Biointerphases (2012) 7:2
DOI 10.1007/s13758-011-0002-x

IN FOCUS: NANOMEDICINE - REVIEW

Self-Assembled Peptides: Characterisation and In Vivo Response

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Received: 12 September 2011 / Accepted: 9 November 2011 / Published online: 11 February 2012

Abstract The fabrication of tissue engineering scaffolds is a well-established field that has gained recent prominence for the in vivo repair of a variety of tissue types. Recently, increasing levels of sophistication have been engineered into adjuvant scaffolds facilitating the concomitant presentation of a variety of stimuli (both physical and biochemical) to create a range of favourable cellular microenvironments. It is here that self-assembling peptide scaffolds have shown considerable promise as functional biomaterials, as they are not only formed from peptides that are physiologically relevant, but through molecular recognition can offer synergy between the presentation of biochemical and physio-chemical cues. This is achieved through the utilisation of a unique, highly ordered, nano- to microscale 3-D morphology to deliver mechanical and topographical properties to improve, augment or replace physiological function. Here, we will review the structures and forces underpinning the formation of self-assembling scaffolds, and their application in vivo for a variety of tissue types.

This article is part of the Topical Collection "In Focus: Nanomedicine".

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1 Introduction

Nanotechnology has been identified as having the potential to benefit many aspects of society, from health care to electronics [1]. Inspiration within this field often comes from functional nano-architectures discovered within the biological world, where seemingly simple rules and forces underpin the formation of complex systems [2, 3]. A key challenge presented to researchers is to selectively utilise these principles; most notably within the field of tissue engineering, where there is a pressing need for the formation of biologically relevant 3-D scaffolds for regenerative medicine and defined cell culture matrices [4]. The recent advancement of interdisciplinary research that involves nanotechnology, cellular and molecular biology and biomaterial science provides an optimistic outlook for the development of adjuvant tissue engineering scaffolds for in vivo repair. Ideally, such scaffolds should have the capacity to concomitantly utilise mechanical, topographical and biochemical properties to stimulate the cellular milieu and promote endogenous regeneration. Understanding features of the cellular niche, biological function, associated pathophysiology and the cascade of regenerative event for each individual application are crucial for engineering adjuvant scaffold that promote repair. Thus far, engineered scaffolds cannot appropriately recreate tissue analogues due to the intricate complexity and hierarchical nature of physiological tissue.

Recently, the non-covalent self-assembly of relatively simple peptide based molecules has gained increasing attention for the formation of nanostructured, biologically functional materials. Here, inherent structural properties allow the simple self assembly of these peptides to yield ordered nanostructures (e.g. tubes, rods, and sheets) [5], and this organisation enables the noncovalent presentation of the peptides chemical functionality to the surface of

these nanoscale features. When multiples of these structures come together, a supramolecular network is established. These have the benefit of biocompatibility and ease of synthesis not necessarily found in polymeric or covalently linked systems [6]. Once robust mechanisms for the assembly of fibrillar networks are established, cell-interactive stimuli (both physical and chemical) can be easily introduced and presented by the supramolecular ordering of the assembly process. This then allows the customisation of these materials to activate the innate regenerative cascade of specific cell types; through tuning the peptide sequence, controlling the rate of formation, and introducing biologically relevant molecules.

2 Self-Assembling Peptides as Biomaterials for In Vivo Applications

For successful tissue reconstruction/regeneration it is necessary to provide a cellular microenvironment that facilitates cell migration and/or transplantation, their 3-D organisation and, where appropriate, extracellular matrix (ECM) deposition. To this end, the development of adjuvant tissue engineering scaffolds have considerable promise, as they potentially offer synergy between the delivery/recruitment of cells, presentation of biochemical and physio-chemical cues, 3-D morphology and mechanical and topographical stimuli to improve or replace physiological function [7].

Within the past decade, a variety of 3-D tissue engineering scaffolds have emerged; for instance, fibrous scaffolds produced from a diverse range of polymers, both natural and synthetic, have been fabricated using a variety of methods, notably electrospinning [8–10]. These fibrous networks, through subsequent biochemical and physio-chemical modification, may present appropriate stimuli for in vivo repair [11, 12]. However, the deployment of such scaffolds will be disease/injury specific, as while the fibrous morphology can encourage cell migration, differentiation and elongation their potential to encourage regeneration within lesions is debatable, as they cannot readily interface with the surrounding tissue. Conversely hydrogels have shown utility toward these applications due to their inherent high water content, the ability to tune their mechanical properties, biocompatibility and, perhaps most importantly, their ability to fill irregularly shaped voids [13, 14]. However, whilst they can be produced from both natural and synthetically derived polymers they generally lack the fibrous morphologies reminiscent to the native ECM. Therefore, an ‘off-the-shelf’ material with the ability to concurrently incorporate the advantages of both of these types of scaffolds, and that can be engineered and optimised for a variety of different applications will be of clinical relevance. The nanoscale fibrillar structure and subsequent hydrogels produced by the controlled self-assembly of peptides (and peptide derivatives) are therefore excellent candidates to provide some of the critical aspects to the in vivo cellular microenvironment [15]. This class of material not only provides the stable 3-D microenvironment required to mimic some of the features of the ECM (morphological and dimensional), but the nanoscale presentation of the peptides within the fibre scan also encourage interaction with endogenous cells and the native ECM [16]. This enables the scaffold, through their bioinspired motifs, (both chemical and physical), to influence cell signaling, which is responsible for coordinating their function (i.e. survival, proliferation, migration, differentiation and the like). As the scaffolds are formed from peptides they are also inherently biocompatible. For the purposes of this review, we define biocompatible as not producing a chronic inflammatory response showing the materials are well-tolerated upon implantation, with minimal scar tissue formed. The self-assembled synthetic microenvironments are ideal vectors for exogenous cell transplantation, allowing host cell migration and proliferation within the matrix [17], and, in the case of some tissue types, enhance natural ECM deposition [18].

A key advantage to this approach is the facile synthesis of the peptides via manual or automated synthetic processes from biologically relevant molecules. Whilst many other tissue engineering scaffolds have employed animal derived products to fabricate a favourable niche with some success, clinically this is not ideal due to unknown contamination and the possibility of rejection and pathogen transmission. There are also issues of reproducibility in regards to protein content that will have a direct impact on cell function. The use of self-assembling peptide scaffolds avoids this limitation, as they have no batch-to-batch variability, and the stepwise manner of their synthesis allows the incorporation of bioactive moieties [19–21]. In addition, they have a long shelf life being stable at room temperature for many years, which is critical for translation to the clinic.

As tissue engineering scaffolds the systems can be delivered in a variety of minimally invasive ways, chiefly via (micro)injection. The physical hydrogels undergo a profound gel/solution/gel transition upon application and removal of shear, allowing a preformed hydrogel to be delivered as a liquid [22, 23]. Alternatively, the peptides can be injected in vivo as a solution with the assembly/gelation triggered by physiological conditions such as temperature, biocatalysis and ionic strength [24–26]. This allows spatially defined application directly to the site of therapeutic need; unlike the covalently linked, polymeric fibrous scaffolds mentioned above, these materials can be injected into, diffuse throughout, and assemble within irregular shaped lesions (Fig. 1c). This flexibility is crucial for a variety of applications as it allows intimate contact with the surrounding tissue

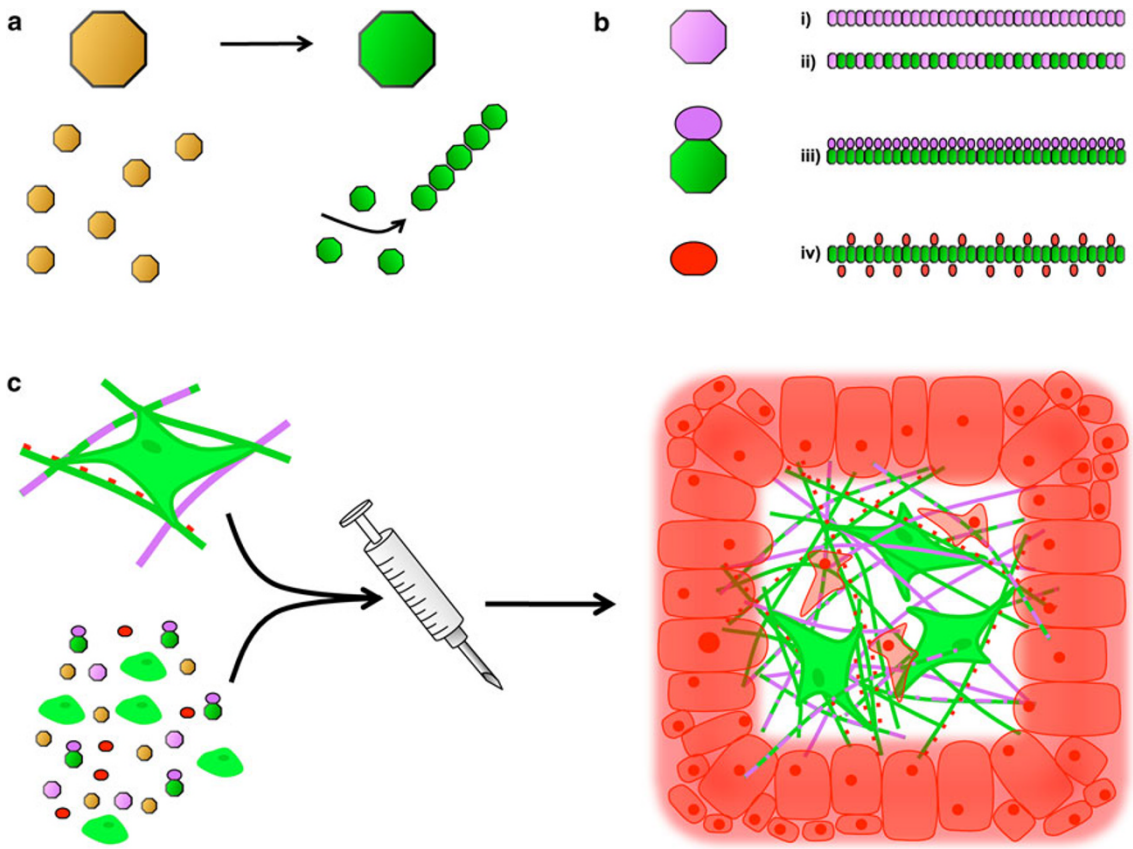

Fig. 1 **a** In order to trigger the self-assembly process, peptides freely in solution must be stimulated (temp, pH, ionic strength, etc.) to change into a form by which the ordered assembly is more favourable than remaining in solution. **b** In order to functionalise the self-assembled fibrils, signals must be included. Methods for achieving this include: *i* the functional epitope self-assembles (e.g. fmoc-RGD), *ii* doping creating a heterogenous assembly (e.g. fmoc-FF doped with fmoc-RGD), *iii* the assembling motif is capped with the signal, allowing the ordered presentation (e.g. peptide amphiphiles capped with IKVAV) or *iv* the signal is attached to the assembled fibril post assembly (whole proteins or peptide tags). **c** In order for the material to be delivered, the assembly process should either occur in vitro (and have cells pre-cultured on the material if required), or the peptides in solution are mixed with the appropriate signals (and cells), to assemble in vivo. These are then transferred to a delivery vector (e.g. microinjection). Once introduced to the tissue, the peptide assemblies interact with the implanted cells, directly contacting and supporting the host tissue, or, more typically, rely on endogenous cells migrating into the scaffold to promote repair

facilitating cell migration, whilst also physically supporting the stroma/parenchyma. It also provides the scaffolds with the unique ability to control inflammation and avoid scar formation not only through physical support but also biochemically if they are further functionalised with, or used to encapsulate drugs via hydrogelation.

In this review we will discuss the structures and forces underpinning these systems, and recent progress (since 2000) of their application in vivo. As this field is an emerging one, we will provide a perspective of current challenges and future opportunities.

2.1 Self-Assembly of Peptide Materials

Nature uses peptide sequences to yield an array of structural components, with β-sheets, α-helices, random coils, turns and loops being well known structural motifs. These structures are formed by interactions governed by the physicochemical properties of the side chains of the specific amino acid sequences that contribute to these formations, yet the overall fundamental interactions are remarkably conserved [27, 28]. Post-expression interactions drive the formation of protein structures and higher order interactions, governed by the properties of the amino acid residues as presented within the polypeptide chain. These forces include hydrogen bonds (polar amino acids), hydrophobic interactions (non-polar), ionic bonds and electrostatic interactions (acidic and basic), π-stacking, van der Waals interactions (aromatic), induction of turns (conformational constraint), the formation of disulphide bridges (sulphur containing) and especially relevant for biological systems, water mediated hydrogen bonds (all residues) [27, 29].

Several research groups have utilised these properties to pioneer the self-assembly of (oligo-) peptide systems to

form nanostructured fibrous materials. Here, we define molecular self-assembly as the spontaneous organisation of molecules under thermodynamic and kinetic conditions into stable and structurally welldefined arrangements [30, 31]. This process is driven/governed by a range of numerous weak non-covalent interactions. Although each of the bonds or interactions formed are weak, the collective interactions can impart defined intermolecular order resulting in stable structures and materials [32]. These systems operate on a hierarchy of scales; the underlying molecular interactions between peptides give rise to the formation of nanostructures, supramolecular associations between these form microscale networks, which ultimately give rise to macroscale materials that are easily handled. Simply put, individual peptides are completely solvated and only exist individually, or in disordered aggregates. Upon the application of a stimulus (pH, ionic strength, temperature, etc.), the peptides decrease in solubility and increase in attractive force facilitating their interaction (Fig. 1a). Firstly, two or more individual peptides amalgamate to form a nucleating aggregate; additional building blocks then amalgamate and become ordered, based on the innate structural information provided by the peptide chain. This yields a single, supramolecular fibril. Multiple fibrils then interweave, based on their surface properties, to form bundles, which in turn interact to form a branched network (the peptide scaffold). This network is highly porous, and results in the formation of a hydrogel ($\sim$0.1% w/v), through the entrapment of the solvent in which assembly occurs.

Recently, focus has been on developing assembly strategies using the easily accessible structures elicited by fibrillating peptide sequences found in, or inspired by, nature. Robust mechanisms have been developed for the formation of nanoscale spheres, fibres, sheets and tubes. These include: complementary peptide repeats which utilise complementary charges to self-organise [29]; glutamine rich sequences [20]; peptide-amphiphiles, where a hydrophobic alkyl tail is attached to a peptide driving tubular micelle formation [33]; aromatic N-terminally capped peptides or aromatic residues which take advantage of π-stacks or aromatic motifs [5, 32]; proline containing hairpin forming oligopeptides [34]; and α-helical coiled coil peptides utilising the self recognition inherent in leucine zippers [35] (see Table 1 and Fig. 2 for more examples).

A wide range of techniques have been used to characterise these systems spectroscopically, optically and mechanically at each stage of the assembly process [23, 30, 32, 36, 37]. Individual subunit interactions have been characterised by UV–vis spectroscopy, in which the emission wavelength changes during aggregate formation. Circular dichroism has been employed to gain insight towards the chiral ordering of the molecules, with additional characterisation of peptide interactions being monitored by infra red measurement of the amide I band, and the spacings between the molecules observed by small and wide angle X-ray scattering. Small angle neutron scattering and circular dichroism is utilised in combination to determine supramolecular interactions between the assembled structures. The nanoscale assemblies formed can be directly visualised by (cryo-) transmission electron microscopy, and the microscale networks through (cryo-) scanning electron microscopy and (wet) atomic force microscopy. It should be noted that cryo techniques are more desirable than the utilisation of a metal stain/coating, as the structures are embedded in vitrous ice, and therefore bear more relevance to the actual assembled morphologies. The mechanical properties of the resultant hydrogels are analysed primarily through parallel plate rheometry, which provides information on the elastic properties of the networks, which arise from the interactions and organisation of the supramolecular structures.

2.2 Functionalisation of Peptide Materials

The first examples of self assembling peptide (SAP) scaffolds were initially engineered to produce robust, controllable nanoscale assemblies [4]. More recently, next generation systems are being modified to introduce functionality, potentially at the expense of homogeneous assembly, as depicted in Fig. 1 [38, 39]. This approach generally attempts to mimic the ECM to provide structural and biochemical support to control and augment tissue repair. The ECM contains proteins rich in structure, such as

Table 1 Structure and bonding of different classes of SAP scaffolds

Entry	Class	Bond	Structure	References
1	Tripeptide repeats $(RAD)_n$	Hydrophobic/complementary charge	ß-sheet tapes	[17, 18, 24, 75–81]
2	Glutamine-rich	Hydrophobic/π–π stack	β-sheet ribbons	[82–88]
3	Peptide Amphiphiles	Ionic/amphiphillic	Cylindrical micelles	[19, 33, 54, 58, 89–96]
4	N-terminally capped peptides	π–π-stack, H-bonds	Nanotubes, nanofibres	[5, 26, 30, 32, 38, 52, 97, 98]
5	Beta hairpin	Ionic/hydrophobic/H bond	β-sheets tapes	[34, 99–102]
6	Coiled coils	Hydrophobic/complementary charge	α-helical rods	[35, 103–108]

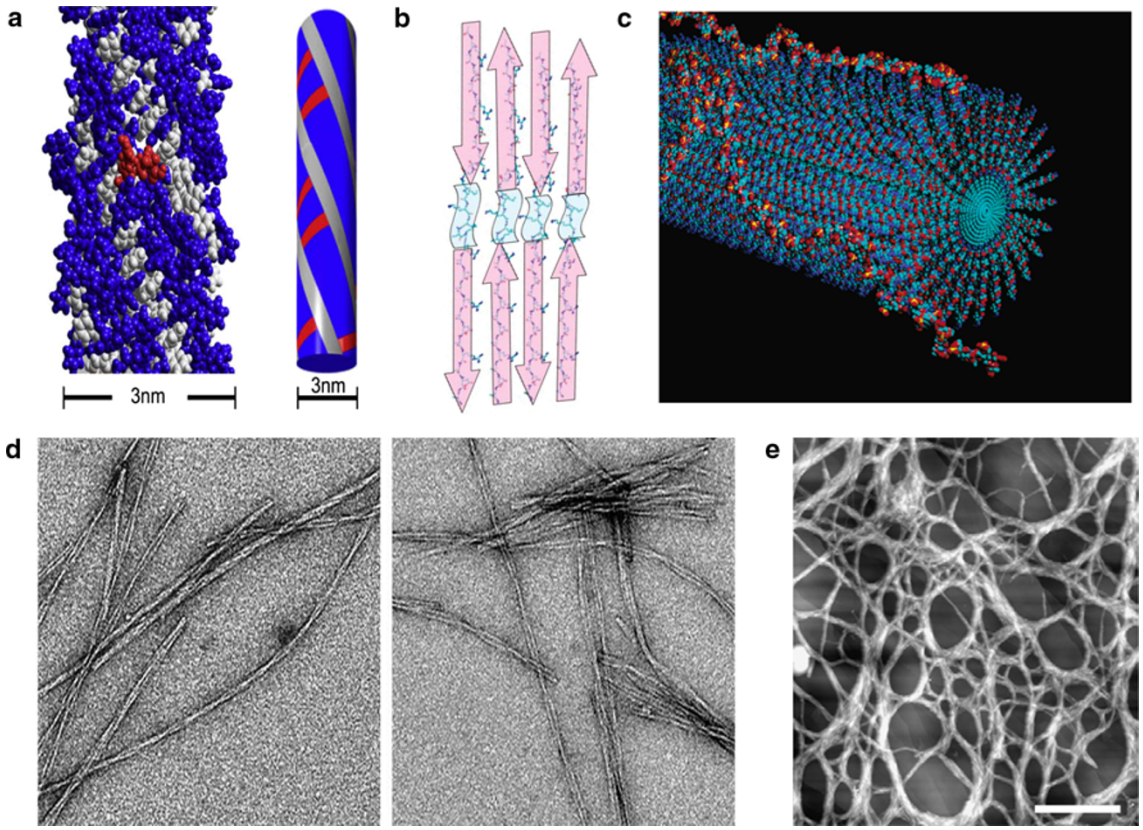

Fig. 2 **a** Shows the molecular arrangement of a π–β fibril formed from fmoc-FF. *Red* indicates the inclusion of the fmoc-RGD motif. Reprinted from Ref. [38]. Copyright (2009), with permission from Elsevier. **b** The anti-parallel β-sheet motif of $(RADA)_3$ (*pink arrows*) allows the bioactive epitope of IKVAV (*blue*) to be ordered within the nanoscale structure. Reprinted with permission from Ref. [21]. **c** Through the inclusion of a heparin-binding motif to a peptide amphiphile, heparin is recruited post-assembly to the surface of the nanostructure. Reprinted with permission from Ref. [73]. Copyright (2006) American Chemical Society. **d** Nanoscale fibrils of Q11-RGDS and Q11-IKVAV peptides visualized by TEM shows the underlying fibrillar structure of the hydrogel. Reprinted from Ref. [20]. Copyright (2009) with permission from Elsevier. **e** Individual fibrils intertwine to form a scaffold as evidenced here by atomic force microscopy image of an fmoc-peptide/protein hybrid matrix. Reprinted from Ref. [23]. Copyright (2011), with permission from Elsevier

laminin and fibronectin, which can be attached to artificial biomaterials either covalently or physically. Whole proteins have been incorporated into SAP scaffolds: permanently through the use of hydrophobic fibrils to allow stable inclusion of whole basement membrane proteins [23]; or temporarily, utilising the electrostatic interactions of charged residues, producing zero order slow release system [40]. Peptide amphiphile systems have been shown to self-assemble in the presence of, and subsequently actively bind, heparin through the inclusion of a heparin binding motif, whilst retaining its bioactivity [41]. In order to avoid the problems associated with the use of biologically derived whole proteins, some bioactive peptide sequences from oligopeptides have been utilised to engineer SAP scaffolds, such as LAIKNDNLVYVY to shorter motifs such as IKVAV, and RGD [42]. Indeed, tripeptide motifs represent an important class of biological signals, both in structure induction and functionality [43]. As such, these are an ideal target for inclusion within self-assembled scaffolds, both as a SAP and bioactive signal. Studies suggest that the tripeptide motif is retained in both structure and function within the self-assembled construct, but the mechanism by which it is included is important (Fig. 1b). For example, the bioactive sequence fmoc-RGD self-assembles into a well ordered π–β nanoscale assembly and a clear hydrogel [44]. However, doping of fmoc-RGD into a system predominantly consisting of fmoc-FF results in the disruption of the ordering of the system but an increase in the biofunctionality [38]. The presentation of RGD has also been observed to be a function of the aromatic side-chains flanking the epitope, where fmoc-RDGF has a greater bioavailability than fmoc-FRGD, possibly through increased rigidity provided by the aromatic residue at the C terminal [45]. When the RGD peptide was included as a pendant molecule on an oligopeptide, the biological response was increased without disrupting the mechanism of assembly as the pendant signal was not integral to the assembly process [20]. Incorporating bioactive sequences

by supramolecular decoration of SAP scaffolds has also been achieved. The forces responsible for assembly have been shown to be available for the non-covalent decoration of these networks. Peptide 'tags' containing oppositely charged functional sequences (at physiological pH) have been included on the surface of fibrillar assemblies, facilitating stable decoration in a spatially defined way [46]. Bioconjugation has also been possible using Click chemistry, where side chains of lysine, included within the SAP scaffold backbone, were modified with azide moieties to allow post assembly modification with no apparent decrease in order [47]. This highlights the potential for larger molecules and sequences to be conjugated. However, when decorating a self-assembled network, consideration of the method of functionalisation is essential to avoid overwhelming the assembly mechanism; the addition of an antibiotic has been shown to disrupt the scaffolds by providing an alternative supramolecular structure through a more favourable ligand/receptor binding event [48].

2.3 Tailoring Mechanical Properties

The viscoelastic properties of a hydrogel matrix can have a profound effect on the behaviour of encapsulated cells, and the endogenous cell response within adjacent tissue [7, 49]. Having a system in which these properties can be tuned is therefore desirable. The stiffness of the self-assembled hydrogels is a combination of the peptide sequence, morphology of the fibrils, their supramolecular ordering, and the strength of the bonds between them. For example, the minimalist SAP fmoc-VLK form linear networks, whereas the same residues in the sequence fmoc-KLV form branched networks with an order of magnitude increase in stiffness [50], showing that the relative position of single residues can have a profound effect on the macroscopic properties. The rate at which the peptides self-assemble has been shown to be a controlling factor over the material properties of the physically underpinned system. This process is directly related to the kinetics of the solution/gel transition [34, 36], and as such is determined by the type, extent and conditions of the stimulus. If the fibrils rapidly coalesce, non-ideal interactions lead to entanglements and stiffer gels, whereas slow controlled formation results in increased fibre alignment and weaker gels. When formed from identical peptides, this is directly related to the type, and extent of the stimulus, and the conditions under which assembly occurs. This effect is particularly obvious when using enzymes as a trigger [31]. One way reactions allow variable stiffness from identical peptides by altering the enzyme concentration and therefore the rate of formation, which has been exploited to control cell fate [26, 51]. Conversely, reversible mechanisms under thermodynamically controlled conditions result in identical gels, as they achieve more ordered interactions via component selection [30, 52]. However, such systems are also useful for tissue engineering as they can be employed to incorporate whole proteins for in vivo delivery [23, 40].

Once the matrix has been formed based upon the supramolecular properties of the fibrils, efforts have been made to improve the stiffness post assembly. In one example, chemical crosslinking via native chemical ligation (NCL) has been used to introduce covalent bonds between fibrils [53]. Importantly for biological applications, this is a mild, aqueous reaction that allows the linkage of the N terminal Cys residue with a C terminal thioester on 11 residue peptides, yielding a fivefold increase in storage modulus without disrupting the fibril. Ligation resulted in increased proliferation of human umbilical vein endothelial cells cultured within the peptide hydrogels and increased expression of platelet endothelial cell adhesion molecules on the cell surface. This demonstrates the importance of tunable storage modulus for in vivo applications [53]. Remodelling of the material in response to the biological environment is also important, which has been achieved by the introduction of degradation sequences that have proteolytic susceptibility. For instance, peptide sequences allowing cleavage specific to matrix metalloproteinase-13, important to tissue remodeling during wound healing, have been incorporated into SAP hydrogels, demonstrating varying degrees of proteolytic degradation [17, 54, 55]. Such sophisticated systems that control the degradation kinetics are critical for the manipulation and tuning of biological responses, as well as for facilitating dynamic changes to the material in response to the changing environment; properties particularly relevant to in vivo applications.

3 Utilising Self-Assembled Peptide Materials In Vivo

The design of the SAP scaffolds must support the survival of specific cellular phenotypes (application dependant) and their subsequent integration, proliferation and differentiation etc., as shown in Fig. 1c. This has been demonstrated extensively in vitro, for instance a variety of stem cells have been cultured on different SAP scaffolds, demonstrating a capacity to modulate fate specification [56, 57]. Results from such studies are now being translated in vivo. However, this presents a raft of complications that are not experienced in the static in vitro environment. Chief amongst these is the inflammatory cascade. This complex biological phenomenon occurs as a response to harmful stimuli, which in the case of utilising SAP scaffolds in vivo may include a foreign body reaction and local cell damage. Among other things, which are specific to each tissue type, the initial stages of inflammation are designed to protect

the integrity of surrounding tissue and prevent collateral injury through attenuating injurious stimuli. This reaction is critical to tissue repair. However, one of the ironies is that in order to achieve this, the phagocytosis of damaged cells and foreign material must occur, but the same process may also lead to the rapid degradation of implanted biomaterials. However, despite such complications associated with the deployment of SAP scaffolds in vivo, foundational research has demonstrated repair of a variety of different tissue types upon implantation, highlighting the exciting potential to further engineer these systems to produce hierarchical tissue engineering scaffolds. Below we will discuss several tissue types where administration of SAP scaffolds has shown significant potential for reparative medicine.

3.1 Neural Applications

Neural applications have typically relied upon scaffolds formed from covalently linked polymers such as poly (L-lactic acid), which require functionalisation, or biological polymers such as collagen, gelatin or fibrin, which then carry the possibility of activating a foreign immune response. The scaffolds are generally prefabricated 2- or 3D scaffolds which are post functionalised and seeded with cells prior to implantation [58]. SAP scaffolds that have predominately been employed for neural tissue engineering applications contain the laminin derived peptide epitope IKVAV. Through the decoration of the C terminal of a peptide amphiphile with IKVAV, these nanofibrous systems have been demonstrated to promote regeneration of descending and ascending axons across a compression spinal cord injury in a mouse model, resulting in significant improvement in the animals motility [59]. Here, self-assembly was initiated in response to the ionic strength of the tissue. The network, upon assembly, thereby displayed IKVAV at extremely high densities, and encouraged a number of desirable effects. These included reduced astrogliosis (associated with the inflammatory response within the central nervous system that can result in tissue destruction and necrosis, foiling the preceding trophic phases) and reduced cell death, possibly by the improvement of the cellular microenvironment at the wound site. Therefore, it is likely that inflammation attenuation was a major contributor to the behavioral improvement observed. In another example, after sciatic axotomy, a 10 mm gap between the nerve stumps was filled with RADA16-1 hydrogels [60, 61]. The supramolecular nature of the gels allowed the lesion to be completely filled and facilitated full contact with the 'stump', promoting the two-way migration of cells (Fig. 3b, c). This scaffold was then used as a carrier for tissue specific cell types; green-fluorescent protein (GFP) positive neural progenitor cells and Schwann cells, achieved by the preconditioning of cells within the hydrogels for 7 days prior to implantation. The delivery of cells to the injury site increased axonal regeneration, possibly due to the cells becoming acclimatised to the artificial environment and secreting trophic molecules that augment the regenerative process. An important observation was that if the hydrogel did not contain culture media, cavities and cysts formed around the implant due to its low pH, a consequence of the mechanisms used to trigger the assembly process. Scaffolds based on the RADA16-1 peptide have also been implanted into the injured cortex of adult rats where regeneration was also observed [62]. This was evident by the absence of cavities after 6 weeks, in addition to a reduction in recruited microglia/macrophages and astrocytosis compared to the sham (Fig. 3d). Again, this demonstrates and potential of the RADA based peptide system to minimise collateral injury through attenuation of the immune response. An important functional milestone was reached when this material demonstrated an ability to restore vision in hamsters post severance of the optical tract [24], demonstrating the promotion of neurite elongation and appropriate reconnection (i.e. synapse formation). The next step will be to expand the current work by characterising the associated functional recovery with behavioural analysis and electrophysiological studies.

3.2 Restoring Muscle Function

The deployment of SAP scaffolds for the treatment of Duchenne's muscular dystrophy (DMD) by the distribution of an otherwise depleted protein has shown considerable promise. DMD is a degenerative disease in which laminin is missing from the basement membrane. As a result, the muscle fibres fail to attach appropriately. Some therapeutic activity has been observed by injecting free laminin, but this undergoes rapid diffusion away from the site of therapeutic need. SAP scaffolds have been deployed to treat this condition by distributing laminin within the tissue and retaining it at the site of therapeutic need. Here, a 3-D scaffold formed from an enzymatically triggered fmoc-tripeptide π–β system was employed [23]. The use of an enzyme permitted the formation of the network to be placed under thermodynamic control, allowing the assemblies and protein to interact in a supramolecular fashion. The hydrophobic leucine residues expressed on the surface of the fibrils interact with structural binding domains on the laminin. This results in a peptide/protein matrix, which has enhanced material properties arising from the additional structural contribution of the protein. The therapeutic benefit of such a scaffold was tested in a zebrafish model, where the scaffold was delivered in vivo using microinjection, which allowed precise delivery adjacent to the cell wall (Fig. 3a). The peptide/protein matrix was observed to

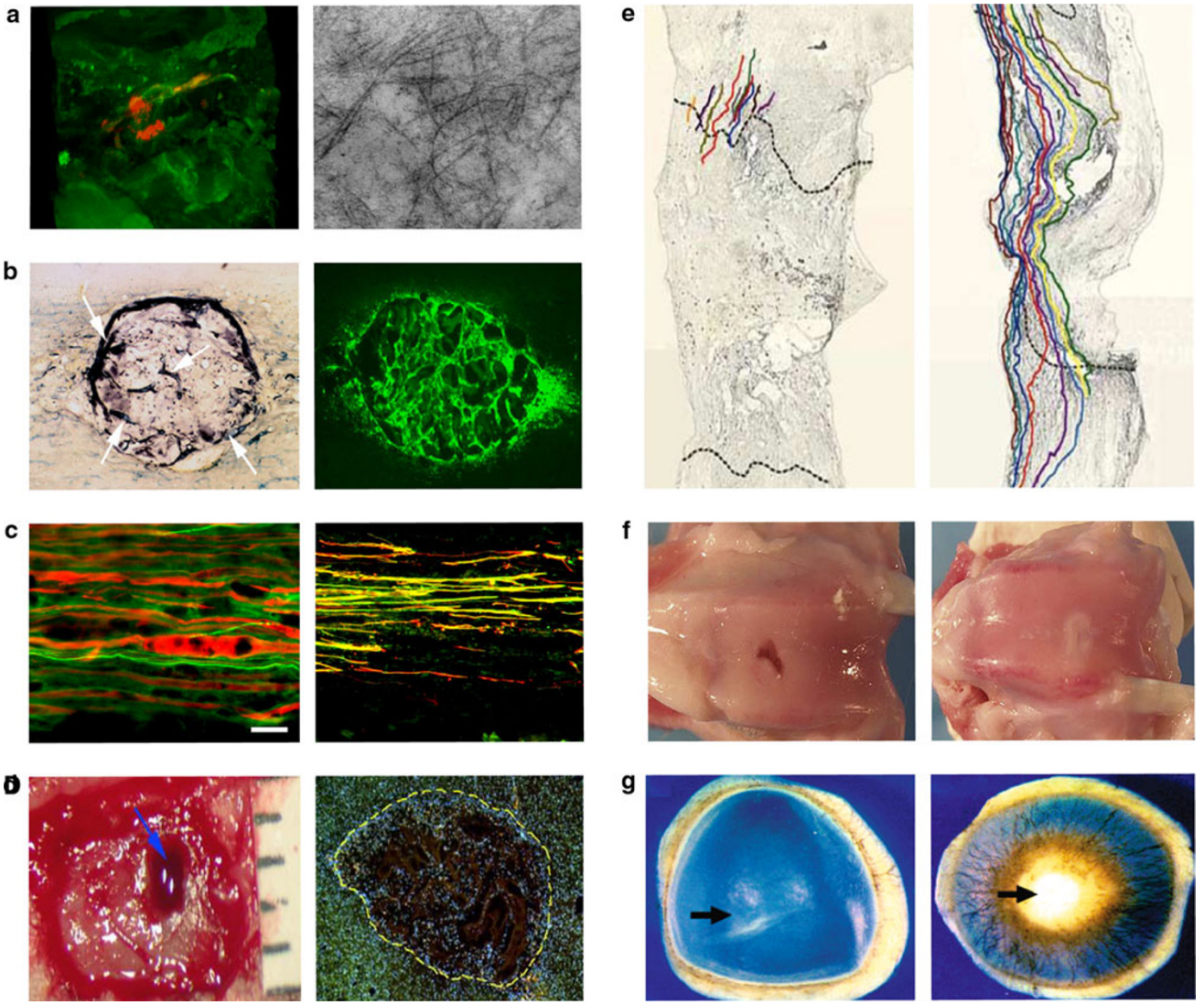

Fig. 3 **a** Confocal image of a peptide/protein hydrogel (*red*) injected into the muscle of a dystrophic zebrafish (*green*) showing the localisation of the hydrogel within the tissue. TEM image shows the existence of the self-assembled fibrils in vivo. **b** Shows the integration of self-assembled peptide hydrogels within an injured spinal cord. AP staining (*arrows*) showed that blood vessels grew into the implants, whereas the fluorescent image shows the distribution of co-implanted GFP neural precursor cells within the matrix. Reprinted from Ref. [61]. Copyright (2007), with permission from Elsevier. **c** Longitudinal sections through the membrane conduit and the distal nerve end, respectively, at 3 weeks following sciatic nerve injury and repair. Regenerating axons are shown immunostained for βIII-tubulin (*green*) along with S100-positive Schwann cells (*red*) after transplantation of BD hydrogel with Schwann cells midconduit, and in the distal stump. *Scale bar* represents 100 and 25 μm. Reprinted from Ref. [60]. Copyright (2010), with permission from Elsevier. **d** *Arrow* shows a 1 × 2 × 2 mm void cut into the right hemisphere of a rat brain, which, post bleeding, was filled with preformed SAP. Nissl and DAPI staining shows the lesion sites of the SAP. The *dashed area* indicates the extent of the SAP scaffold 6 weeks post implantation, demonstrating good integration with the brain tissue with no visible voids. Reprinted from Ref. [62]. Copyright (2009), with permission from Elsevier. **e** Scaffolds formed from IKVAV peptide amphiphiles promote the regeneration of motor axons after spinal cord injury. Neurolucida tracings of BDA-labeled descending motor fibers within a distance of 500 μm rostral of the lesion in vehicle-injected and IKVAV peptide amphiphile-injected animals, respectively, clearly showing the fibres crossing the injury in the presence of the self-assembled scaffold. The *dotted lines* demarcate the borders of the lesion reprinted with permission from Ref. [59]. Copyright (2008). **f** Shows a full-thickness osteochondral defect of a femoropatellar joint 12 weeks post injury. The defect is seen to regenerate when treated with a direct injection of SAP hydrogel. Reprinted from Ref. [65]. Copyright (2010), with permission from Elsevier. **g** Micrographs of the in vivo angiogenesis of rat cornea. Neovascularisation is not observed with growth factors alone, nor collagen and growth factors (not shown), whereas an injected hydrogel of heparin bound to a SAP networks (containing growth factors) shows an extensive effect. Reprinted with permission from Ref. [73]. Copyright (2006) American Chemical Society

maintain its nanostructure over a number of days with no significant inflammation. This shows that the biostability of the scaffolds can be tuned by performing the assembly step at the environmental conditions found in vivo under thermodynamic control. Although the stable in vivo presentation of the missing protein is an important step, further work will need to be done to confirm that the signals are biologically available. Such SAP systems also have significant potential to be further developed and optimised to mimic features of the basal lamina for wider reaching tissue engineering applications.

3.3 Bone Formation

Hydroxyapatite coatings are the standard method employed in a clinical setting to encourage bone ingrowth and regeneration for dental and orthopaedic implants. Whilst such coatings enhance osteoconduction and osteoinduction they are not easily biofunctionalised with growth factor and inhibit vascularisation due to their condensed nature. Therefore, extensive research is being conducted to improve such coatings and/or develop new methods to encourage the implants to directly integrate with the surrounding tissue. To this end, SAP scaffolds have been utilised to provide biofunctionality to inert titanium foam implants, whereby a peptide amphiphile nanofibre matrix was assembled on and within the surfaces of the scaffold pores [63]. It was shown that the scaffold was capable of encapsulating mouse calvarial pre-osteoblastic cells, demonstrating the feasibility of using such a hybrid scaffold for cell transplantation. The assembly of nanostructures from a highly penetrating fluid phase was useful in this case, as it allows the resultant fibres to completely penetrate the titanium support. This strategy yielded a phosphorylated, anionic nanofibre gel matrix, which was used as a strongly nucleating template to spatially confine the formation of hydroxyapatite crystals yielding nanostructured artefacts [64]. These composite scaffolds were implanted within the femur of adult rats using a bone plug model. After 4 weeks, highly mineralised bone was observed around the implant and within the pores, along with some evidence of vascularisation. These results suggest that composite materials formed in this way are orders of magnitude stronger than the hydrogels alone, well tolerated, biocompatible and, importantly for implants, facilitate host cell migration as evidenced by this osteoconductive effect.

3.4 Repair of Cartilage

Degeneration of the cartilage, either through aging and/or injury is a debilitating condition leading to chronic pain, loss of mobility and a reduced quality of life. In order to utilise SAP scaffolds to stimulate the formation of new cartilage, bone marrow stem cells (BMSC) along with transforming growth factor-β1 (TGF-1), dexamethasone, and insulin-like growth factor 1 (IGF-1) have been premixed into a solution of trimeric KLD and injected into a full sized cartilage defect model in skeletally mature rabbits [65]. The assembly of the matrix was triggered by the ionic strength in the defect and filled the area completely (Fig. 3f). When the SAP matrix was injected into the defect, improved collagen II and Safranin-O staining was observed. Upon inclusion of the growth factors and drugs within the matrix there was an increased aggrecan production, a proteoglycan found within the ECM of cartilage. Interestingly, there was no beneficial effect from the inclusion of the growth factors within the scaffold compared with the administration of the SAP scaffold alone. Therefore, the effect observed when the matrix is injected into the defect site, as discussed previously, could well be due to stabilisation of the wound site via full contact with the surrounding tissue. However, it should be noted that co-injection with BMSC lead to an undesirable increase in fibrous tissue formation, possibly through the lack of appropriate signaling that may have been influenced by growth factors secreted from the transplanted cells. As growth factors easily diffuse out of the matrix, in this instance the sequestration of these molecules may be highly desirable. In order to test the effects of free growth factor versus bound, IGF-1 and streptavidin were mixed with KLD before assembly to facilitate weak adsorption, or were strongly tethered through binding biotinylated IGF with the peptide [66]. As expected tethering of factors lead to improved retention times over soluble or adsorbed, but surprisingly decreased bioactivity. The delivery of growth factor from these systems therefore depends on the specific growth factor, the method of delivery and the location of the tethering site to the growth factor binding domain, which can be optimised by the freedom afforded by the mechanism of attachment. Control over growth factor delivery is essential for a vast range of tissue engineering application. For instance, it would be possible to combine the regenerative mechanism of this system with the reported visco supplementation of the synovial fluid (found in the cavities of synovial joints) using the unique properties of the SAP systems. Current treatments for osteoarthritis involve using regular injections of hyaluronic acid (HA). The physical and shear responsive peptide hydrogels are therefore good potential candidates, as they spontaneously assemble upon injection, permeate throughout the area of degeneration, and exhibit the capacity to form and reform over time, reducing the needed frequency of injection. The ability to mimic HA has been demonstrated by the network of ribbons and tapes that was formed from alternating polar and apolar 11-mer peptides [67]. When the solutions of the (unassembled) peptides were delivered

to sites of damage the viscous fluids and hydrogels formed were effective in reducing friction and allowing lubrication, in some cases showing improved performance over HA.

3.5 Improved Recovery Post Glaucoma Surgery

Glaucoma surgery requires postoperative filtering to lower intraocular pressure (IOP) for effective recovery, but this process attracts significant scarring. There are other associated risks with such surgery that arise from iatrogenic injuries, such as bleeding, infection and pain. However, the most common complications are associated with the proliferation of fibroblasts around bleb sites following trabeculectomy surgery. When this occurs an implantable drain device must be used to relieve IOP, which adds additional complication. Therefore, it is advantageous to inhibit fibroblast proliferation around the bleb to increase the success rate of trabeculectomy surgery. To this end many growth factors and drugs have been delivered to attempt to inhibit fibroblast proliferation. Recently SAP materials have been employed for this purpose. In this instance, fmoc-FFRGDF peptide derivatives self-assembled to form a biocompatible scaffold containing the RGD fibronectin motif [68]. The hydrogel formed was used to achieve time resolved drug release of the anti-proliferative drug 5-fluorouracil (5-Fu), as an attempt to prevent excessive scarring that results from fibroblast proliferation. The drug itself was incorporated during the assembly process, and the loaded hydrogel injected during surgery. This proved to be effective for the reduction of fibrosis and therefore IOP, as the biocompatible peptide scaffold facilitated slow release (via diffusion) of the drug to the surrounding tissues, limiting toxicity and increasing its effective half-life. This accentuates the potential of such systems to increase the success rate of trabeculectomy surgery, limiting the need to utilise invasive implantable drainage devices.

3.6 Regeneration of Myocardium

Heart disease is the leading cause of death and a major cause of disability within the western world. Myocardial infarction results from the reduction in blood supply of a proportion of the myocardium that ultimately leads to irreversible necrosis and if severe, heart failure. In order to treat myocardial infarction, cell transplantation is of great interest, as mammalian cardiomyocytes have limited regenerative capacity, even if blood supply is restored. Many different types of tissue engineering hydrogels have been investigated to improve the survival of transplanted cells for myocardial repair, which include SAP hydrogel scaffolds. Myocardial cells have been shown to tolerate a peptide hydrogel microenvironment formed from RAD16 peptides. These hydrogel microenvironments were reproducible and non-inflammatory when injected into the myocardium [69]. They were easily identifiable within the tissue, and were observed to allow the ingress of host endothelial progenitor cells, resulting in the formation of smooth muscle cells, an important step toward functional vascular structures. In addition, when neonatal cardiomyocytes were co-injected with the hydrogel, cell recruitment was increased. This natural migratory effect is of particular interest, as the constituent peptides were unfunctionalised, allowing the potential for improved functionality through the use of various factors. Therefore, the properties of the SAP scaffold in creating functional, injectable microenvironments that promote the survival of implanted cells, and encouraging the migration and revascularisation of damaged tissue is a promising new mechanism for the treatment of heart disease by allowing spatially controlled regeneration of the damaged tissue.

3.7 Haemostasis

Inevitably surgery results in iatrogenic injury, which results in bleeding. This must be stopped not only to minimise blood loss from the patient, but for a variety of other reasons including reducing the need for postoperative drainage, saving operative time and expense and decreasing the length of hospital stay. It is common practice to apply electrocautery and topical haemostats, such as synthetic sealants, gels and fabrics. Each of these systems has associated advantages and disadvantages, providing stimulus for continuing research investigating the use of SAP scaffolds for this purpose. The space-filling properties of an SAP scaffold that can assemble in vivo have been shown to provide haemostatic properties [70]. The injection of a solution of RADA based peptides resulted in the formation of an integrated nanofibrous barrier that quickly and effectively stopped bleeding in a range of tissue types including liver, brain, spinal cord, artery and skin [71]. Here, self-assembly occurred rapidly in response to the ionic strength of the blood, resulting in complete cessation of bleeding (<15 s). This mechanism was not attributed to clotting, as platelet formation was not observed until several minutes after application of the SAP solution. Although not directly tested, it is hypothesised that the solution permeates throughout the wound, allowing the resultant nanofibres to penetrate all regions to provide a flexible barrier, which illustrates the excellent contact between tissue and scaffold upon self-assembly in situ. The rapid rate at which the flow of blood is arrested suggests that only a small amount of nanofibres closely associated with the tissue are required, as the time scale is too short to achieve full assembly of the peptide. The gel also

prevented the lysing of red blood cells and allowed surrounding cells to migrate into the hydrogel. Somewhat counter intuitively, this phenomena is most effective in 'soft' hydrogels, as opposed to those which form a stiffer matrix, as these were found to fail along fractures, thereby allowing bleeding to recommence. Such preliminary works shows the potential of SAP scaffolds to be developed as superior haemostatic agents that are clinically translational.

3.8 Angiogenesis

Imparting angionesic properties to the SAP scaffolds is of great interest for tissue engineering to achieve rapid revascularisation of damaged tissues and to introduce blood flow to the biomaterial to provide a favourable cellular milieu. However, even for materials containing ECM proteins and peptides prevascularised in vitro, the ingrowth of new vasculature is a real concern [72]. In order to induce the formation of new vessels into peptide amphiphile nanostructures, a heparin binding sequence was added to the C-terminus [73]. These signals presented in a high density on the surface of the nanostructure resulted in the co-assembly and presentation of heparin, as shown in Fig. 2c. The heparin binding peptide amphiphile scaffold, heparin and appropriate growth factors were injected to the cornea of adult rats, a tissue that is free of vascularisation, and thereby allows facile visual analysis. Here, the heparin binding system was shown to have considerable propensity for the generation of new vascular tissue, interestingly more so than if the scaffold was exchanged for a more 'traditional' scaffold formed by collagen (Fig. 3g). In an extension of this work, percutaneous injections of the preformed hydrogels were applied to a mouse model. Here, the hydrogel re-formed to yield a stable mass [74]. Importantly for their potential use as an implant, there was no evidence of the formation of a fibrotic capsule, nor penetration of immune cells. Post implantation (10 days) the scaffold remained stable, and had induced the penetration of host fibroblasts, which had begun to form vascularised tissue. This process was accompanied by the reduction and replacement of the SAP scaffold leading to a conversion toward well vascularised connective tissue, with a high density of blood vessels, important for the long-term survival of tissue formed within the implant.

4 Perspectives

The examples discussed above represent a number of diverse approaches toward tissue engineering utilising the unique properties of materials formed via peptide self-assembly. This reflects the strength of the approach in allowing a range of supramolecular structures to be formed from a solution of molecules modularly synthesised from a common and easily accessible toolbox of physiological building blocks. The macroscopic material properties are easily governed by the stimulus and environmental conditions under which the assembly process occurs. The assembly process can be triggered by stimuli found upon injection into host tissue; either preformed or an injection of precursors, which begin to assemble as a response to the environmental chemical milieu, or triggered by the biocatalytic action of enzymes. The self-assembly of peptides is therefore an approach which allows access to a variety of systems with rationally designed material characteristics. First amongst these is the ability to easily manufacture robust nano- to macroscale structures of varying mechanical strength, fibre diameter and surface property. These underlying motifs provide structures with the ability to provide a skeleton upon which the chemical signals can be decorated. A considerable amount of research has been focused toward forming networks that present a variety of signals and further development of these will allow tuning by the end user to illicit tissue specific regenerative cascades. The presentation of such signals can be achieved in a variety of ways; built into the scaffold, or tethered to it, and examples exist demonstrating the selective binding, and/or controlled release of growth factors, along with the recruitment of bioactive molecules including peptides and proteins. What is clear is the potential and requirement for a variety of multiple epitopes of various densities co-assembled into a single heterogeneous assembly, which can be rationally tuned toward a specific biological niche. A key development will be the programmed ability of the scaffold to modulate its structure at predetermined and variable rates to allow the material to respond to the dynamic environment of a regenerating tissue. The inclusion of cleavable sites allows some control over this, as does a slow degradation observed as an effect of the external forces presented by a dynamic, living system. However, to date, these effects are currently an observation, rather than designed phenomenon.

Therefore, whilst the biological outcomes of all of the in vivo studies mentioned previously generate optimism, for these technologies to be truly transferred beyond academic interest to the clinic, progress must be made in regards to controlling and understanding precisely how the nano- and microstructures of the individual scaffolds provide mechanical, topographical and biochemical properties to interact with the tissue. While it is clear that the scaffolds influence inflammation, cell migration, angiogenesis, etc., such responses are currently (relatively) uncontrolled; hence their influence must be fully characterised, and this may require the development of new analytical methods and the adaption of existing techniques to fully visualise. Inherent in this class of system is the propensity to

disassemble, which is a desirable property in a long-term implant that encourages tissue regeneration. Precise control over the degradation rate is essential, because if the rate of ECM formation is slower than that at which the scaffold degrades, undesirable and harmful void formation will result. The mechanisms driving degradation will most likely result from the aqueous, enzymatic and/or the cellular environment in vivo, and the largest dynamic induction of these arise through the inflammatory process. A natural part of the healing inflammation is associated with a cascade of biochemical events that can either promote tissue regeneration or prevent it through chronic inflammation, tissue destruction, fibrosis and necrosis. Therefore, when engineering an 'ideal' SAP scaffold for in vivo deployment it must be recognised as 'self' by the immune response, avoiding, or at least limiting the rate of phagocytosis. In addition the inflammatory cascade post implantation must be controlled and optimised to encourage acute inflammation to be trophic and promote repair and reconstruction. Scaffolds formed from peptides therefore present enormous potential for regenerative medicine since they are assembled using structural motifs that are physiologically present, they simulate nature in regards to the molecular recognition of epitopes, and thereby conduct biochemical activity using the physicochemical properties inherent in the amino acids of which they are formed.

5 Conclusions

The use of man made scaffolds that effectively integrate with biology for controlled tissue engineering holds significant promise for regenerative medicine. Currently, the need for region specific physical and biochemical cues (which are essential for instructing, stimulating and/or augmenting the cellular microenvironment) have not been realised in vivo. While tissue engineering scaffolds have the potential to be therapeutic, they do not define regenerative events but rather through their interaction with cells, whether endogenous or transplanted, instruct cells to behave tropically for the repair and reconstruction of tissue. It is obvious that the rapidly evolving field of SAP scaffolds is beginning to fulfill their rich promise by moving toward being a functional biomaterial, as demonstrated by preliminary success in encouraging the regeneration of selected tissues in vivo. The key advantages of these systems arise from the versatility imparted by the constituent peptides; peptide synthesis is a common and easily performed synthetic technique, allowing researchers to readily ultilisea range of molecules. As peptides are the structural motif used by nature, they have the inherent ability to form biologically relevant supramolecular structures on the nanoscale, but also have the benefit of structure and chemical functionality. Therefore, by utilising these properties, the scaffolds formed can not only physically and mechanically support cells, but have the potential to provide the enduser with and ability to tailor tissue specific multicomponent scaffolds through manipulating the structural hierarchy. The nano- to macro-ordering of these scaffolds also offer the possibility of delivering various combinations of growth factors, proteins, cytokines and hormones that are tailored to control and harness the endogenous response, spatially and temporally. The examples explored in this review show the feasibility of these features, and serve as inspiration; the challenge presented to researchers in this field is to take this understanding and perform systematic studies of the outcomes of rational design to yield a more quantitative understanding of the biological response to these systems. Although none of these systems have FDA approval, and are therefore a long way from the clinic, we envisage that exploiting the increasing complexity of SAP scaffolds will ultimately enable bioengineers to actively modulate the regenerative cascade for specific cellular microenvironments, offering an effective next-generation treatment to some of the most debilitating disease states.

Acknowledgments DRN is funded by an Australian Research Council Postdoctoral Fellowship. RJW is funded by an Alfred Deakin Research Fellowship.

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