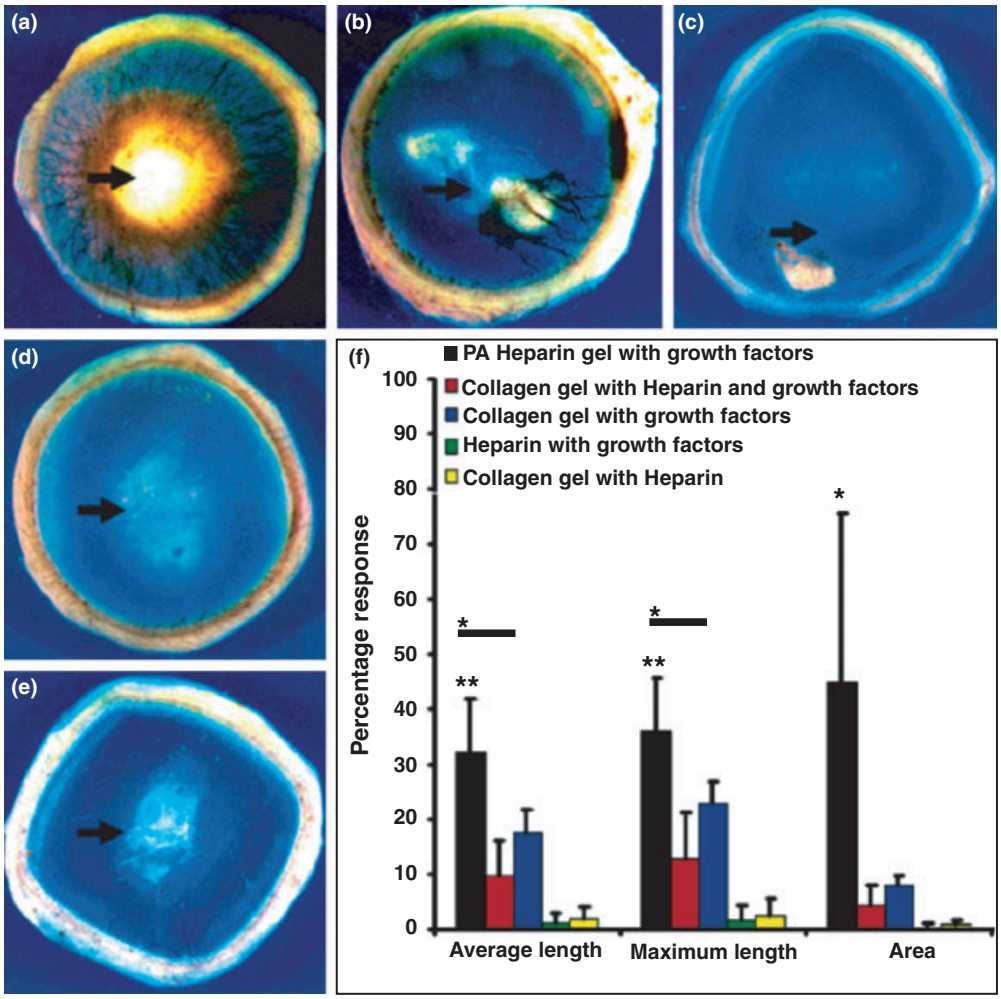

Fig. 5 *In vivo* angiogenesis assay. Rat cornea photographs 10 days after the placement of various materials at the site indicated by the black arrow. (a) Heparin-nucleated PA nanofibre networks with growth factors show extensive neovascularization. Controls of collagen, heparin, and growth factors (b) and collagen with growth factors (c) show some neovascularization. Heparin with growth factors (d) and collagen with heparin. The bar graph (f) contains values for the average and maximum length of new blood vessels and the area of corneal neovascularization. A 100% value in the area measurement indicates that the cornea is completely covered, and a 100% value in the length parameters indicates that the new vessels are as long as the diameter of the cornea (bars are 95% confidence levels, $*P < 0.05$ when PA-heparin gel was compared to collagen gel with growth factors, $**P < 0.005$ when PA-heparin gel with growth factors was compared to all of the other controls). PA nanofibres with heparin, PA solution with growth factors, and growth factors alone did not result in measurable neovascularization (values not shown in graph). Reprinted with permission from Rajangam *et al.* [37]. Copyright © 2006 American Chemical Society.

of the tissue reaction using a skinfold chamber model demonstrated no adverse effects of the material on the microcirculation, and also confirmed the biocompatibility and vascularization seen in the subcutaneous model. This points to the potential to use the HBPA system for the healing of chronic wounds or to enhance efficacy of skin grafts, as both of these could benefit from the extensive granulation tissue observed.

Islet transplantation

Treatments currently being explored for type I diabetes mellitus, a condition that results from autoimmune

destruction of insulin-producing β-cells, involve transplantation of donor islets, the pancreatic cell aggregates that contain β-cells. The efficacy of this strategy is limited by poor islet viability and engraftment following transplant [39]. To improve islet engraftment, the angiogenic potential of the HBPA-heparin nanofibre system was explored *in vivo* to deliver donor islets into a diabetic mouse model [40]. Currently, donor islets are transplanted into the liver due to its high degree of vasculature. HBPA, combined with FGF-2 and VEGF, was found to significantly enhance vasculature in the omentum, an intraperitoneal fat mass that in principle would be a more desirable site for transplantation of islets. When islets were transplanted with HBPA-heparin nanofibre networks and angiogenic growth factors into the omentum, the cure percentage of diabetic mice restored to a normoglycemic state was significantly enhanced relative to untreated diabetic mice. This was not the case for the administration of islets with HBPA-heparin alone or with growth factors alone.

NOx release for neointimal hyperplasia

Atherosclerosis affects nearly 80 million people in the US alone, and whilst there are a variety of possible treatment modalities such as angiogplasty and stenting, these procedures have limited efficacy due in large part to secondary complications of neointimal hyperplasia. Nitric oxide has long been recognized as a possible solution to prevent the onset of these secondary complications. Peptide amphiphiles presenting heparin were mixed with diazeniumdiolate nitric oxide donors to prepare nitric oxide releasing nanofibre gels [41]. Mixing with the nanofibre gel extended the release of nitric oxide over a period of 4 days *in vitro*, significant considering the half-life for release from the small molecules alone is on the order of seconds to hours. This prolonged release profile suggests advantages for the delivery of nitric oxide in the treatment of neointimal hyperplasia, further supported by demonstrating the ability to decrease smooth muscle cell proliferation whilst leading to greater cell death *in vitro*. When applied to a rat carotid artery balloon injury model, the nitric oxide releasing PA nanofibre gels led to a reduction in neointimal hyperplasia by up to 77% compared with the controls and also limited inflammation in the injury site (Fig. 6).

Hard tissue replacement and regeneration

Regeneration or replacement of hard tissue in the body has proven to be a challenge in part due to the mechanical properties of these tissues. One possible

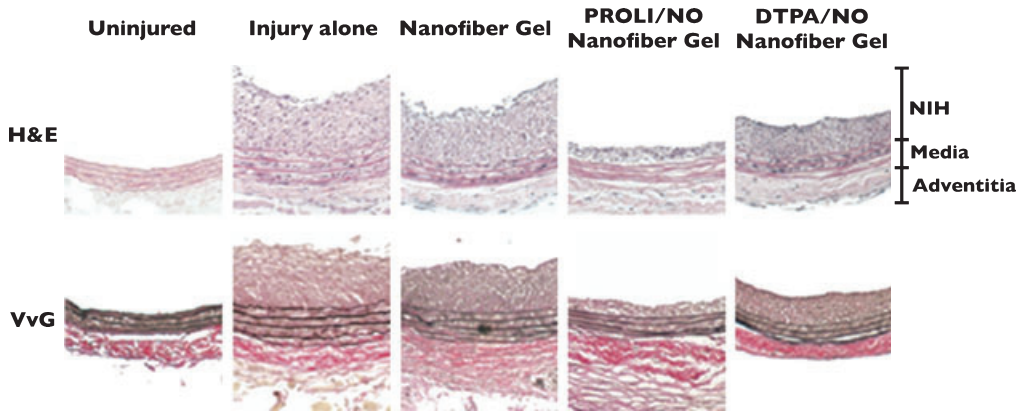

Fig. 6 Rat carotid artery sections from uninjured, injury alone, nanofibre gel, PROLI/NO nanofibre gel, and DPTA/NO nanofibre gel animals killed at 14 days (n = 6 per group). Displayed are representative sections (100× original magnification) from each group using routine staining with haematoxylin and eosin (top) and Verhoff-van Gieson (bottom). PROLI/NO and DPTA/NO are two small molecule diazeniumdiolate nitric oxide donors that were mixed with the PA nanofibre gel. Reprinted with permission from Kapadia *et al.* [41].

approach has focused on replacement of the tissue with a permanent metal implant. Whilst traditional metal implants made from titanium and its alloys possess good mechanical properties and reasonable biocompatibility, these materials do not incorporate a bioactive component. PA nanofibres were explored as a means to functionalize these metal implants to enhance bioactivity and prompt tissue growth around the implant to assist in long-term implant fixation. A nickel-titanium (NiTi) alloy that is frequently used for stents, bone plates, and artificial joints was modified through covalent attachment of PA nanofibres using standard silanization and cross-linking chemistry [42, 43]. Modifying the metal with RGDS-presenting PAs led to a significant increase in the number of adhered preosteoblastic cells cultured *in vitro*, whilst cells did not attach to the nonfunctionalized NiTi. Porous titanium implants are frequently used to encourage tissue ingrowth and assist with implant fixation. RGDS-presenting PA nanofibre gels have been prepared within the pores of such scaffolds [44, 45]. The PA molecules were triggered to gel by the introduction of counterions within the interconnected pores of titanium foam. Preosteoblastic cells seeded within these PA-titanium foam hybrids were viable, proliferative, and exhibited signs of osteogenic differentiation. These results point to the potential to use these PA-functionalized metal constructs *in vivo*, and a pilot study in a rat defect model has been undertaken [44].

Enamel, the outermost coating of vertebrate teeth and the hardest tissue in the body, remains a formidable challenge in regenerative medicine. The ameloblast cells responsible for the production of enamel during development subsequently apoptose, preventing enamel regeneration during adulthood. Branched RGDS-presenting PA nanofibres have been used as scaffolds for ameloblast-like cells and primary enamel organ epithelial (EOE) cells that initiate the process of enamel formation [46]. When treated with branched RGDS PA nanofibres *in vitro*, these cells showed an enhancement in proliferation and increased their expression of amelogenin and ameloblastin, two proteins secreted by ameloblasts during enamel formation. In an organ culture model, the RGDS PA was injected into embryonic mouse incisors. Again, EOE proliferation and ameloblastic differentiation was observed, evidenced by an increased expression of enamel specific proteins.

Hartgerink *et al.* have used PAs as an *in vitro* scaffold for dental stem cells. They found that stem cells from human exfoliated deciduous teeth proliferate and secrete a soft collagen matrix when encapsulated within the PA whilst dental pulp stem cells differentiate into an osteoblast-like phenotype and deposit mineral when encapsulated within the gel [47]. They propose that this PA system combined with dental stem cells could be used as a treatment for dental caries, enabling regeneration of both soft and mineralized dental tissue.

Templating biomineral

The ability to template biologically relevant material holds great promise for the treatment of bone loss, osteoporosis, or dental caries. The surface of PA nanofibres can be customized to create templates for this inorganic mineralization. The first PAs designed by our group incorporated phosphorylated serine residues with the aim of templating biomimetic hydroxyapatite crystals similar to those found in bone and dentine [2, 48]. Phosphorylated serine is a nonstandard amino acid that is found frequently in proteins that template calcium phosphate in mineralized tissues [49–51]. Using this amino acid to template mineral produced hydroxyapatite with the crystallographic axis aligned with the length of PA nanofibres, mimicking the crystallographic orientation of hydroxyapatite in bone with respect to the long axis of collagen fibres. The enzyme alkaline phosphatase was later determined to be critically important for the biomineralization of PA networks in three dimensions [52]. Temporal control provided by enzymatically harvested phosphate ions enables phosphorylated serine-presenting PA nanofibres to nucleate hydroxyapatite on their surface which provides spatial control for biomineralization to occur on the PA nanofibre networks.

Magnetic resonance imaging (MRI) contrast agents

Magnetic resonance imaging is one of medicine's most powerful diagnostic techniques, providing three-dimensional structures of living tissue at near cellular resolu-

tion. However, this technique is currently limited by the timecourse and circulation time of the small molecule contrast agents used for imaging. Previously, we covalently attached a derivative of a molecule (DOTA) that chelates gadolinium to PA molecules to increase the relaxivity of the MR agent [53, 54]. These MR agent-conjugated PA molecules self-assemble into cylindrical nanofibres or spheres under aqueous conditions. The rotational correlation time of the MR agent was increased following self-assembly into nanostructures, suggesting enhanced relaxivity. It was also found that the molecule's relaxivity and imaging properties can be influenced by the position on the PA molecule that the DOTA derivative is attached, showing enhancement when the DOTA/Gd(III) complex was closer to the hydrophobic region. Nanofibre networks comprised of these MR agent-conjugated PAs can be imaged using MRI techniques [54]. Future work will examine these MRI-conjugated PA constructs *in vivo* to track the fate of an implanted PA nanofibre gel and also evaluate the efficacy of IV-administered PAs as a prolonged blood-pool contrast agent.

Other self-assembling peptide technology

β-Sheet peptides

Previous work by Aggeli *et al.* demonstrated the importance of the *β*-sheet structural motif in peptide supramolecular chemistry, exploiting this noncovalent interaction to prepare self-assembling oligopeptides that form semi-flexible nanotapes [55]. This assembly strategy is primarily governed by hydrogen bonding along the peptide backbone and interactions between specific amino acid side-chain constituents. These *β*-sheet peptide nanotapes can further assemble into a hierarchy of supramolecular morphologies (Fig. 7a), including twisted ribbons (double tapes), fibrils (twisted stacks of ribbons), and fibres (entwined fibrils) by changing pH, monomer concentration, amino acid sequence/charge, or the intrinsic chirality of the precursor oligopeptide [56, 57]. These structures can entangle into viscoelastic networks in both aqueous and organic conditions, depending on the selection of amino acids, and can form hydrogels or organogels, respectively, when a critical concentration is reached

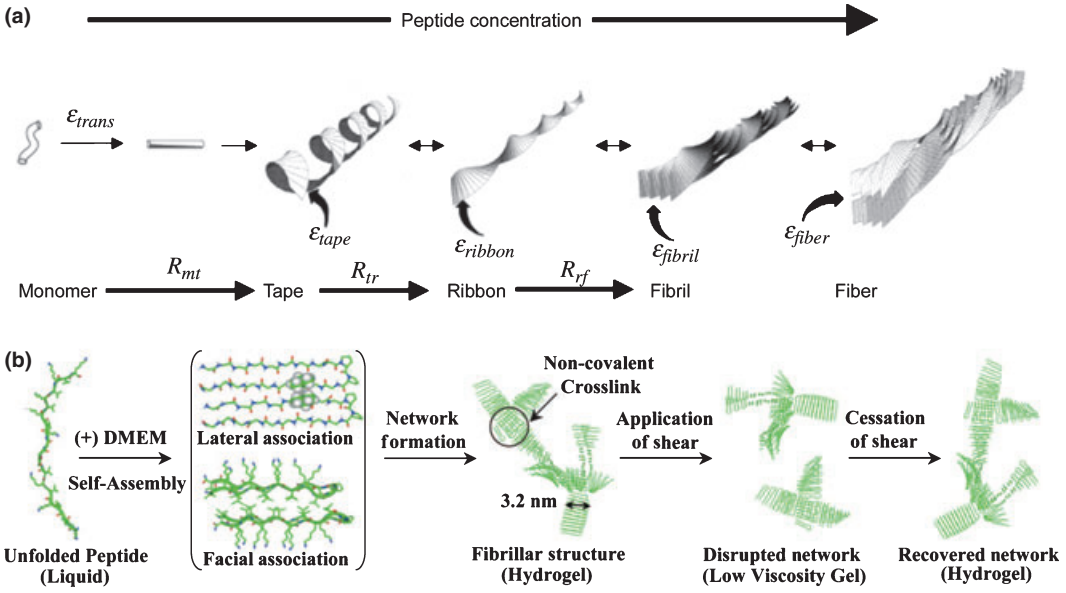

Fig. 7 (a) The hierarchical equilibrium configurations of self-assembly of *β*-sheet forming peptides, which assemble to form tapes, ribbons, fibrils and fibres depending on parameters such as peptide concentration, solution pH, or sequence/charge of the precursor monomer. Reprinted with permission from Aggeli et al. [57]. Copyright © 2003 American Chemical Society. (b) The self-assembly of *β*-hairpin peptides through charge screening in physiological media (DMEM) allows for the formation of self-healing hydrogels that can be syringe-delivered. Reprinted from Haines-Butterick *et al.* [66]. Copyright © 2006 National Academy of Sciences, USA.

[57]. Mixing aqueous solutions of cationic and anionic β-sheet peptides with complementary acidic and basic side-chains results in the spontaneous self-assembly of fibrillar networks and nematic hydrogels that are robust to variations in pH and peptide concentration [58].

In the context of regenerative medicine, these β-sheet peptide nanostructures have been evaluated for the treatment of enamel decay [59]. Extracted human premolar teeth containing caries-like lesions were exposed *in vitro* to multiple cycles of demineralizing (acidic conditions) and remineralizing (neutral pH conditions) solutions. Applying an anionic peptide designed for β-sheet assembly to the defects both decreased demineralization during exposure to acid and increased remineralization at neutral pH, resulting in significant gains of net mineral within the lesions over the 5 day study. The peptide gels also nucleated the formation of *de novo* hydroxyapatite when incubated in mineralizing solutions [59].

In another application towards regenerative medicine, these peptides were evaluated as an injectable joint lubricant for the treatment of osteoarthritis [60]. An array of peptides were designed with variations in charge and hydrophilicity to determine which supramolecular assembles would have properties similar to those of HA, the main contributor to the lubricating properties of synovial fluid. One such β-sheet peptide with molecular, mesoscopic, and rheological properties most closely matching those of HA performed similarly in healthy static and dynamic friction tests; however it did not perform as well as HA when friction tests were performed with damaged cartilage [60]. One advantage of these systems is that they assemble *in situ*, but have very low viscosity prior to and during injection, making them easier to handle than the highly viscous HA. This preliminary work points to the possibility of using self-assembled peptides as a synthetic joint lubricant in the treatment of degenerative osteoarthritis.

β-Hairpin peptides

Another peptide design that captures the self-assembling potential afforded by the β-sheet is prepared from monomers of alternating hydrophilic and hydrophobic residues, lysine and valine, respectively, flanking an intermittent tetrapetide designed to mimic a Type II' β-turn, termed a β-hairpin peptide [61–66]. These peptides are designed to be hydrated in pure water, adopting a random coil conformation. In charge screening conditions, such as in the presence of ions in physiological fluid or through transitions to basic pH, the electrostatic repulsion between charged residues is diminished, allowing the peptide to fold into its β-hairpin conformation. This molecular folding produces a monomer with extensive intermolecular hydrogen bond formation and with one face of the β-hairpin exposing the hydrophobic valine residues, whilst the other has exposed hydrophilic lysines, organization that results from the alternating sequence in the unfolded molecule. Subsequent self-assembly of the individual hairpins due to hydrophobic interactions and intramolecular hydrogen bond formation leads to a highly entangled hydrogel (Fig. 7b). In cases where pH is increased to induce hydrogel formation, unfolding of the hairpins and dissociation of the hydrogel can be triggered by lowering the pH below the pK_a of the lysine side-chains, restoring electrostatic repulsion [61]. If a lysine residue is replaced by a negatively charged glutamic acid, the overall peptide charge is decreased and the peptide can be more easily screened, resulting in much faster self-assembly [66]. The changes in the hydrogelation kinetics were found to significantly improve the homogenous distribution of encapsulated cells within these self-assembling gels, an important characteristic for an injectable cell scaffold material. Studies *in vitro* have found that these β-hairpin hydrogels can support survival, adhesion, and migration of fibroblasts [64, 66, 67], and can be used to encapsulate MSCs and hepatocytes [66]. These gels have also been found to have inherent antimicrobial properties; showing selective toxicity to bacterial cells compared with mammalian cells [68].

Ionic self-complementary peptides

Another class of self-assembling peptide molecules developed by Zhang *et al.* was designed based on β-sheet-rich proteins from nature, prepared from

sequences of alternating hydrophobic and hydrophilic residues [69]. The first such molecules were based on a segment of the yeast protein, Zuotin, and formed an insoluble macroscopic membrane [70, 71]. Variations in peptide sequence, whilst maintaining the alternating ionic hydrophilic and hydrophobic residues, have utilized mixed charged residues, such as repeat units of Arg-Ala-Asp-Ala (RADA) or repeat units of RARA-DADA. These peptide sequences demonstrate stable fibrillar nanostructure self-assembly driven by spontaneous β-sheet formation and complementary ionic interactions between the oppositely charged ionic residues (Fig. 8a). With the addition of counterions or physiological media, these fibrillar nanostructures form entangled hydrogels [71, 72]. Other studies have found that oligopeptide length [73] and side-chain hydrophobicity [74] are important variables that affect both the peptide self-assembly and the mechanical properties of the resulting gel.

These ionic self-complimentary peptides have advanced quite far towards being applied as therapies for regenerative medicine. With respect to *in vitro* evaluation, there has been several studies examining the ability of these self-assembled scaffolds to support cell attachment [71], to promote the survival, proliferation, differentiation and neurite growth for neural cells [72, 75], to promote the differentiation of liver progenitor cells into hepatocyte spheroids [76], to serve as scaffolds for human endothelial cells [77–80], as well as scaffolds for chrondrocytes [81] and for osteogenic differentiation of human embryonic stem cells [82, 83].

Several studies have been carried out *in vivo* examining these peptide scaffolds for the treatment of cardiovascular disease, focusing on the delivery of growth factors and the recruitment of progenitor cells [84–87]. For the treatment of myocardial infarction, the peptide scaffolds were injected into rat myocardium and were found to enhance the recruitment of endothelial progenitor cells and vascular smooth muscle cells into the injection site, appearing to form functional vascular structures [84]. Nanofibres displaying biotin were subsequently used to deliver streptavidin-linked insulin-like growth factor 1 (IGF-1) along with neonatal cardiomyocytes and this therapy was shown to significantly improve systolic function after

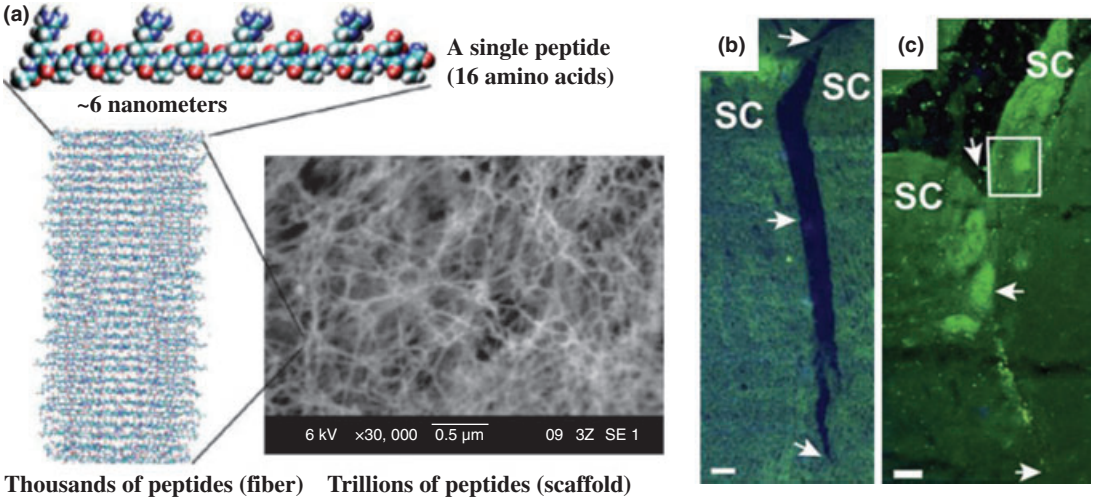

Fig. 8 (a) Ionic self-complimentary peptides self-assemble by hydrogen bonding into fibres consisting of thousands of peptides which further entangle into scaffolds that are ~99.5% water and ~0.5% peptide. Reprinted from Gelain *et al.* [69]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission. (b, c) View of the superior colliculus (SC) 30 days following the formation of a tissue gap by deep transection of the optic tract in the hamster midbrain following a control treatment with saline (b) or treatment with ionic self-complimentary peptide nanofibres (c). With nanofibre treatment, the site of the lesion has healed and axons have grown through the treated area and reached the caudal part of the SC. Reprinted from Ellis-Behnke *et al.* [88]. Copyright © 2006 National Academy of Sciences, USA.

myocardial infarction [85]. Other studies evaluated the delivery of platelet-derived growth factor (PDGF) using these self-assembling peptide nanofibres in a rat myocardial infarct model, demonstrating decreased infarct size corresponding to reduced cardiomyocyte death and functional improvement in cardiac performance after infarction [86, 87].

In applications towards regeneration of the central nervous system, these peptide scaffolds were evaluated in an acute model severing the optic tract within a hamster brain, demonstrating the ability to prompt axon regeneration and the reconnection of target brain tissues, eventually resulting in restored vision (Fig. 8b,c) [88]. These scaffolds, when implanted with NPCs and Schwann cell into a transected dorsal column of a rat spinal cord, also promoted cell migration, blood vessel growth, and axonal elongation through the injury site, demonstrating the potential to perhaps use these materials to reverse spinal cord injury [89].

Aromatic-terminated peptides

An emerging class of self-assembling peptides use conjugated aromatic groups such as carbobenzyloxy, naphthalene, or fluorenylmethyloxycarbonyl (Fmoc) on the N-terminal end of di- and tri-peptides, demonstrating the formation of very stable, highly tunable hydrogels [90, 91]. Frequently, these aromatic groups have been attached to a di-phenylalanine peptide, and gel formation from these molecules can be induced by altering pH [90] or by incorporating domains which are sensitive to enzymatic activity [92]. Assembly of these small molecules occurs through two simultaneous attractive forces, antiparallel β-sheet formation between the oligopeptide domain and π–π stacking of the aromatic rings [93]. A number of these sheets then twist together to form nanotubes. Studies examining these peptide hydrogels *in vitro* indicate that these materials can support chondrocyte survival and proliferation in both two and three dimensions [90, 94]. Adding chemical functionalities to these Fmoc-peptides made these materials selective to the culture of different types of cells and affected the mechanical properties of the formed hydrogel [95].

Future prospects

We have here described recent progress in the development of peptide-based nanotechnology as a novel therapeutic strategy for regenerative medicine. There remains work to be done in developing this technology. The field so far has involved the design of peptidic molecules for assembly into nanostructures by different mechanisms, yielding great variability in the degree of precision in supramolecular structure. Precision in shape and dimensions of the peptide nanostructures needs to be part of future work in the field. It is also important to multiplex biological functions, either through their integration into single nanostructures or by creating arrays of different nanostructures. Designs could also include incorporation of extracellular and intracellular bioactivity targets to directly control signal transduction pathways. The use of bioactive peptide nanostructures in hierarchical structures with sophisticated function is also an important area for future growth. This could yield innovative constructs for controlled three-dimensional cell culture, particularly for stem cells, or novel methods for cell transplantation or cell delivery. Another fertile direction is the design of peptide nanostructures that can be delivered systemically and targeted to specific compartments of the body to promote regenerative processes, including crossing of the blood-brain barrier.

The technologies we have highlighted are just beginning the process of translation into disease therapeutics. This is evident by the recent number of small animal studies that are being undertaken by our group and others. If this technology is to advance as a therapeutic option for treating human ailments, studies like these are essential. We have described some very promising *in vivo* data using PAs as a therapy for spinal cord injury, islet transplantation, prevention of neointimal hyperplasia, and induction of angiogenesis. Ongoing work will evaluate these PAs in a variety of other disease models, from cancer to myocardial infarction to Parkinson's disease, and we hope to continue to show therapeutic efficacy in these evaluated models. In addition, Zhang *et al.* have extensively evaluated their ionic self-complimentary peptides in a

number of small animal models. The results from the *in vivo* studies described in this review are encouraging for the future use of peptide-based nanotechnology as a therapy for humans, though hurdles remain. However, we hope that the efficacy observed by these preliminarily evaluated technologies, combined with the continued development of new technologies, will lead to a new therapeutic niche based on self-assembling peptides that could be a realistic therapeutic option for humans in the coming years.

Conflict of interest statement

No conflict of interest was declared.

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