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Research review paper

From short peptides to nanofibers to macromolecular assemblies in biomedicine

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ABSTRACT

In the last few years, a variety of self-assembling short peptides that consist exclusively of simple amino acids have been designed and modified. These peptides exhibit self-assembling dynamic behaviors. At the molecular structural level, they form α-helical, β-sheet and β-hairpins structures in water. These structures further undergo spontaneous assembly to form nanofibers which aggregate into supramolecular scaffolds that entrap large volumes of water. Furthermore, nanostructures and supramolecular structures that self-organized from these short peptides also have a broad spectrum of biotechnological applications. They are useful as biological materials for 2D and 3D tissue cell cultures, regenerative and reparative medicine, tissue engineering as well as injectable drug delivery matrices that gel *in situ*. We have endeavored to do a comprehensive review of short peptides that form nanofibrous hydrogels. In particular, we have focused on recent advances in peptide assembly motifs and applications.

Contents

1. Introduction

Peptides are versatile building blocks for fabricating supramolecular architectures. Their ability to adopt specific secondary structures, as prescribed by amino acid sequence, provides a unique platform for the design of self-assembling biomaterials with hierarchical three-dimensional (3D) macromolecular architectures, nanoscale features and tunable physical properties. To date, synthetic membranes, multilamellar structures, amphiphilic micelles, tubules and fibrillar networks have been obtained from the self-assembly of various peptide motifs (Zhang, 2003). In this review, we will focus on recent advances in the design of short peptides that self-assemble into nanofibrous networks capable of entrapping water—hydrogels.

Through probing various protein motifs found in nature, scientists have been able to elucidate the molecular interactions that govern peptide self-assembly. Peptide self-assembly is highly specific—the intermolecular interactions such as hydrogen bonding, ionic, electrostatic, hydrophobic and van der Waals interactions are mediated by molecular recognition. This understanding of molecular and structural biology has inspired the design and synthesis of increasingly complex self-assembled biomaterials for biomedicine and bionanotechnology. By engineering the amino acid sequence, the secondary structure of peptides

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(β-sheets, β-hairpins and α-helices) can be manipulated to optimize the interactions between adjacent peptides. Long-range organization of peptide monomers produces nanofibrils which aggregate into 3D fibrous networks.

From self-assembly motifs derived from naturally occurring proteins, scientists have moved towards designing *de novo* short self-assembling peptides that are amendable to functionalization. Functionalized hydrogels have been developed for various biomedical applications. In the latter part of this review, we have highlighted recent progress made in applying short self-assembling peptide hydrogels to the delivery of bioactive therapeutics and as biological scaffolds in regenerative medicine.

2. Peptide motifs that favor self-assembly to nanofibrous hydrogels

2.1. β-Sheet peptides

Pioneering work by Zhang in the early 1990s, a serendipitous discovery of a natural protein motif that self-assembled in water, utilized β-sheet peptide motifs as peptide scaffolds. The first member of this class of soft biomaterials, AEAK16-II (AEAEAKAKAEAEAKAK), was serendipitously discovered in a yeast protein, Zuotin (Zhang et al., 1993). Subsequently designed members are characterized by periodic repeats of ionic hydrophilic and hydrophobic amino acids. This motif causes the peptides to fold into β-sheet secondary structures with distinct hydrophobic and hydrophilic surfaces (Zhang et al., 1993) (Fig. 1A). During assembly in aqueous conditions, the hydrophobic alanines form overlapping hydrophobic interactions, while on the hydrophilic aspect, positive and negative charges of adjacent peptides pack together through intermolecular ionic interactions in a checkerboard-like manner. Consequently, the β-sheets stack to form nanofibers of approximately 10 nm in diameter as illustrated in Fig. 1B. The nanofibers aggregate into scaffolds that are extremely hydrated, containing more than 99% water (5 to 10 mg/mL w/v of peptide in water). The propensity for self-assembly into nanofibers is retained when the L-amino acids are replaced with the corresponding D-chiral isoform, although peptides consisting of hetero-chiral amino acids can only form non-structured nano-aggregates (Luo et al., 2008a, 2010). This suggests that β-sheet self-assembly requires homo-chirality. Hydrogel formation is also influenced by peptide sequence, concentration, and salt concentration. In the 16-amino acid peptides RADA16-I (Ac-RADARADARADARADA-NH_2) and RADA16-II (Ac-RARADADARARADADA-NH_2), arginine and aspartate residues (substituting lysine and glutamate in AEAK16) facilitate nanofiber scaffold formation in the presence of salts. By substituting alanine with isoleucine (in IKIE), valine (in VKVE) or phenylalanine (in FKFE), peptides with more hydrophobic residues are formed, and required lower critical concentrations for β-sheet formation. Reducing the number of repeats from 4 to 2, as demonstrated by comparing 16-amino-acid peptides with 8-mers, also lowers the critical gelation concentration (Ulijn and Smith, 2008). High salt concentrations inhibit gelation by masking the charges on the β-sheet while low salt conditions enhance gelation by limiting random interactions at low peptide concentrations. In general, the resulting β-sheet structures are stable across a broad range of temperature, wide pH ranges in high concentration of denaturing agent urea and guanidium hydrochloride. Interestingly, mechanical disruption by sonication disrupts the macromolecular structure temporarily but not the supramolecular β-sheet structures. The longer micron-length $RADA_4$ fibers reassemble after 2 hours, demonstrating

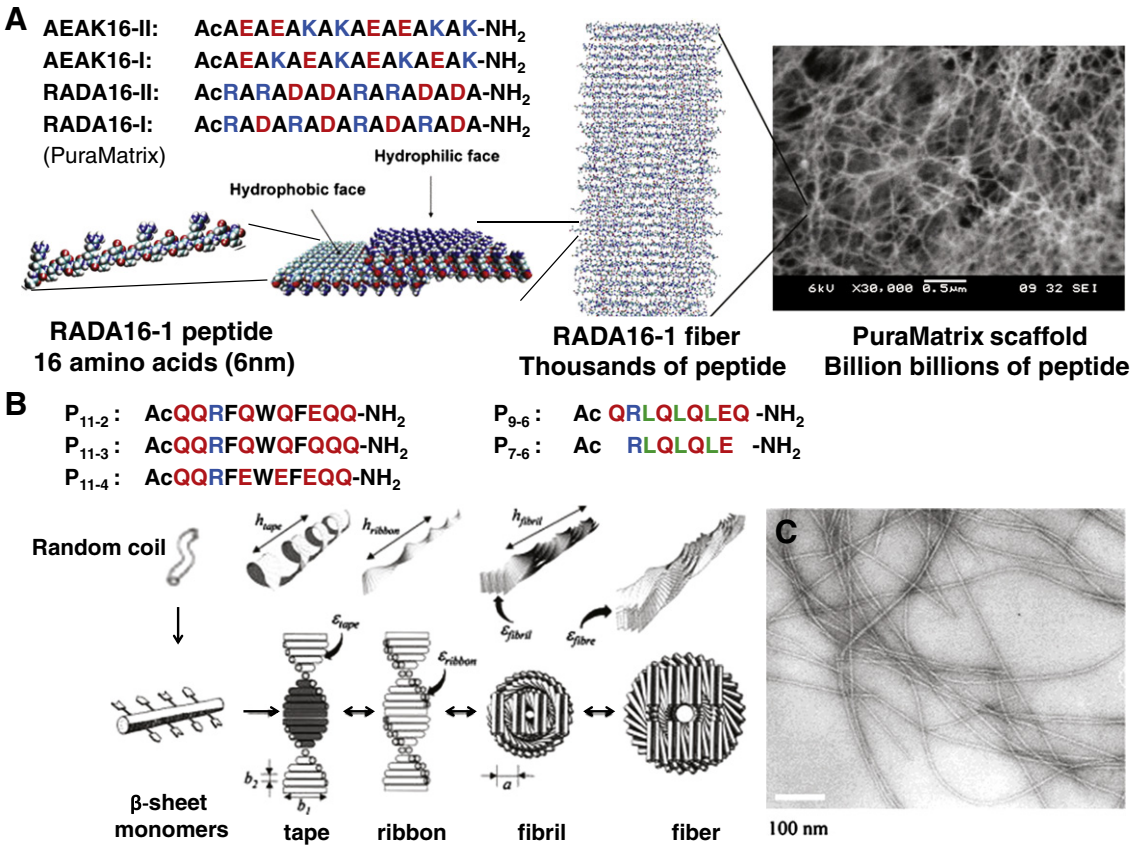

Fig. 1. (A) β-Sheet forming short peptides with alternating ionic complementary properties: peptide sequences of 4 β-sheet 16-mer peptides, including the commercially available RADA16-I (PuraMatrix™). Structure and assembly of RADA16-1 peptide into fibers and nanofibrous scaffolds (electron microscopy image of RADA16-I is shown). (B) Short amphiphilic β-sheet peptides that self-assemble into anti-parallel nanotapes and further aggregate into ribbons and higher order structures. In a recent paper, shorter sequences (P_{9-6} and P_{7-6}) with aliphatic hydrophobic resides (in green) were demonstrated to form fibrillar structures. (Reproduced with permission from reference (Aggeli et al., 2001).) (C) Transmission electron micrograph of a P_{11-4} gel in water (6.3 mM, pH 3) showing semirigid fibrils and fibers. (Reproduced with permission from reference (Aggeli et al., 2003). Copyright 2003 American Chemical Society.)

the stability of the system. The stability of RADA fibers bodes well for *in vivo* biomedicine applications. In particular, the attachment of peptide ligands does not disrupt self-assembly, allowing the formation of scaffolds with cell-attachment peptides that facilitate cell adhesion and proliferation (Gelain et al., 2007; Horii et al., 2007; Kumada and Zhang, 2010), as well as matrix metalloprotease cleavage sites to accelerate biodegradation (Chau et al., 2008; Kumada et al., 2010).

β-Sheet peptides with a similar alternating hydrophobic and hydrophilic amino acid motif but bearing either a cationic or anionic residue do not form hydrogels due to repulsive forces. However, hydrogels spontaneously form by mixing oppositely charged peptides, such as 10-amino-acid N-terminus acetylated peptides Ac-WKVKVKVKVK-amide and Ac-EWVEVEVEVE-amide designed by Yu et al. (Ramachandran et al., 2005). Nanofibers in the scaffold are stabilized by electrostatic interactions between adjacent β-sheet peptides. Such peptides are sensitive to changes in pH, salt conditions and exposure to shear forces, giving rise to stimuli-responsive hydrogels.

Nanofibrous hydrogels are also obtained when peptides with a β-sheet motif flanked by charged residues self-assemble in response to pH changes. Hartgerink's group designed a series of ABA multi-domain short peptides—Ac–KK(QL)$_6$KK–NH$_2$, Ac–E(QL)$_6$E–NH$_2$, Ac–KK(SL)$_6$KK–NH$_2$, Ac–E(SL)$_6$E–NH$_2$ and Ac–E(CLSL)$_3$E–NH$_2$, in which self-assembly is driven by hydrophobic interactions in the β-sheet B-domain when the flanking charged residues are neutralized (Aulisa et al., 2009). Cross-linking via disulphide bonds is viable through the incorporation of cysteine residues in the β-sheet domain. Enzymatic cross-linking is also feasible when lysine residues are present (Bakota et al., 2011a). In addition, like RADA peptides, the strong assembly forces permit the incorporation of bioactive motifs such as matrix metalloprotease cleavage sites and cell adhesion sequences (Galler et al., 2010), for tissue engineering applications.

Aggeli et al. developed a novel class of fibrillizing peptides that form β-sheet nanotapes (Aggeli et al., 1997). These amphiphilic peptides undergo one-dimensional self-assembly from random coil monomers to nanotapes of anti-parallel β-sheets, in response to physio-chemical changes (Aggeli et al., 2001), as illustrated in Fig. 1B. Members of this class of β-sheet nanotapes contain between seven and eleven amino acids, of which the 11-mers P$_{11}$ are the most extensively studied. To illustrate the molecular basis of assembly, P$_{11\text{-}4}$ (Ac-QQRFEWEFEQQ-NH$_2$) adopts β-sheet conformations when the pH is lowered to less than 2, due to the net positive charge on arginine (Aggeli et al., 2003). The terminal glutamines are uncharged, and can thus facilitate hydrogen bonding and hydrophobic interactions between adjacent anti-parallel β-sheets. The resulting micron-range assemblies form single-molecule thick nanotapes. Pairs of nanotapes stack to form ribbons due to the π–π interactions of the aromatic residues in aqueous conditions. Higher order structures such as fibrils and fiber networks capable of entrapping water are obtained from aggregation of ribbons. Depending on peptide sequence, hydrophobicity and overall charge, isotropic or liquid crystalline hydrogels containing 10 to 30 mg/mL of peptide can be obtained. In a recent paper, Davies and Aggeli designed and evaluated peptides P$_{9\text{-}6}$ (Ac-QRLQLQLEQ-NH$_2$) and P$_{7\text{-}6}$ (Ac-RLQLQLE-NH$_2$) which also formed fibers (Davies and Aggeli, 2011). By substituting aromatic with aliphatic hydrophobic residues, shorter sequences of 7–9 amino acids could still produce fibril networks that trap water to form hydrogels.

β-Sheet peptide motifs derived from silk $(GAGAGS)_m(GVGVP)_n$ and elastin $(VPGVG)_m(VPGXG)_n$ proteins are also the basis of many hydrogels utilized in biomedicine. However, as the silk and elastin-like polymers are significantly longer, they are out of the scope of this review.

2.2. β-Hairpin peptides

β-Hairpin secondary structures (two β-strands linked by a kink) can be rationally designed to self-assemble into fibrillar macromolecular scaffolds. Most notably, Pochan and Schneider et al. developed a series of short amphiphilic peptides with a central tetrapeptide β-turn (V^{D}PPT) flanked by alternating valine (hydrophobic) and lysine (hydrophilic) residues (Pochan et al., 2003;Schneider et al., 2002). Examples include 20-mer MAX1 (VKVKVKVKV^{D}PPTKVKVKVKV-NH$_2$) and MAX8 (VKVKVKVKV^{D}PPTKVEVKVKV-NH$_2$) peptides. These peptides transition from random coil to β-hairpin conformations in response to specific stimuli—light (Haines et al., 2005), pH (Rajagopal et al., 2009), ionic (Micklitsch et al., 2011; Ozbas et al., 2007) and temperature (Pochan et al., 2003) changes. The β-hairpins pack in an orderly fashion along their hydrophobic faces, forming bilayers that further aggregate to form cross-linked fibrils, illustrated in Fig. 2A and B. Lateral assembly is driven by intermolecular hydrogen bonding and van der Waals forces, while hydrophobic interactions dominate facial assembly (Lamm et al., 2005; Rajagopal and Schneider, 2004; Rajagopal et al., 2006). Subsequent development was inspired by classical domain swapping mechanisms that cause fibrillogenesis in proteins (Hule et al., 2009; Nagarkar et al., 2008, 2010). SSP2 (VKVKVKV^{D}PPTKVKVKVKVKV-NH$_2$) and SSP3 (VKVKVKVKVKV^{D}PPTKVKVKV-NH$_2$) are complementary β-hairpin peptides that strand swap during self-assembly. In their random coil conformations, electrostatic repulsions inhibit assembly. However, when heated, β-hairpins form and the exchangeable domains (depicted in blue in Fig. 2C) are projected away from the hairpin core. Adjacent SSP2 and SSP3 β-hairpin peptides strand swap, forming an amphiphilic anti-parallel β-sheet dimer. Two dimers further aggregate along their hydrophobic face to form a bilayer structure, which assembles laterally into fibrils (Fig. 2C). Recently, the group also designed the first three-stranded β-sheet peptide, TSS1 (VKVKVKVKV^{D}PPTKVKVKVK^{D}PPKVKVKVKV-NH$_2$), that self-assembles into an extensive network of fibrils leading to hydrogelation (Rughani et al., 2009).

2.3. α-Helical peptides

The coiled-coil peptide motif is of great interest as building blocks in self-assembled fibers, since the secondary structure and oligomerization pattern are rigorously dictated by amino acid sequence. Most notably, α-helical coiled-coil peptides that self-assemble into hydrogelating self-assembling fibers (hSAFs) have been extensively studied and developed by Woolfson et al. (Banwell et al., 2009; Bromley et al., 2009; Moutevelis and Woolfson, 2009; Papapostolou et al., 2007). Taking inspiration from leucine-zipper motifs, 28-amino-acid peptides were rationally designed with a coiled-coil heptad sequence repeat, $(g_Pa_Hb_Pc_Pd_He_Pf_P)_n$ where the subscript H denotes hydrophobic and P, polar residues. (Fig. 3A) Isoleucine and leucine residues in the *a* and *d* positions respectively enable adjacent peptides to dimerize by forming an inter-helical hydrophobic core. Dimer formation is further stabilized by asparagine residues (which preferentially pair with each other) at specific *a* sites. Hence two peptides with complementary sequences (such as Ac-IAALKAKIAALKAEIAALEAE**N**AALEA and IAALKAK**N**AALKAEIAALEAEIAALEA) are needed to form these parallel heterodimer fibrils. Oppositely charged residues in the *e* and *g* positions ensure the heterodimer adopts a staggered parallel fibril formation. The resulting extensions, "sticky ends," enable lengthwise extension of fibrils. Polar residues in the *b*, *c* and *f* positions facilitate fibril aggregation into mature fibers (shown in Fig. 3B) via electrostatic interactions. Replacing these with residues that generated weaker hydrophobic and hydrogen-bond interactions, more flexible and thinner fibers were formed (Banwell et al., 2009). By incorporating a third "blunt end" helix, branched fibrillar networks can be obtained (Ryadnov and Woolfson, 2003a, b). Biotin and peptide antigen "tags" can also be introduced as branches into linear hSAFs (Mahmoud et al., 2010). As this method was highly inefficient (due to the inability of the highly ordered self-assembly to tolerate modified peptides), Mahmoud et al subsequently substituted specific residues in position *f* with synthetic amino acids containing azido and

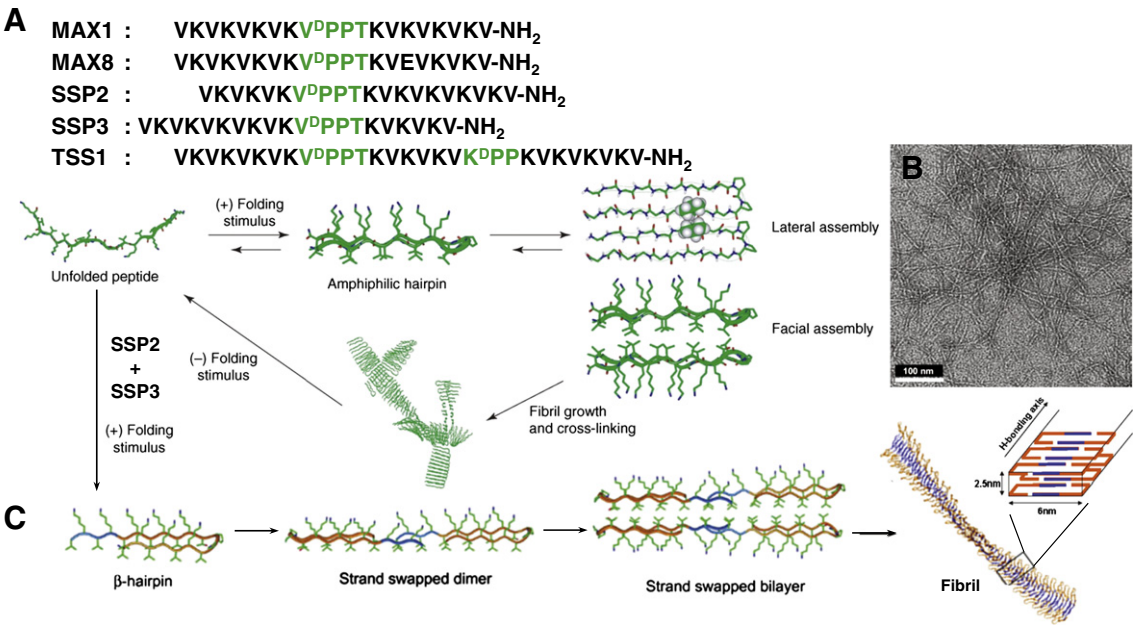

Fig. 2. (A) Short peptides that fold into β-hairpin structures which can subsequently assemble laterally or facially to form long-range, cross-linked fibrils. The tetrapeptide V^DPPT highlighted in green forms a β-turn in response to changes in the microenvironment, resulting in stimuli-responsive self-assembly. (Reproduced with permission from reference (Rajagopal and Schneider, 2004). Copyright 2004 Elsevier.) TSS1 is the first known peptide to fold into a three-stranded β-sheet capable of self-assembly into fibrils. (B) Transmission electron microscopy image of negatively stained MAX8 fibrils. (Reproduced with permission from reference (Altunbas et al., 2011). Copyright 2011 Elsevier.) (C) Peptides such as SSP2 and SSP3 adopt β-hairpin structures that domain-swap to form bilayers. Adjacent bilayers form hydrogen bonds, building fibrils. (Reproduced with permission from reference (Nagarkar et al., 2008). Copyright 2008 American Chemical Society.)

allyl moieties that can be exploited for copper(I)-catalyzed azide–alkyne, and thiol-ene click reactions (Mahmoud et al., 2011). The resulting hSAF peptides self-assembled into fibers with bioorthogonal dual functionalization. The development of functionalization techniques opens this class of hSAF peptides to various applications in biomedicine.

Hartgerink et al. recently demonstrated that "sticky ends" are not integral to the aggregation of coiled-coil peptides into nanofibers (Dong et al., 2008). Instead, a critical minimum concentration is required for fiber formation. Like peptides designed by Woolfson's group, these 21-mer peptides contain isoleucine and leucine in the *a* and *d* positions of the α-helical heptad, enabling the dimerization of adjacent peptides. Unlike Woolfson's peptides, glutamic acid residues are used in the *e* and *g* positions to confer pH-sensitive assembly, while lysine, glutamine, serine and tyrosine residues in the *b*, *c* and *f* positions modulate length and fiber diameter. For instance, lysine residues in the peripheral end of the coil (EI**K**QLESEIS**K**LEQEIQSLE**K**) stabilize 4 nm fibers via charge repulsion; peptides with glutamine and serine (EI**S**QLESEISQLEQEIQSLE**S**) continue to aggregate until the fiber diameters exceed 20 nm, probably due to non-covalent interactions between adjacent fibrils. This result presents an alternative mechanism to generate coiled-coil based nanofibers.

Hartgerink's group is also working on collagen-mimetic peptides derived from natural collagen sequences (X–Y–glycine, where X is usually proline and Y hydroxyproline). During self-assembly, three peptides come together to form a triple helix. $(POG)_{10}$ and $(PPG)_{10}$ form stable homotrimeric (using a single peptide sequence) helices (Inouye et al., 1982). Heterotrimeric systems are also feasible—Hartgerink et al. successfully utilized electrostatic interactions between oppositely charged peptides to drive self-assembly of $(PKG)_{10}$, $(DOG)_{10}$ and $(POG)_{10}$ ABC heterotrimers (Fallas et al., 2009;Gauba and Hartgerink, 2007a, b), as

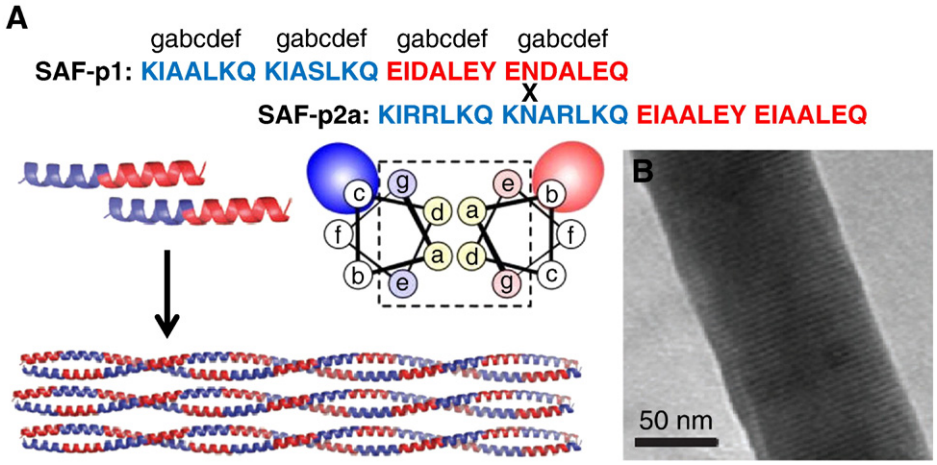

Fig. 3. (A) Complementary α-helical peptides that dimerize into hydrogelating self-assembling fibers (hSAFs). (Reproduced with permission from reference (Woolfson and Mahmoud, 2010). Copyright 2010 Royal Society of Chemistry.) The peptide sequence is a (B) high resolution image of a single fiber, demonstrating the 4.2 nm banding pattern associated with crystallization of α-helical building blocks. (Reproduced with permission from reference (Banwell et al., 2009). Copyright 2009 Nature Publishing Group.)

well as $(PRG)_{10}$ and $(EOGPOG)_5$ AAB triple helices (O'Leary et al., 2011; Russell et al., 2010). All the above-mentioned triple helical systems aggregate at high concentrations to form mesh-like assemblies. However, no fibrils or fibers are observed, possibly due to the lack of "sticky ends" for lengthwise propagation (Fallas et al., 2010). Strategies to drive fiber formation in collagen-mimetic peptides include the design of sticky ended trimers (Rele et al., 2007), the incorporation of hydrophobic residues at the periphery (Cejas et al., 2008;Kar et al., 2009), cysteine residues to form cysteine knots (Yamazaki et al., 2008), and metal-binding ligands at the termini for metal-triggered fibril assembly (Pires et al., 2009;Przybyla and Chmielewski, 2008). To date, there is only limited success in designing collagen-mimetic peptides that self-assemble into triple helical fibrillar structures and form hydrogels (Fallas et al., 2010;Pires et al., 2009).

2.4. Ultrashort peptides that form β-turn fibrils via α-helical intermediates

Significantly shorter than other helical peptides listed above, Hauser et al. discovered ultrasmall linear peptides with 3–7 natural aliphatic amino acids that self-assemble to helical fibers within supramolecular structures (Hauser et al., 2011;Mishra et al., 2011). These peptides belie the general view that short linear peptides (of less than 7 residues) cannot form α-helical structures in aqueous conditions as there are insufficient amino acids to form a complete turn of the helix. The amphiphilic peptide motif – a hydrophobic tail and a hydrophilic head group – facilitates self-assembly via parallel–antiparallel α-helical pairs and subsequent stacking into β-turn fibrils (Hauser et al., 2011), as illustrated in Fig. 4. Aggregation of fibrils into fibers results in the formation of nanofibrous scaffolds capable of entrapping up to 99.9% water—hydrogels. The ultrashort peptide hydrogels demonstrate high mechanical stiffness, thermal stability and biocompatibility (Mishra et al., 2011). Their short length also lowers the cost of synthesis, making them attractive for various biotechnological and industrial applications.

3. Biotechnology applications stemming from unique material properties

3.1. Delivery of bioactive therapeutics

Short self-assembling peptides are of great interest as injectable drug delivery matrices due to their propensity to gel *in situ* under physiological conditions or in response to specific stimuli. Their well-defined chemistry (sequence, molecular weight and stereochemistry) can be harnessed to control their biodegradability, porosity and drug release kinetics.

Bioactive therapeutics are typically loaded into the hydrogel by mixing with the peptide during gelation, as illustrated in Fig. 5A. Studies with β-sheet RADA16 hydrogels demonstrated that the release kinetics of various dyes is influenced by the structure and availability of charged groups (which interact with the self-assembling peptides) on the molecule of interest (Nagai et al., 2006). Peptide concentration also affects drug diffusion via the scaffold porosity—most biomolecules range from a few to hundreds of nanometers, comparable to the spaces between nanofibers in a hydrogel. By tailoring the peptide sequence and concentration, the diffusion of drugs from the peptide matrix can be modulated. Building on this, functional proteins of various size and charge densities ranging from lysozyme (14.3 kDa) to IgG (150 kDa) were encapsulated in RADA16 hydrogels (Koutsopoulos et al., 2009). Protein diffusion from the hydrogel depended largely on their size and peptide nanofiber density. Encapsulation and their subsequent release did not denature the proteins as evident from secondary and tertiary structure analysis and functionality assays, which bodes well for the use of RADA16 hydrogels as injectable depots for localized, sustained delivery of therapeutic proteins. To date, RADA16 has been used to deliver epidermal growth factor to accelerate cutaneous wound healing, platelet-derived growth factor, stromal cell-derived factor-1 and insulin-like growth factor to the post-infarction myocardium (Davis et al., 2005, 2006). RADA16 hydrogels can also be used to deliver growth factors such as human fibroblast growth factor, vascular endothelial growth factor and brain-derived neurotrophic factor for cell substrate and tissue engineering applications (Gelain et al., 2010).

Multi-domain β-sheet hydrogels developed by Hartgerink et al. have been used as a delivery agent for factors secreted by stem cells (Bakota et al., 2011b). These nanofibrous hydrogels released growth factors and cytokines which revived glomerular epithelial cells that simulated diabetes-induced kidney injury.

MAX8 β-hairpin hydrogels are also capable of encapsulating small molecules and proteins for applications in localized injectable therapies. In particular, hydrophobic drugs such as curcumin have been loaded and released over a fortnight, without compromising drug activity (Altunbas et al., 2011) or hydrogel stiffness. Like RADA16,

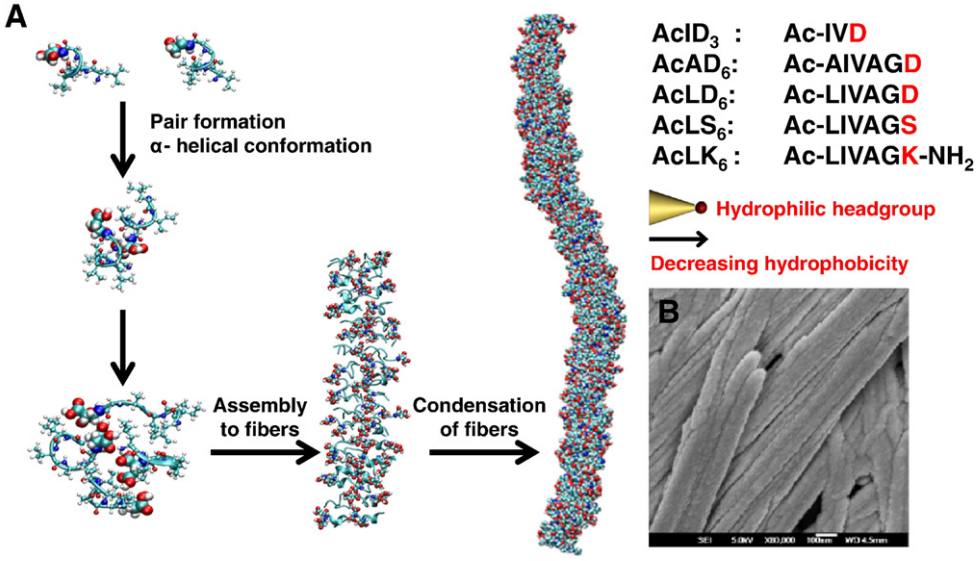

Fig. 4. (A) Hypothesis of self-assembly from peptide monomers to supramolecular networks of condensed fibers. Self-assembly is initiated with antiparallel pairing of two peptide monomers by changing to α-helical conformation. Subsequently, peptide pairs assemble to fibers and nanostructures and condense to fibrils resulting in hydrogel formation. (B) Ultrasmall peptide sequence motif and (C) FESEM image of fibers formed from $AcLD_6$ peptide. (Reproduced with permission from reference (Mishra et al., 2011). Copyright 2011 Elsevier.)

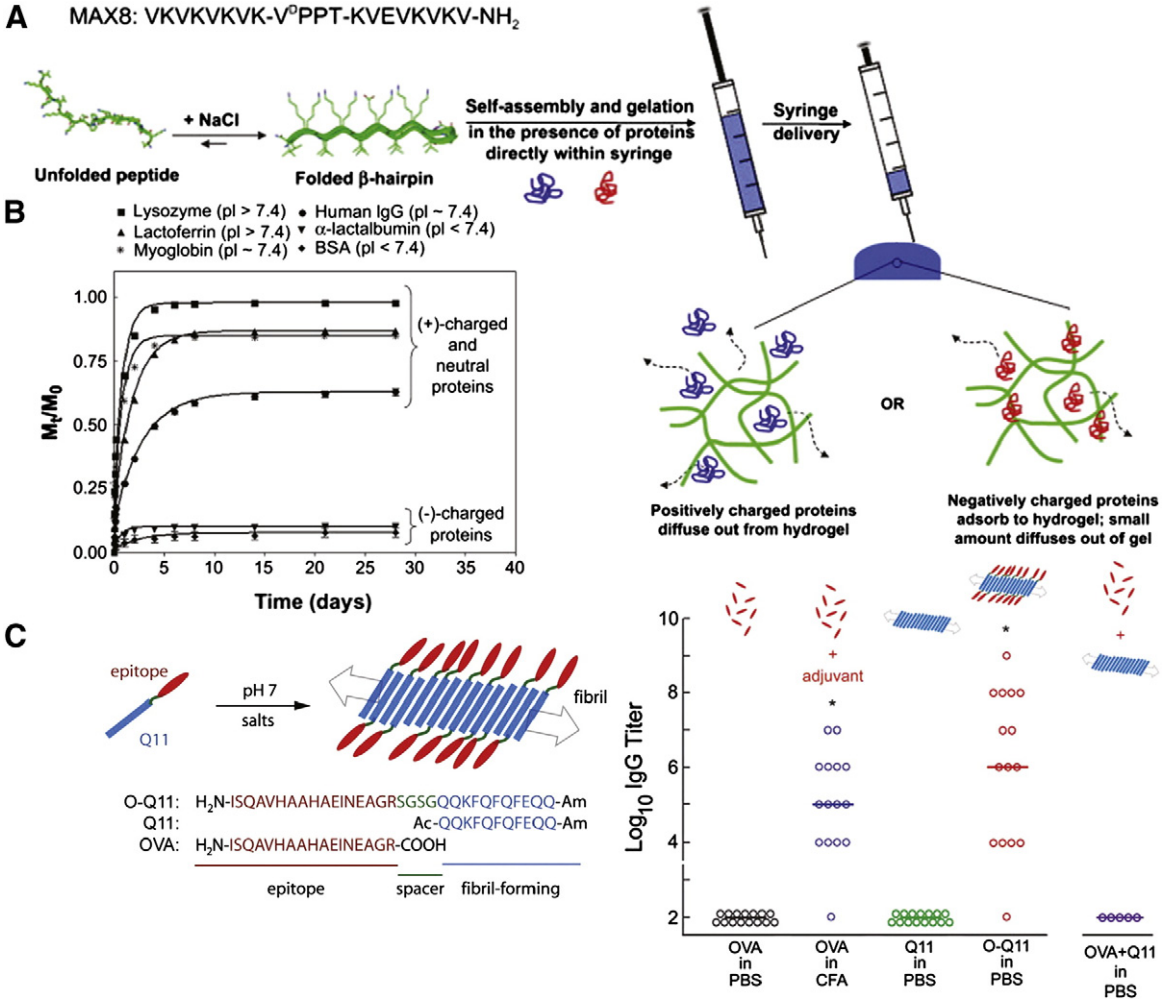

Fig. 5. (A) Self-assembly mechanism of MAX8 into hydrogel directly in a syringe, subsequent delivery, and expected interactions between differently charged proteins and positively charged MAX8 hydrogel network. (B) Cumulative release profiles (M_t/M_0) of different proteins from 1% MAX8 hydrogels (Reproduced with permission from reference (Branco et al., 2010). Copyright 2010 Elsevier.) (C) Q11 β-sheet fibrillizing peptides as chemically defined adjuvants. When ovalbumin (OVA) antigenic epitopes are added to the Q11 sequence, the modified peptides form β-sheets that optimally present the antigen to stimulate high antibody production. (Reproduced with permission from reference (Collier et al., 2010). Copyright 2010 Royal Society of Chemistry.)

drug release is a function of peptide concentration. Similarly, proteins have been directly encapsulated without significantly compromising gelation or hydrogel mechanical strength (Branco et al., 2010). Unlike RADA16, the positively charged MAX8 preferentially released neutral and cationic proteins, with kinetics governed by steric interactions; negatively charged proteins such as bovine serum albumin and α-lactalbumin were too tightly bound, resulting in negligible release even after a month (Fig. 5B). This suggests that protein release can be modulated by peptide sequence. On a side note, some β-hairpin peptides are also bioactive—MAX1 demonstrated inherent antibacterial activity without causing concurrent hemolysis (Salick et al., 2007). Such peptides could thus function as both a bioactive therapeutic and a controlled drug release matrix.

β-Sheet fibrillizing, glutamate-rich peptides developed by Collier et al. also demonstrate bioactivity when used in vaccine delivery. The peptide Q11 (Ac-QQKFQFQFEQQ-NH$_2$) presents antigenic epitopes to the immune system to generate a good antibody response (Rudra et al., 2010). Q11 is non-immunogenic when unmodified or modified with RGD sequences, and the ovalbumin epitope (OVA) in solution elicits no antibody response. However, when OVA was conjugated to the N-terminus of Q11, the modified peptide forms β-sheet fibrillar hydrogels that stimulate high antibody production in mice (Fig. 5C). This promising result opens new application avenues for β-sheet peptides as chemically defined adjuvants.

The forces that drive self-assembly in β-structured peptides are not significantly disrupted by the presence of proteins and other small molecules, enabling bioactive molecules to be loaded into hydrogels for drug delivery applications. In contrast, to date, α-helical peptide hydrogels have not been reported in sustained drug release studies.

3.2. Regenerative medicine

Self-assembled peptide hydrogels are attractive candidates for tissue engineered scaffolds, as their nanofibrous microarchitecture is biomimetic, providing spatial and temporal regulation (Shastri, 2009). The peptide hydrogels can also be tuned to optimize their mechanical and physiochemical properties to match the tissue of interest. A variety of studies using peptide amphiphiles as scaffolds have demonstrated the induction of biomineralization (Hartgerink et al., 2001), reducing glial-scar tissue formation (Tysseling-Mattiace et al., 2008) and controlling neuronal progenitor cell differentiation (Silva et al., 2004).

These new self-assembling peptide nanofiber biological scaffolds have become increasingly important not only in studying 3D spatial behaviors of cells, but also in developing approaches for a wide range of innovative medical technologies including regenerative medicine. One example is the use of RADA16-I peptide scaffolds (Fig. 6), developed by Zhang et al. and now commercially available as PuraMatrix™ to support neurite growth and differentiation

(Holmes et al., 2000), neural stem cell differentiation (Gelain et al., 2006), *in vivo* brain damage repair (Ellis-Behnke et al., 2006b), osteoblast differentiation (Bokhari et al., 2005), *in vivo* bone regeneration (Misawa et al., 2006), and cartilage cell cultures (Kisiday et al., 2002). RADA16-I and RADA16-II formed nanofibrous scaffolds in physiological solutions that stimulated extensive rat neurite outgrowth and active synapses formation on the peptide scaffold (Holmes et al., 2000). Furthermore Ellis-Behnke et al. have demonstrated that peptide nanofiber scaffold can repair hamster brain lesions by reconnecting severed nerve fibers (Ellis-Behnke et al., 2006b). The same RADA16-I peptide hydrogel can also facilitate wound healing and arrest bleeding in a few seconds (Ellis-Behnke et al., 2006a). Their observations have been reproduced by others including in chiral D-form peptide nanofiber scaffolds (Luo et al., 2008a, b, 2010). Considering the lack of signaling motifs in RADA16-I, these studies implicate that the intrinsic 3D architecture promotes cell growth, proliferation and migration. Comparing the ultra-structures of extracellular matrix (Matrigel) and RADA16-I hydrogels (Gelain et al., 2006), the dimensions of the nanofibers and porosity are comparable. It is clearly visible in the SEM images that the cells embedded in the peptide nanofiber biological scaffolds are in a truly 3D environment.

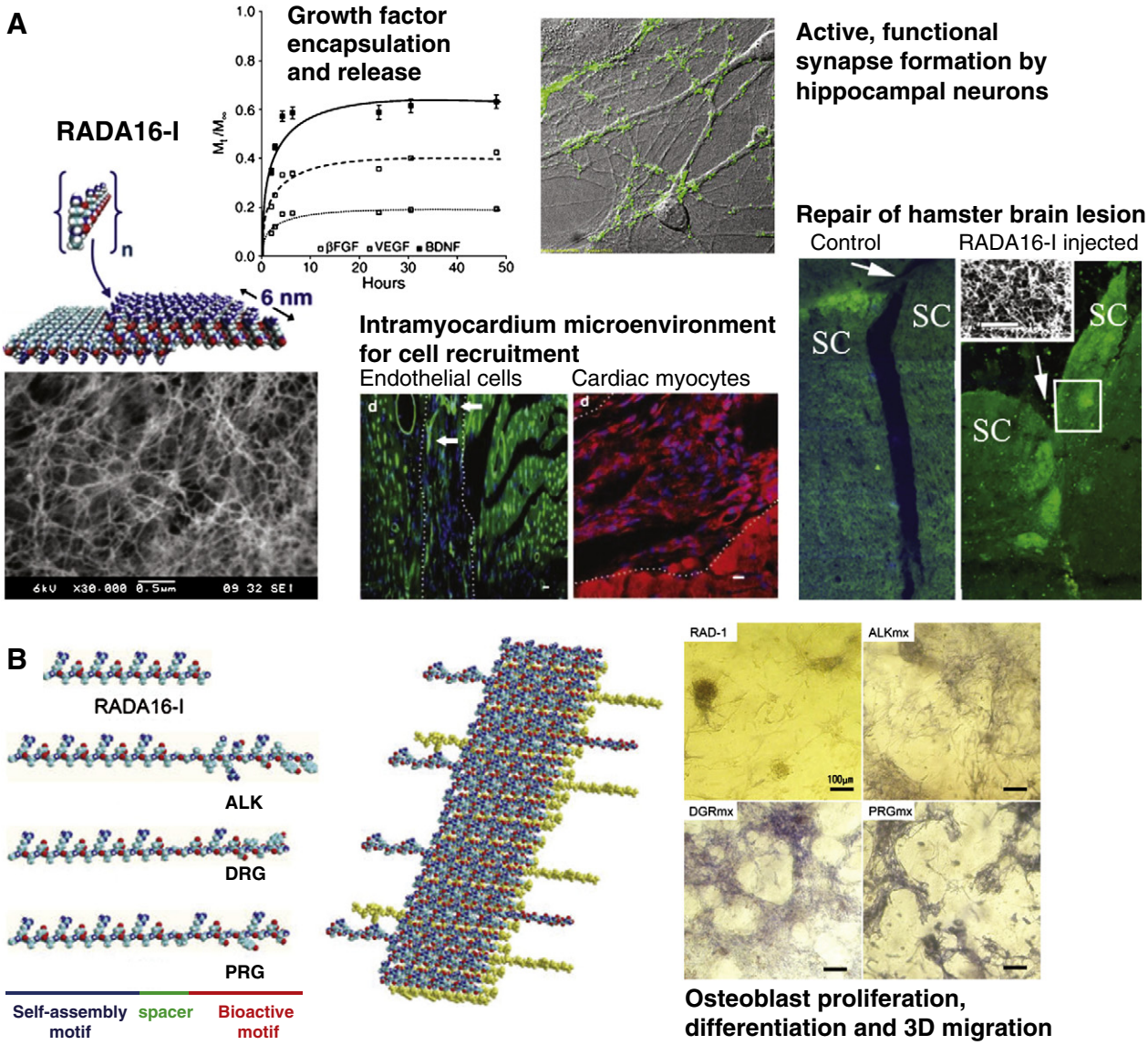

Fig. 6. From designer peptides to nanofibrous scaffold for regenerative medicine. (A) RADA16-I peptide hydrogels have been used for encapsulation and release of cytokines (human fibroblast growth factor, vascular endothelial growth factor and brain derived neurotrophic factor), without compromising their bioactivity. (Reproduced with permission from reference (Gelain et al., 2010). Copyright 2010 Elsevier.) When used as a synthetic cell culture substrate, primary rat hippocampal neurons form active, functional synapses on RADA16-I scaffolds. The confocal images were obtained following after incubation of neurons with the fluorescent lipophilic probe FM-143 which selectively traces synaptic vesicle turnover during the process of synaptic transmission. When RADA16-II was injected into mice myocardium, endothelial cells (identified by endothelial cell marker isolectin-FITC (green)) spontaneously migrated into the cell-free peptide microenvironment (within 7 days) and organized into distinct capillary-like structures (arrows) after 28 days. Putative myocyte precursors (identified using α-sarcomeric actin (red)) infiltrated the peptide microenvironment with a later time course. (Reproduced with permission from reference (Davis et al., 2005). Copyright 2005 Lippincott Williams & Wilkins.) Brain damage repair of lesion in optic tract of the hamster midbrain was also observed following injection of Puramatrix™. The lesion was sealed by the migrating cells after 2 days, wherein a great number of neurons form synapses (image courtesy of Rutledge Ellis-Behnke). (B) RADA16-I can be modified with functional domains to create bioactive scaffolds that display cell adhesion or signaling motifs. The functional motif is typically added to the C-terminus of the β-sheet assembling domain and separated by a short spacer. The motifs included osteogenic growth peptide ALK (ALKRQGRTLYGF) bone-cell secreted-signal peptide, osteopontin cell adhesion motif DGR (DGRGDSVAYG) and 2-unit RGD binding sequence PGR (PRGDSGYRGDS). Cells aggregated and attached poorly to unmodified RADA16-I. The modified peptide hydrogels significantly enhanced osteoblast proliferation, differentiation and 3D migration, as observed using alkaline phosphatase staining. (Reproduced from reference (Horii et al., 2007).)

Similarly, $P_{11\text{-}4}$ have been investigated by Aggeli et al. as injectable scaffolds for treating bone defects, dental hypersensitivity and dental decay (Firth et al., 2006). It was proposed that the peptides formed a fibril network within the pores of the lesion, where the anionic groups of the side-chains could attract calcium, leading to the *de novo* nucleation of hydroxyapatite and retardation of demineralization (Kirkham et al., 2007).

α-Helical peptide hydrogels have also been evaluated as synthetic cell culture substrates. Woolfson et al. demonstrated that hSAF hydrogels can support neuronal cell growth and neurite extension (Banwell et al., 2009). Chmielewski et al., who were the first to successfully form interwoven nanofibrous collagen-mimetic peptide scaffolds (via metal-triggered self-assembly), cultured human endothelial cells on these collagen-like hydrogels (Pires et al., 2009). Ultrashort peptide hydrogels developed by Hauser et al. are biocompatible and their physical properties (Fig. 7), particularly their high mechanical stiffness, make them attractive candidates for orthopedic applications (Mishra et al., 2011).

Although self-assembling peptides are promising scaffolds, many do not demonstrate specific cell interactions as their sequences are not naturally found in living systems. As such, various groups began to engineer biologically active peptide motifs onto β-peptides to create designer second generation of scaffolds. The simplest way to incorporate functional motifs is by appending to the C-terminus of the self-assembling domain during peptide synthesis (Fig. 6B). A spacer comprising 2-glycine residues is typically added to maintain the flexibility of the functional domain. Different functional motifs in various ratios can be incorporated in the same scaffold by simply mixing them. Upon exposure to solution at neutral pH and in physiological salt environment, the functionalized peptides undergo self-assembly with the biologically active motifs on nanofiber. This gives rise to nanofibrous microenvironments that provide specific biological stimuli.

Zhang et al. have produced different designer peptides from a variety of functional motifs with different lengths (Gelain et al., 2006; Horii et al., 2007; Kumada and Zhang, 2010). The addition of motifs to the self-assembling peptide RADA16-I did not significantly inhibit self-assembling properties and nanofiber formation, and simulations have been performed to demonstrate the motifs are displayed on the surface of the nanofibers (Fig. 6B) (Horii et al., 2007; Kumada and Zhang, 2010). Modified peptides can be mixed with RADA16-I to control the degree of ligand display. Although their nanofiber structures appear to be indistinguishable from the RADA16-I scaffold, the appended functional motifs significantly influenced cell behavior. Though experimentation with different peptides, novel peptide ligands which influenced cell behavior were discovered—a class of bone marrow homing peptides (Gelain et al., 2006, 2010) stimulated adult mouse neural stem cells adhesion and differentiation. In a separate study, Kumada and Zhang found that 2-unit RGD binding sequence PRG (PRGDSGYRGDS) and laminin cell adhesion motif PDS (PDSGR) coupled to RADA16-I facilitated fibroblast proliferation and migration, and stimulated collagen production (Kumada and Zhang, 2010; Kumada et al., 2010). This observation suggests a new class of designer self-assembling peptides for 3D cell biology studies. Several peptide nanofibrous scaffolds were designed specifically for osteoblasts (Horii et al., 2007). Short biologically active motifs such as osteogenic growth peptide ALK (ALKRQGRTLYGF) bone-cell secreted-signal peptide, osteopontin cell adhesion motif DGR (DGRGDSVAYG) and 2-unit RGD binding sequence PGR (PRGDSGYRGDS) significantly promoted mouse pre-osteoblast MC3T3-E1 cell proliferation. Moreover, alkaline phosphatase (ALP) activity and osteocalcin secretion, which are early and late markers for osteoblastic differentiation, were also significantly increased, thus demonstrating that the designer, self-assembling peptide scaffolds promoted the proliferation and

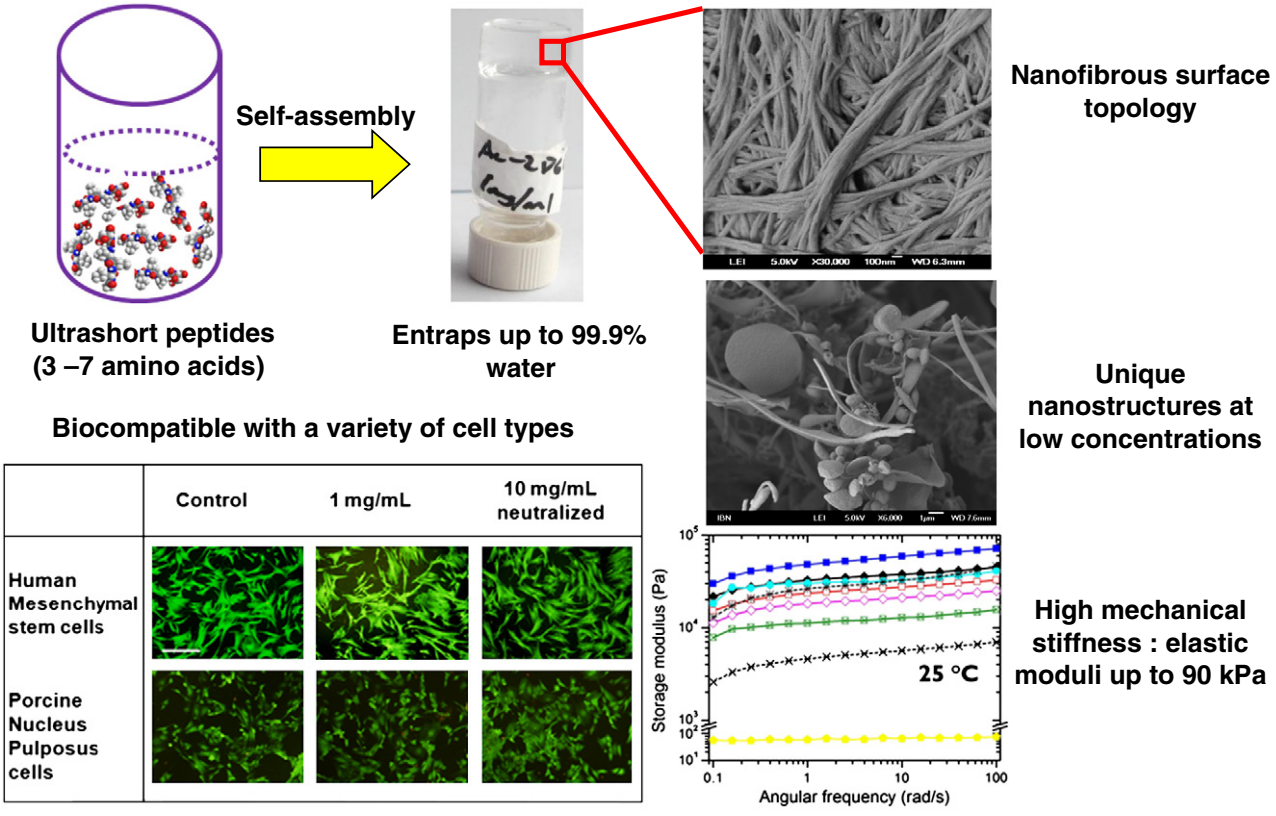

Fig. 7. Properties of ultrasmall peptide hydrogels favorable to drug delivery and tissue engineering applications. Minimally invasive injectable therapies can be designed with peptides that self-assemble into hydrogels within minutes. The nanofibrous surface topology may facilitate cell growth and differentiation. The high mechanical stiffness is a distinguishing characteristic, which makes these peptides attractive candidates for orthopedic applications. (Reproduced with permission from reference (Mishra et al., 2011). Copyright 2011 Elsevier.)

osteogenic differentiation of MC3T3-E1. Under the identical culture conditions, confocal images unequivocally demonstrated that the designer PRG peptide scaffold stimulated cell migration into the 3D scaffold. These modified designer scaffolds can also be used to encapsulate growth factors which further promote cell differentiation (Gelain et al., 2010).

Matrix metalloprotease (MMP) cleavage motifs are frequently incorporated into short self-assembling β-sheet and β-hairpin peptides to enhance scaffold biodegradability and facilitate cell infiltration (Galler et al., 2010; Giano et al., 2011; Kumada et al., 2010). The modular nature of β-sheet peptide hydrogels developed by Hartgerink et al. accommodates bioactive domains such as RGD cell adhesion motifs and MMP-2 sites (Galler et al., 2010). The resulting biofunctional scaffold demonstrated increased cell viability, spreading and encouraged cell migration. These multi-domain scaffolds can be concurrently loaded with growth factors via heparin binding (Galler et al., in press). The cytokines described in Galler et al. remained bioactive and promoted vascularization and connective tissue formation *in vivo*. As the construct resembled dental pulp tissue, this bioactive scaffold is of great interest as a biomaterial for regenerative endodontics.

β-Hairpin peptides can also be functionalized with bioactive motifs. Gungormus et al. incorporated a heptapeptide (MLPHHGA) into MDG1, forming nanofibrous hydrogels that direct hydroxyapatite mineralization (Gungormus et al., 2010). Mineralization was achieved both biochemically, and via secretion by cementoblast cells encapsulated in the biocompatible hydrogel.

The short peptides described in this review self-assemble into nanofibrous scaffolds which mimic the nanoscale dimensions and microarchitecture of natural extracellular matrix. The encapsulation of growth factors promotes cell viability, proliferation and/or differentiation. Incorporating bioactive motifs enhances cell adhesion and cell-matrix interactions. 3D cell growth and migration within the hydrogels can be facilitated by the addition of MMP sequences. Such recent advances make short self-assembling peptides promising candidates as scaffolds for cell delivery and tissue regeneration.

4. Future perspectives

Motifs derived from Mother Nature formed the basis of the first generation of self-assembling peptide and polypeptide biopolymers (Kopecek, 2003). These discoveries led to a deeper understanding of the relationship between peptide structure and function, particularly with respect to self-assembling and biorecognition properties. Based on our new-found knowledge of the rules and interactions governing self-assembly, significant progress has been made in designing novel (not found in nature) self-assembling domains as building blocks for biomaterials.

The modular nature of short β-structure self-assembling peptides and the strong forces that govern self-assembly makes them amendable to modifications to incorporate functionality and increases the complexity of the resulting macromolecular structures. This implies that various molecular domains can be varied independently or in conjunction with each other, without significantly affecting their assembling and physical properties. To date, a lot of effort has been made to add bioactive motifs for cell adhesion, differentiation and migration. The resulting hydrogels have widespread applications as synthetic cell culture substrates and biological scaffolds for *in vivo* tissue regeneration. Continuing progress will be facilitated by insights into how the heterogeneity and modularity of functionalized peptide scaffolds impact cell behavior *in vitro* and *in vivo* (Jung et al., 2009).

With the discovery of novel biofunctional motifs, self-assembling peptides that form fibrillar structures can be used to display these cues in an optimal fashion three-dimensionally, to achieve a desirable therapeutic effect. Furthermore, the modular nature of self-assembling β-structured peptides also lends itself to the simultaneous display of a well-defined combination of functional motifs. This is an advantage that is not fully utilized to date—there are very few studies which have demonstrated that several bioactive functional domains can be displayed simultaneously (Collier et al., 2010; Jung et al., 2009). The combinatorial display of bioactive motifs and MMP cleavage sites, as well as incorporation of soluble factors, is particularly integral to developing nanofibrillar networks that mimic native extracellular matrix—a complex microenvironment in terms of both structural and biochemical cues. The nanofibrillar topography and porous microarchitecture, combined with cytokines encapsulated in the hydrogel environment, provide a multi-prong approach for enhancing cell proliferation and differentiation in regenerative medicine.

Although to date only peptide sequences have been utilized as functional motifs, the ease and flexibility of solid phase peptide synthesis can potentially open up the variety of short self-assembling peptidomimetics, as exemplified by Pochan and Schneider's β-hairpin structures (Pochan et al., 2003; Schneider et al., 2002). We also anticipate the increasing integration of synthetic amino acids to facilitate cross-linking and biofunctionalization (Haines et al., 2005; Mahmoud et al., 2011), modulate biodegradation and immune recognition, and generate new secondary structures. In particular, α-helical motifs would benefit from such alterations, as they are less modular and the addition of bioactive peptides to the self-assembly sequence would most likely destabilize the assembly process. Likewise, lipids, glycoproteins and proteoglycans can theoretically be chemically conjugated to the self-assembling motif to facilitate self-assembly or cell-substrate interactions. Most notably, Stupp et al. have put in significant efforts in developing peptide amphiphiles with long hydrophobic alkyl chains that facilitate self-assembly into nanofibers and hydrogels (Bull et al., 2005; Hartgerink et al., 2001; Silva et al., 2004; Zhang et al., 2010). Self-assembling peptide domains have also been conjugated to polymers to create hybrid peptide–polymer hydrogels that are stimuli responsive (Tang et al., 2001; Wang et al., 2001). These chemically well-defined composite systems combine the best characteristics of self-assembling peptides (including modularity and reproducible assembly) and polymers (including low cost of synthesis and flexibility) for applications in drug delivery (such as stimuli-responsive drug release, *in situ* gelation drug depots) and tissue engineering.

Although there are a number of biological materials on the market, in clinical studies and in medical applications, there is still room for improvement. The ideal three dimensional biological scaffolds should meet several important criteria: 1) the basic building blocks should be derived from biological sources, namely, amino acids, lipids, nucleic acids and sugars; 2) basic units should be amenable to design and modification at the single molecular level to achieve specific needs; 3) the scaffolds should exhibit a controlled rate of material biodegradation; 4) the materials should have no cytotoxicity; 5) they promote cell-substrate interactions; 6) the materials afford economically scale up and reproducible material production, purification, processing and long-term storage; 7) the materials should be readily transportable; 8) they should be chemically compatible with aqueous solutions and physiological conditions; 9) they do not elicit immune responses and inflammation if used in human therapies; 10) the materials should integrate with other materials and especially tissue in the body. The designer self-assembling peptide scaffolds have above-mentioned attributes. Thus they present a class of promising biological scaffolds for a wide range of biomedical applications.

5. Conclusion

Since the serendipitous discovery of a self-assembling peptide in yeast, we have come a long way, from initial surprise, puzzlement, no understanding at all to, in outline, not only gradually understand the design principals at the molecular level, the molecular and

ultra-fine material structures, interactions of the peptides, and the dynamic self-assembly behaviors, but also how to further improve their design. From there, we have proceeded to optimize their sequence for delivering bioactive therapeutics such as drugs and growth factors. Recent advances in functionalization have also led to the development of better synthetic cell culture substrates, as well as bioactive scaffolds that promote cell proliferation, migration and differentiation for regenerative medicine. As some of non-functionalized self-assembling peptide scaffolds proceed through clinical trials, it is our hope that in the not too distant future, they will open the door for more clinical applications in biomedicine.

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