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Bioinspired Strategies for Hard Tissue Regeneration

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1. Introduction

Synthesis of biomaterials has seen tremendous growth in the past decade and protein templates have emerged as a high utility scaffold to facilitate cell-matrix interactions. The development of new biomaterials play a central role in modern regenerative medicine and tissue engineering. Protein-engineered biomaterials have recently received much attention. The diversity of peptide modules available throughout the proteome broadens the horizon that protein engineered biomaterials can achieve (1). The current challenges for developing protein-based scaffolds for tissue engineering lies in the fabrication of three-dimensional hierarchical materials that mimic the structural complexity of the natural ECM. The natural ECM is a hierarchically structured composite material that is able to furnish several cues for cellular proliferation and differentiation (2, 3). Complex protein-engineered scaffolds can be synthesized by designing scaffolds that self-assemble into specific hierarchical structures.

Molecular self-assembly presents a very attractive strategy to construct nanoscale materials at multiple length scales due to its simplicity in application (4-6). Self assembly can be defined as the spontaneous organization of individual entities such as molecules under thermodynamic equilibrium conditions into coherent, well-defined and stable arrangements without human interventions. In nature, peptides and proteins have the ability to self-organize hierarchically and precisely into well-defined two or three dimensional structures. The building blocks that can undergo the self-assembly process undergo stepwise aggregation through the formation of the hydrogen bonds, electrostatic interactions, hydrophobic interactions, van der Waals interactions and π- stackings (7-10). It is the collective strengths of these weak interactions that form the basis for fabrication of very supramolecular architectures and bioinspired nanomaterials with chemical complimentarity and structural compatibility (7, 9, 11).

In nature we find myriad examples of peptides and proteins that fold with great precision into three dimensional structures. Researchers have identified different protein domains for various end use applications in tissue engineering. Manipulation of the protein domains can be used to control the material properties of the hydrogel. Tailor-made protein scaffolds should provide mechanical properties similar to the extracellular matrix. Further, self-

assembling protein scaffolds are capable of presenting multiple cell-interactive components in spatially resolved networks via supramolecular self-assembly. Self-assembled materials provide several advantages: namely they can be multifunctional, possess high multifunctionality and control over the nanoscale positioning of ligands and other biomolecular features. Self-assembling based peptide materials can be used for regenerative medicine and drug-delivery technologies as they are found to be minimally immunogenic, inherently biocompatible and biodegradable. Thus, protein based scaffolds would be highly beneficial to guide spatially-and temporally-complex multicellular processes of tissue regeneration.

Peptide and protein based biomaterials have emerged as a new class of biomaterials that possesses unique and often superior properties when compared to conventional materials and are widely used for regenerative and reparative medicine (2, 12-14) . The main application of peptide-based biopolymers for tissue engineering is an injectable scaffold. Such hydrogels can serve as a three-dimensional artificial extracellular matrix to provide embedded cells with structural integrity and functionality for tissue repair and regeneration. There are many protein domains and peptide motifs available for tissue engineering applications particularly for hard tissue regeneration. However, peptide-based biomaterials have limitations such as short shelf-life and thermal instability.

2. Hydrogels

Hydrogels are hydrophilic polymers that can retain a significant amount of water while maintaining a distinct three-dimensional structure (15, 16). Based on the nature of the crosslinking, hydrogels can be categorized as chemical or physical gels. The structure and properties of the primary chains as well as the crosslinking density, contribute to the overall properties of chemical hydrogels. Physical gels are networks held together by molecular entanglement or secondary molecular interactions. These interactions can be disrupted by changes in the environment, such as temperature, pH, ionic strength, presence of specific solutes and stress; consequently, the formation of physical hydrogels may be reversible(16). The fact that properties of a peptide domain can be imposed on the whole hybrid hydrogel suggests that the supramolecular organization of hybrid materials is driven mainly by the biorecognition of peptide segments (17).

3. Rheological properties

The mechanical properties of the peptide hydrogels are important for determining their suitability in various tissue engineering applications. As protein-based scaffolds in tissue engineering are used as a structural component to support cell growth, it is therefore important that they possess sufficient mechanical strength. Cellular responses to ligand-presenting materials are strongly influenced by the material's stiffness. In order to elucidate a relationship between the molecular structure of a polypeptide and its rheological properties it is necessary to determine G′ the elastic modulus which indicates the solid-like component of a viscoelastic hydrogel and the viscous modulus G″ represents the liquid-like component (13). To maintain the hydrogel in a solid-like state it is necessary to have G′ larger than G″(13). Therefore, modifying substrate stiffness influences cell morphology, proliferation and differentiation and is an important factor in tailoring matrices for regenerative medicine.

4. Hard tissue regeneration

Bone and dentin are biological composites of organic and inorganic phases have a microstructure that provides an unusual combination of toughness and fracture resistance. Cartilage is composed of specialized cells called chondrocytes that produce a large amount of extracellular matrix composed of Type II collagen, proteoglycans and elastin fibers. The rapidly emerging field of tissue engineering holds great promise for the generation of functional bone and cartilage tissues. To this end molecular self-assembly presents a very attractive strategy to construct nanoscale materials for hard tissue engineering. This free energy-driven process spontaneously organizes molecules into ordered structures at multiple-length scales.

Molecular Biology techniques can be employed to synthesize protein domains and peptide motifs to create responsive protein for tissue engineering bone, dentin and cartilage. A critical requirement for materials designed to interact with cell receptors is the organization of multiple ligands on the surface of a scaffold in order to engage the receptors more effectively. Structures forming α-helices and β-sheets have been used to mediate self-assembly into hydrogels of peptides. In this review we review these processes on a few peptides that possess self-assembling properties and their use in hard tissue engineering.

5. Genetically engineered polypeptides in hard tissue engineering

(a) Self-Assembly of Elastin: Elastin is the major extracellular matrix protein which is responsible for the properties of extensibility and elastic recoil of many tissues such as the large arterial blood-vessels, lung parenchyma and skin (18). Elastin is synthesized as a monomer, tropoelastin, which is subsequently assembled into a stable, polymeric structure in the extracellular matrix (19). This self-assembly property of full-length tropoelastin can also be mimicked by smaller polypeptides.

Elastin-like polypeptides (ELPs) have the ability to undergo organized self-assembly into network structures through a process of temperature-induced phase separation or coacervation (20) . Elastin-like polypeptides are derived from a repeating motif within a hydrophobic domain of mammalian tropoelastin: the most common motif has the sequence $(VPGXG)_m$, where X can be any amino acid other than proline, and m is the number of repeats (1). There are many other variants of ELPs that range from other pentapeptides with the repeat sequence KGGVG (21) or LGGVG (22) to heptapeptides with the sequence LGAGGAG and nonapeptides with the sequence LGAGGAGVL. All of these elastin analogues appear to exhibit elastin-like properties.

Wright et al. and Nagapudi et al.(23, 24) have synthesized self-assembling elastin-mimetic triblock polypeptides. The copolymers composed of a plastic domain VPAG as the end blocks and an elastomeric domain VPGVG as the middle block. The single substitution of an alanine residue for a glycine residue in the third amino acid position of the repeating sequence converts the blocks mechanical behavior from elastic to plastic. This change is caused by the structural change from the Pro-Gly type II β-turn structure to the Pro-Ala type I β-turn structure (23). For ELPs the important biophysical characterization is the determination of the inverse temperature transition behavior and is usually represented by the lower critical solution temperature (LCST) or transition temperature (T_t). Rheological measurements of an aqueous triblock copolymer solution as a function of temperature showed that the copolymers would be well-suited for biomedical applications.

Fabrication of these covalently cross-linked aggregates of ELPs into membrane-like matrices has been exploited for cartilage tissue engineering. Betre et al. have demonstrated that chondrocytes can be encapsulated in the gel-like material formed by aggregated ELPs (25, 26). These chondrocytes maintained their characteristic morphology and synthesized phenotypic markers such as collagen type II and sulphated glycosaminoglycans.

A critical requirement for materials designed to interact with cell receptors is the organization of multiple ligands on the surface of a scaffold in order to engage the receptors more effectively. Kaufmann et al. have demonstrated a new approach for the preparation of bioactive elastin-mimetic hydrogels (27). Osteoblast adhesion was dependent on the ligand type, ligand density and the use of a spacer. Nettles et al. have used ELP as an injectable peptide into osteochondral defects and demonstrated cell infiltration and cartilage matrix synthesis in critically sized defects (28).

(b) Self-assembly of Leucine Zipper-based triblock proteins: The DNA binding leucine zipper proteins contain a self-assembling leucine zipper domain. Leucine zippers are a structural motif commonly found in transcription factors. The leucine zipper domain is a reversible self-assembly domain (29-31). Hydrophobic forces drive the assembly of the coiled-coil bundles as the hydrophobic planes along the length of the α-helices are buried. The leucine zipper domains are composed of a repeating heptad motif designated *abcdefg* where *a* and *d* are hydrophobic amino acids (leucine is preferred at position d) and *e* and *g* are charged amino acids (glutamic acid is common). The repeating domain has an α-helix structure and easily forms inter-and intra-chain coiled coil dimers due to the hydrophobic interaction between the *a* and *d* residues, which are positioned on a single face of the helix. The charged e and g residues positioned on the opposite phase of the helix impart pH-sensitivity to the coiled-coil dimers. Upon elevation of the pH, temperature or ionic strength, the leucine zipper domains reversibly dissociate and create a viscous polymeric solution (13). The reversible assembly makes the leucine zipper domain to facilitate the formation of physical crosslinks in hydrogel structures. The motif's name reflects the predominance of leucine residues at the *a* and *d* positions. Hydrogels are usually based on physical or chemical crosslink's of hydrophilic gelators to form a three-dimensional network (32). It is able to immobilize and entrap large amounts of water resulting in tissue-mimicking environment.

Petka et al. have demonstrated that genetically synthesized triblock copolymers consisting of leucine zipper helix endblocks and water-soluble polyelectrolyte midblock will self-assemble into pH and temperature-sensitive hydrogels upon dimerization of the leucine zipper coils (29). Wang et al. described the use of leucine zipper domains in a hybrid synthetic polymer-protein material (17). The hybrid material undergoes a volume change in response to temperature change as leucine zipper coiled coils dissociate at high temperature (15, 33).

In order to exploit the use of leucine zipper polypeptides in hard tissue engineering, Gajjeraman et al. have designed a leucine zipper polypeptide with motifs from the hydroxyapatite nucleating domain and cell-adhesive motifs from dentin matrix protein 1 (DMP1) (34). Although, DMP1 was initially isolated from the dentin matrix and was thought to be unique to dentin and named accordingly, it has now been found to be present in all mineralized tissues of the vertebrate system (35, 36). The C-terminal polypeptide of DMP1 contains the HAP nucleating domain as well as an RGD motif for cell-adhesion which makes it a highly desirable polypeptide for *in-vivo* applications requiring calcified tissue formation (36).

In this system a modular design was used to genetically engineer de *novo* self-assembled chimeric protein hydrogels comprising leucine zipper motifs flanked by the C-terminal domain of DMP1. Results from this study showed that the leucine zipper hydrogel exhibited both osteoconductive and osteoinductive properties. Recently Huang et al. (unpublished data) have introduced several cysteine residues in the leucine zipper construct to enable the formation of intermolecular disulphide bonds which would effectively crosslink the nanofibers into a high molecular weight polymer (Fig 1). Cryo SEM showed that the introduction of cysteines was effective in promoting nanofiber networks. Integration of RGD domains in this construct facilitated cell attachment and proliferation (Fig 3). Thus, integration of biological self-organization and cell-attachment components are important to synthesize complex materials that exhibit order from the molecular to the macroscopic scale. Such hydrogels from self-assembled peptides have a potential to serve as synthetic extracellular matrices.

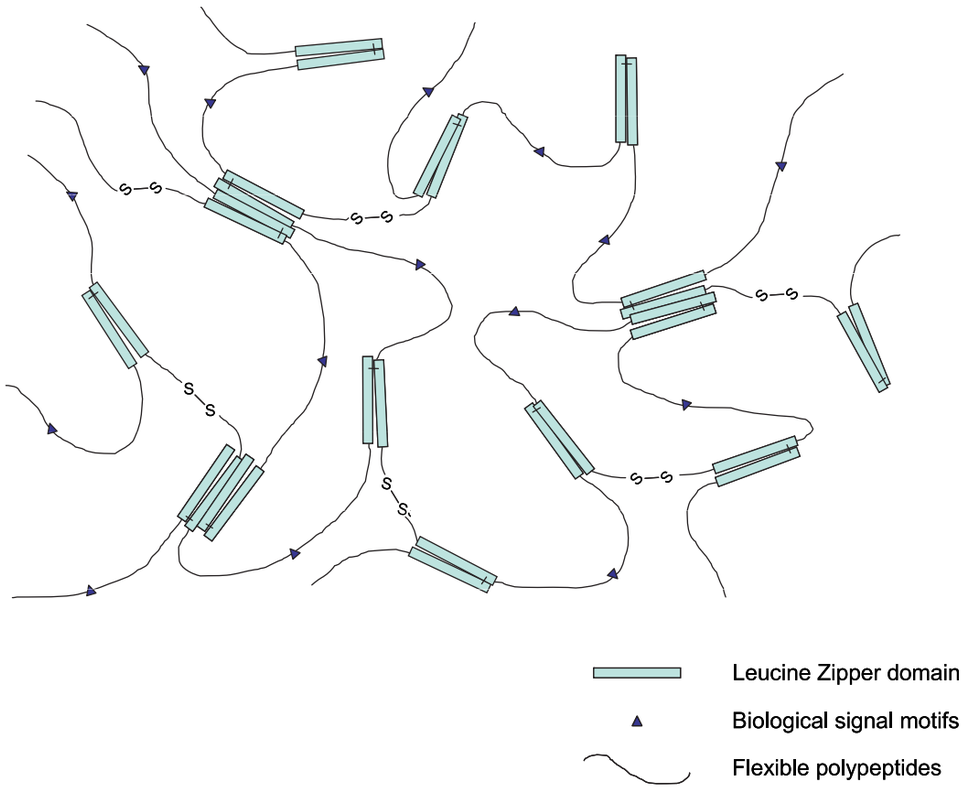

Fig. 1. A schematic representation of the Leucine zipper construct designed for bone and dentin regeneration.

(c) Self-Assembling peptide MDG1 (Mineral Directing Gelator): In a recent study Gungormus et al. described the synthesis of an *in situ* forming self-assembling peptide hydrogel that is capable of directing the mineralization of calcium phosphate (37). The peptide construct MDG1 is a 27 residue peptide designed to undergo triggered intra-molecular folding and subsequent self-assembly to form a fibrillar network resulting in a mechanically rigid gel. This peptide folds in a solution containing calcium chloride and beta-glycerophosphate and in pH buffered water at low ionic strength the peptide remains

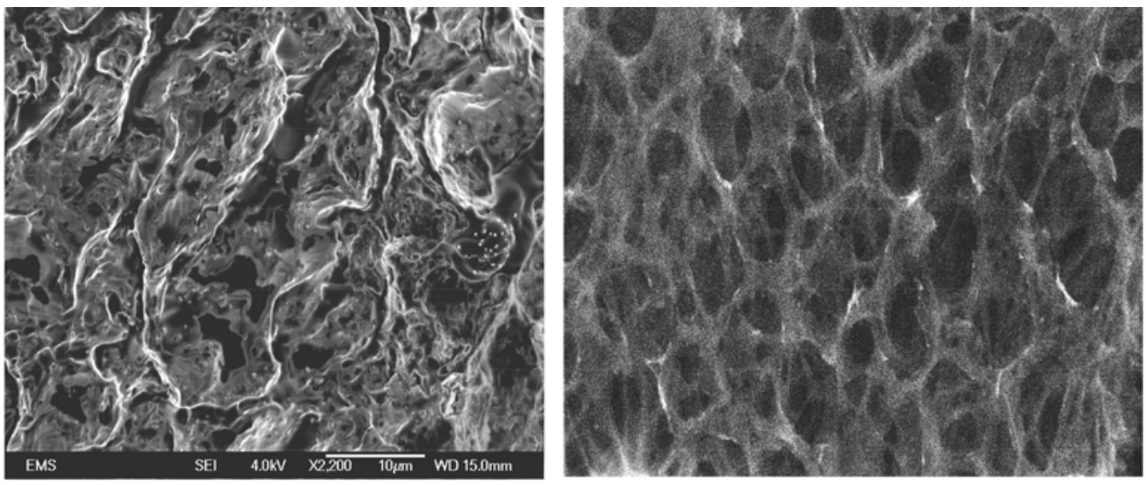

Fig. 2. SEM image of the self-assembled leucine zipper hydrogel

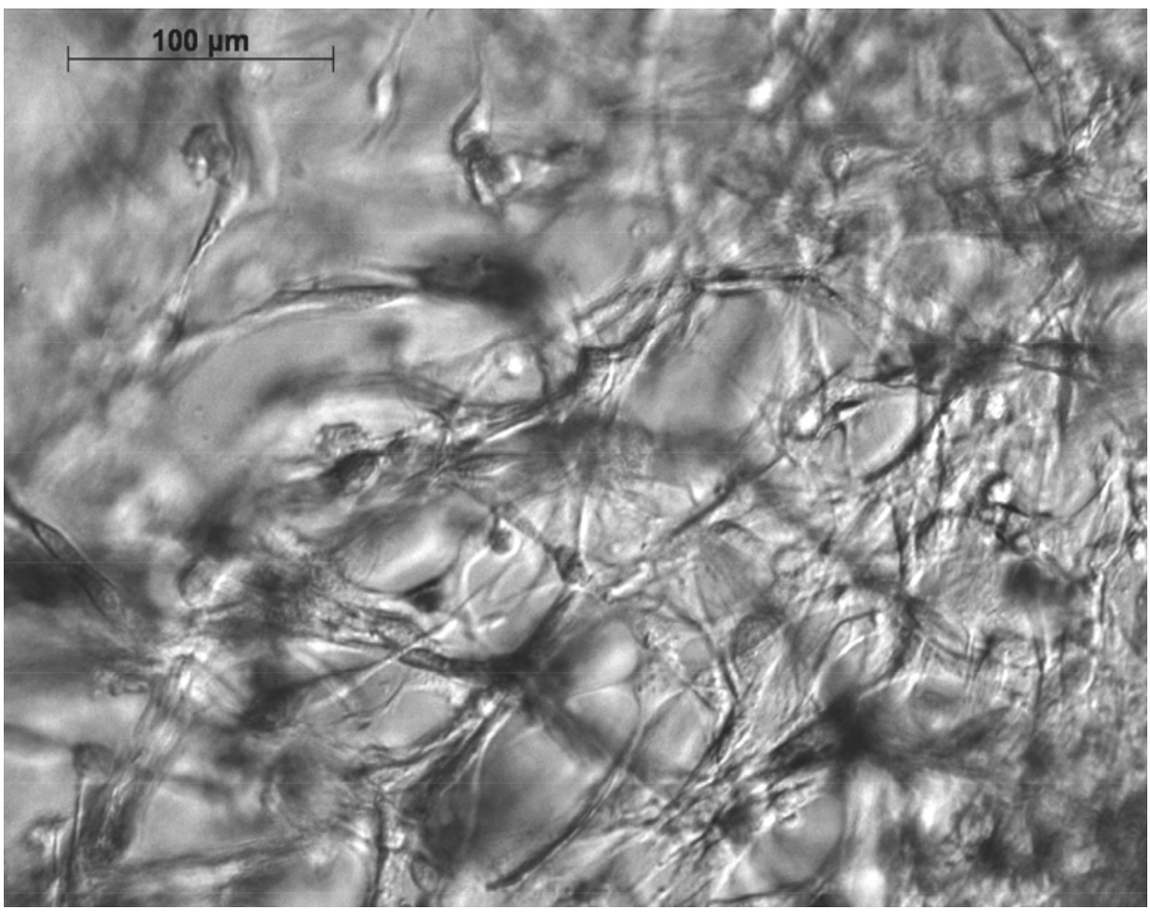

Fig. 3. Attachment and spreading of the human mesenchymal stem cells on the leucine zipper hydrogel at 2 days.

unfolded. The N-terminal twenty residues of MDG1 are designed to adopt an amphiphilic β-hairpin when the peptide folds. The N-terminal portion contains 2 β-strands connected by a four residue sequence (-V^{D}PPT-) known to adopt a type II′ β-turn (38). The β-strands are composed of alternating hydrophobic and hydrophilic residues that give the hairpin its amphiphilic character in the folded state. The complete N-terminal peptide has been reported in the literature as MAX8 and contains the sequence VKVKVKVKV^{D}PPTKVEVKVKV-CONH$_2$ (39) . The C-terminal seven residues of MDG1 contain the sequence MLPHHGA and this sequence directs mineralization. The C-terminal peptide slows the mineralization rate and accelerates the transformation of amorphous calcium phosphate into crystalline octacalcium phosphate during mineralization. Hydrogels for mineralization were formed by the addition of calcium chloride solution containing alkaline phosphatase directly in the cassette. At the end of 2 hrs of gelation the cassette was immersed in a bath that contained a buffered solution of beta-glycerophosphate and calcium chloride. Such a system enabled controlled mineralization of the scaffold as the calcification process occurred when the β-GP diffused into the cassette and was cleaved by the enzyme. Characterization of the mineral deposits within the hydrogel showed that they were highly crystalline and elongated resembling biological apatite. Further, this scaffold supported the viability of cementoblasts and was able to produce a calcified matrix.

(d) Self-Assembly of β-sheet fibrillizing peptides: β-sheet fibrillizing peptides have received particular attention recently as scaffolds for tissue engineering due to their ability to form hydrogels (40-43). β-sheets are well known for their ability to assemble into long fibrous structures. The basic motif present in most β-sheets consists of alternating hydrophobic, hydrophilic residues. As a consequence of this alternating pattern, they give rise to a hydrophobic and hydrophilic face when assembled into a sheet. RAD16 peptide which is derived from the self-assembling sequences of laminin is a β-sheet fibril forming peptide that is capable of presenting bioactive ligands on their surface (44-46). Q11 a peptide containing the sequence (QQKFQFQFEQQ) was designed to present ligands such as RGDS or IKVAV at their N-termini (47). The RGDS sequence found in fibronectin, laminin, vitronectin and many other extracellular matrix proteins is an integrin binding peptide and is neutrally charged and hydrophilic (48). The peptide IKVAV is a cryptic sequence found at the carboxy-terminal end of the α1 chain of laminin is known to be a modulator of neuronal cell attachment and growth (49). This peptide is positively charged and comparatively hydrophobic. Stiffness of Q11 gels was dependent on peptide concentration with storage moduli ranging from 1 to 10kPa for gels having peptide concentrations between 5 and 30mM respectively. Jung et al. have recently shown that the co-assembling hydrogel based on Q11 peptides with the RGD containing ligand influenced HUVEC attachment, spreading and growth (47).

Pochan and Schneider have demonstrated that short amphiphilic peptides that fold into β-hairpin structures will self-assemble into injectable hydrogels that can be used for tissue engineering (42, 43, 50-52). Haines-Butterick et al. used β-hairpin molecules with a lower net positive charge to homogenously encapsulate the mesenchymal stem cells within the hydrogel network (53). In the presence of growth factors these cells could be coaxed into an osteoblast lineage.

(e) Self-assembly of chemically synthesized Peptide Amphiphiles: Peptide amphiphiles (PAs) are a class of molecules that combine the structural features of amphiphilic surfactants with the functions of bioactive peptides and are known to self- assemble into a variety of nanostructures (54). The peptide amphiphiles are obtained chemically using an automated

peptide synthesizer and consist of an alkyl tail connected to a short peptide sequence. The peptide sequence always ends in a hydrophilic head group, giving the PA its amphiphilic character. Stupp et al. have synthesized peptide amphiphiles that consist of 4 key structural domains (55). Domain 1 consists of a hydrophobic region typically consisting of a long alkyl tail. Domain 2 consists of a short peptide sequence capable of forming intermolecular hydrogen bonding, typically in the form of β-sheets. Domain 3 contains charged amino acids for enhanced solubility in water and for the formation of networks. Domain 4 is used for the presentation of bioactive signals for interaction with cells or proteins(56). The self-assembly of PAs in water is due to hydrophobic interactions of the alkyl tails, hydrogen bonding among the middle peptide segments and electrostatic repulsion between the charged amino acids. The PAs developed by Stupp and coworkers self-assemble into high-aspect-ratio nanofibers under specific solution conditions (57, 58) . Molecular packing within a cylindrical geometry allows for the presentation of biological signals at very high density on the fiber surface. Control of PA nanostructures and their subsequent gelation could be controlled through the molecular forces that contribute to the self-assembly process. Thus, molecularly designed peptide amphiphile materials are capable of self-assembling into well-defined nanofibers.

The chemistry on the surface of the PA nanofibers can be customized to create templates for mineralization. Hartgerink et al. designed PA templates with phosphoserines to aid hydroxyapatite deposition (55). Interestingly, the crystallographic c-axis of hydroxyapatite aligned with the long axis of PA nanofibers, mimicking the crystallographic orientation of hydroxyapatite crystals in bone with respect to the long axis of collagen fibers. Recently, Mata et. al reported on the *in vivo* osteogenic potential of self-assembling Pas (59). Results from this study demonstrated that a combination of functionalized PAs i.e RGDS-PA along with S (P)-PA (phosphorylated serine) self-assembling gel promoted bone formation in a rat femoral critical-sized defect within 4 weeks. The newly formed bone was comparable to animals treated with a clinically used allogenic bone matrix. Thus, self-assembling nanofibrous PA matrices could promote formation of biomimetic bone crystals.

Shah et al. designed a coassembly system of PA molecules containing epitopes to transforming growth factor beta-1, that were designed to form nanofibers for cartilage regeneration (60). *In-vitro* studies indicated that these materials were able to support the survival and promoted the chondrogenic differentiation of human mesenchymal stem cells. These studies demonstrated the potential of a completely synthetic bioactive biomaterial as a therapy to promote cartilage regeneration.

Varying the design of the molecular structures of PAs as well as manipulation of their self-assembly environment can be exploited to control the self-assembly process and generate novel materials for hard tissue regeneration and repair.

6. Conclusions

Thus, molecular self-assembly can be used as a toolbox to produce functional materials. The rapidly emerging field of tissue engineering holds great promise for the regeneration and repair of hard tissues. There have been a number of successful approaches to tissue engineer bone and cartilage with the use of natural biomaterial scaffolds; however, there are many challenges ahead with these natural scaffolds. Biomaterials for the future could be envisaged to behave dynamically in their environment and facilitate repair and regeneration within a shorter time-frame.

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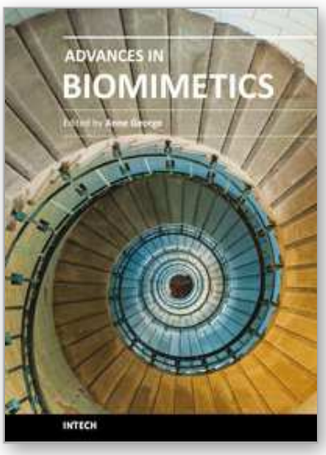

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The interaction between cells, tissues and biomaterial surfaces are the highlights of the book "Advances in Biomimetics". In this regard the effect of nanostructures and nanotopographies and their effect on the development of a new generation of biomaterials including advanced multifunctional scaffolds for tissue engineering are discussed. The 2 volumes contain articles that cover a wide spectrum of subject matter such as different aspects of the development of scaffolds and coatings with enhanced performance and bioactivity, including investigations of material surface-cell interactions.

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