Novel peptide-based biomaterial scaffolds for tissue engineering

Todd C. Holmes

Biomaterial scaffolds are components of cell-laden artificial tissues and transplantable biosensors. Some of the most promising new synthetic biomaterial scaffolds are composed of self-assembling peptides that can be modified to contain biologically active motifs. Peptide-based biomaterials can be fabricated to form two- and three-dimensional structures. Recent studies show that biomaterial promotion of multi-dimensional cell–cell interactions and cell density are crucial for proper cellular differentiation and for subsequent tissue formation. Other refinements in tissue engineering include the use of stem cells, cell pre-selection and growth factor pre-treatment of cells that are used for seeding scaffolds. These cell-culture technologies, combined with improved processes for defining the dimensions of peptide-based scaffolds, might lead to further improvements in tissue engineering. Novel peptide-based biomaterial scaffolds seeded with cells show promise for tissue repair and for other medical applications.

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The extracellular matrix provides a scaffold for organizing tissues. The 3D geometry of tissue is determined both by cell–matrix and cell–cell interactions. Many matrix scaffold proteins and their constituent biologically active adhesive motifs have been identified over the past two decades [1]. Studies in developmental biology have refined our knowledge of the spatial and temporal expression of these motifs – biologically active adhesive or anti-adhesive interactions can occur transiently during tissue development or persist over the lifetime of an organism to maintain the architecture of adult tissues [1,2]. Many of the molecular cues that are responsible for tissue organization during development are absent in the adult animal. Thus, the presence or absence of tissue-organizing cues in the extracellular matrix is a determinant of the tissue regenerative capacity. Such molecular cues are also responsible for specialized cellular functions such as axonal regeneration [2,3]. The need for tissue repair has motivated the development of biomaterial scaffolds that can be used in artificial tissues and for transplantable devices such as biosensors.

Candidate biomaterial scaffolds ideally meet several strict criteria: (1) basic units that are amenable to design and modification; (2) a controlled rate of biodegradation of the materials; (3) lack of cytotoxicity; (4) properties that specifically promote or inhibit cell–material interactions; (5) elicitation of minimal immune responses and inflammation; (6) easy and scalable material production, purification and processing, and (7) chemical compatibility with aqueous solutions and physiological conditions. Failure to meet any of these criteria imposes limits on the potential usefulness of the candidate biomaterial.

Materials ranging from spider silks to extracellular matrix proteins provide excellent examples of biomaterial scaffolds. Naturally occurring biomaterial scaffolds (such as collagen) can be chemically modified to confer desirable properties, however, nature is still the ultimate material engineer and the need for design flexibility motivates human material engineers. Biologically compatible synthetic materials are increasingly used as biomaterial scaffolds. Self-assembling peptides, organic polymers, inorganic materials or mixed co-polymer combinations have been used to create synthetic biomaterial scaffolds. Recent reports in the tissue engineering literature underscore the importance of control over biomaterial geometry. The promotion of desired cell differentiation requires the matrix to have physical properties that facilitate defined geometries of cell–matrix and cell–cell interactions. The geometric distribution of mechanical force transduction from the matrix influences cell shape and even determines whether cells live or die [4]. Strikingly, these phenomena are independent of the type of matrix protein itself [4].

Naturally derived materials

Cell-based therapy is emerging as an alternative therapeutic approach to many diseases. In some cases diseases are resistant to small-molecule drug treatment. Several types of naturally derived animal products, such as collagen-based biological scaffolds, their derivatives and bio-compatible co-polymers can be used as scaffolds for cell attachment [5].

However, one potential problem with all animal-derived biomaterials is that they can carry dangerous pathogens. Transmissible spongiform encephalopathies (TSEs) are among the most feared pathogens capable of jumping species barriers during disease transmission [6]. The appearance of prion-mediated spongiform encephalopathy, which has been accompanied by the cross-species transfer of the prions to humans, highlights this concern. Although many pathogenic agents are destroyed by treatment with extreme pH or temperature, prions (the causative agent of TSEs) are extremely resistant to chemical or physical degradation. Bovine-derived materials are found in >85% of all pharmaceutical products. Accordingly, there are

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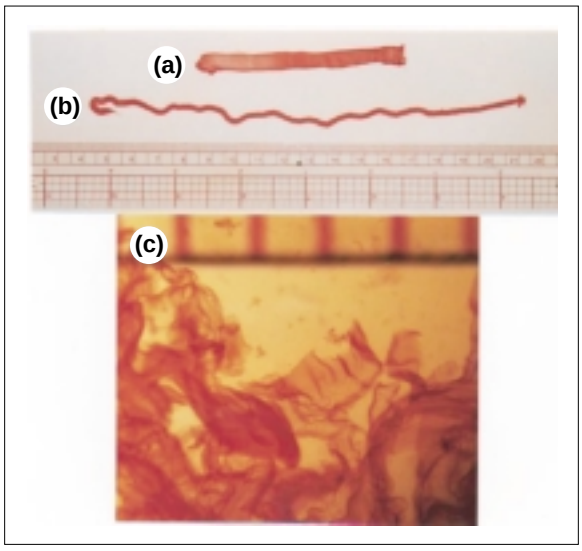

Fig. 1. A RAD16 matrix scaffold dissolved in water was introduced into phosphate-buffered saline to produce (a) tape-shaped, (b) rope-shaped or (c) sheet macroscopic matrix scaffolds. All macroscopic matrix scaffolds are stained with the β-philic dye Congo red. The scale in cm is shown below the sheet structure using a standard lab ruler (adapted from [10], © The National Academy of Sciences of the United States of America).

substantial ongoing efforts to enhance the detection of TSEs in animal-derived products and to create synthetic recombinant collagen [7]. Other viruses might also be carried as pathogens in animal-derived biomaterials.

Synthetic peptide-based biomaterial scaffolds

A benefit shared by all synthetic biomaterials is that they minimize the risk of carrying biological pathogens or contaminants. Synthetic biomaterials exhibit several attractive features for applications in controlled drug release, tissue repair and tissue engineering [8]. Recently developed synthetic biomaterials show promising improvements for *in vivo* biocompatibility. The major benefit of synthetic biomaterials is that they can be designed to meet specific needs. An example of such design flexibility is shown by the incorporation of biologically active motifs that promote cell attachment [e.g. the cell adhesion motif arginine, glycine, aspartic acid (RGD), a ligand for integrin cell adhesion receptors]. In some cases, synthetic biomaterials are composed of polymers of naturally occurring small biological molecules such as amino acids. The basic units of synthetic biomaterials show excellent physiological compatibility and minimal cytotoxicity and the breakdown products of biomaterials that are derived from biological molecules can be incorporated into newly synthesized biomolecules or metabolized in the host organism. Other synthetic biomaterials are composed of molecules that are not found *in vivo* such as ceramic materials. Some of these materials (e.g. bone tissue replacement materials) exhibit desirable properties such as high tensile strength.

A novel class of biomaterials, composed of spontaneously self-assembling oligopeptides, was discovered recently [9–11]. The constituents of these biomaterial scaffolds are self-complementary amphiphilic oligopeptides that have regular repeating units of positively charged residues (lysine or arginine) and negatively charged residues (aspartate or glutamate) separated by hydrophobic residues (alanine or leucine). The self-complementary amphiphilic peptides contain 50% charged residues and are characterized by their periodic repeats of alternating ionic hydrophilic and uncharged hydrophobic amino acids. Examples include RAD16-I, which has the sequence AcN-RADARADARADARADA-CNH_2 (in single letter amino acid code), and RAD16-II, which has the sequence AcN-RARADADARARADADA-CNH_2. Although RAD16-I and RAD16-II share the same length and number of each amino acid, RAD16-I has a spacing modulus of one based on the formula $(RADA)_n$, where n denotes any number of repeats, RAD16-II has a spacing modulus of two based on the formula $(RARADADA)_n$. Representative macroscopic matrix scaffolds processed from RAD16-II are shown in Fig. 1 (adapted from [10]). The isobuoyant (free-floating in solution, neither sinking nor rising to the surface) matrix scaffolds can be fabricated into various geometric forms with relatively even thickness, either as tapes (Fig. 1a), strings (Fig. 1b) or sheets (Fig. 1c). Peptide and salt concentration, as well as the dimensions of the processing apparatus, determine the geometry and dimensions of the macroscopic matrices. Circular dichroism (CD) spectroscopy reveals that RAD-, ELK-, and EAK-based peptides with the representative periodicities described previously exhibit strong β-sheet secondary structure in aqueous solutions. Biomaterials formed from other self-assembling oligopeptides with β-sheet secondary structure have been described by several other laboratories [12–14]. Thus, the self-complementary amphiphilic peptides that follow these simple rules of amino acid composition and sequence spacing, exhibit two distinct polar and nonpolar surfaces.

The measured β-sheet secondary structure of RAD, ELK, and EAK peptides was contrary to expectations that were based on the Chou-Fasman statistical predictions for protein helical preferences. Glutamate, leucine and lysine all have high α-helical propensity in the Chou-Fasman model. Hecht and colleagues provided an elegant explanation of this paradox [12]. In a fascinating study, they looked at the outcome of the competition between local and non-local intramolecular influences for determining secondary structure. These competing influences were tested using a family of self-assembling synthetic peptides. Local influences for determining secondary structure include the intrinsic helical propensity of amino acids (as predicted by the Chou-Fasman model). Non-local influences are exemplified by the periodicity and positioning of amino acids in the context of the peptide sequence – periodicity and amino acid positioning determined secondary structure for all synthetic peptides tested. Thus, non-local effects predominated over local effects.

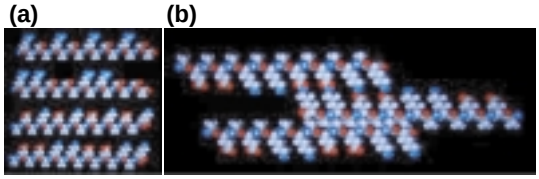

Fig. 2. Molecular models of the amphiphilic self-complementary oligopeptides RAD16-I (upper), RAD16-II (upper middle), EAK16-I (lower middle), EAK16-II (lower) in beta-strand form with distinct polar and nonpolar sites are shown in (a). Proposed inter-peptide side chain electrostatic interactions between the two upper staggered EAK16-II peptides are illustrated in (b). Proposed inter-peptide side chain and peptide backbone hydrophobic interactions between the two lower staggered EAK16-II peptides are also illustrated in (b).

The relative predominance of periodicity and amino acid spacing accounts for the observed β-sheet structural propensity of periodic alternating amphiphilic peptides, rather than the expected α-helical structure. It is worth noting that both local and non-local influences on secondary structure appear to be intramolecular as opposed to intermolecular. This is shown by the relative concentration independence of measured β-sheet secondary structure of EAK16-II by CD [9]. More recently, Broome and Hecht conducted a statistical search for sequences containing periodic alternating polar and non-polar amino acids using a database of 250 514 naturally occurring protein sequences [15]. They found that alternating polar and non-polar amino acid sequences are relatively rare in naturally occurring proteins. Their finding suggests that there are strong selective pressures against the powerful propensity for oligomerization that is conferred by alternating polar and nonpolar amino acid sequences.

Interestingly, the formation of the biomaterial matrix from amphiphilic peptides can be highly condition-dependent. Amphiphilic peptides, such as RAD16 and EAK16, are soluble at low millimolar concentrations in salt-free aqueous solutions. However, when the peptide solution is exposed to physiological media or salt solutions, the amphiphilic peptides form hydrogel-like matrices. Millimolar levels of monovalent cations are responsible for ordered matrix formation. The ordered biomaterial matrix is a hydrogel that has a water content of >99%. In contrast to the ordered biomaterial matrix that spontaneously forms in the presence of monovalent cations, millimolar levels of divalent cations lead to the formation of highly disordered materials from EAK16 and related peptides. How does this salt-triggered molecular switch work? One possibility is that the salt promotes the staggered alignment of individual peptides and matrix formation occurs as a result of electrostatic interactions between the positively and negatively charged residues of adjacent peptides. However, increasing salt concentration does not disrupt matrix stability, which suggests an alternative mechanism. Given that these peptides are self-complementary, monomeric peptides in aqueous solutions might fold to form intramolecular electrostatic interactions. The addition of salt could disrupt intramolecular electrostatic interactions thus allowing the peptides to adopt a conformation that favors intermolecular hydrophobic interactions between adjacent peptides.

Support for this model comes from studies that vary the peptide length and the degree of hydrophobicity of the aliphatic residue. Alanine-containing amphiphilic peptides (such as EAK16) require at least sixteen-mer peptides for salt-induced stable matrix formation [9]. By contrast, leucine-containing amphiphilic peptides (such as ELK8) form salt-induced stable matrices from eight-mer peptides. These results show that increasing the hydrophobicity of the aliphatic residue contributes to matrix formation. A third hierarchal model involves aspects of both previous models. Electrostatic intermolecular interactions between the charged residues of adjacent peptides might contribute to the stabilization of the matrix after the formation of intermolecular hydrophobic interactions takes place. RAD16 peptides and their potential intermolecular interactions are shown in Figure 2. These alternative models of matrix formation and stabilization await direct experimental verification. Self-assembling materials also assemble from peptides that contain synthetic amino acids that are not found in naturally occurring proteins [16] – these molecules are called peptidomimetics. Process conditions (pH, temperature and salt concentration) can be varied to influence the resulting geometry of the self-assembling peptidomimetic-based biomaterials. These results indicate that the design flexibility of peptide-based biomaterial scaffolds is not limited to naturally incorporated amino acids.

So what are these peptide-based matrices good for? The sequence of the RAD-based amphiphilic peptides shares similarity to the cell adhesion receptor integrin ligand RGD. Some extracellular matrix proteins contain RAD sequences that bind to integrin isoforms [17]. The first hypothesis tested was whether cells attach and grow on peptide-based matrices in an integrin-dependent fashion or not. Cell attachment to EAK- and RAD-based matrices is integrin-independent [18], and both EAK- and RAD-based matrices support cell attachment and growth. Whereas the RAD sequence can bind to certain integrin cell adhesion receptors, the EAK sequence does not bind to integrins. Furthermore, high concentrations of RGD peptides have no effect on cell attachment and growth to EAK- and RAD-based matrices, thus confirming that integrin-based adhesion is not essential for cell attachment to these peptide-based matrices. The EAK- and RAD-peptide matrix scaffolds support cell attachment of a variety of mammalian and avian primary and transformed tissue culture cells [16].

More recently, cell attachment, differentiation, neurite outgrowth and the formation of functional synapses by primary and cultured neuronal cells on peptide matrix scaffolds have been examined [10,11]. Such neuron-laden matrix scaffolds can be transported between different environments. This is

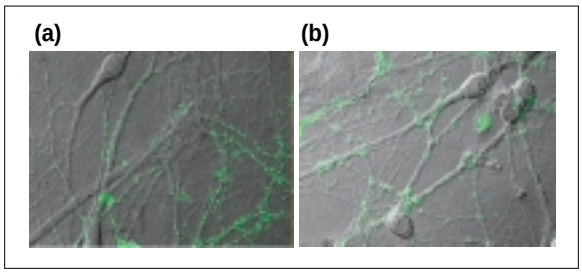

Fig. 3. Primary rat hippocampal neurons form active synapses on peptide matrix scaffolds. The confocal images show discrete bright green labeling indicative of synaptically active membranes following FM1-43 incubation of neurons. Cell diameters range between 3 and 8 μm. (a) Active synapses on the peptide surface. (b) Active synapses on glass coverslips coated with Matrigel™. The active synapses on these different materials are hard to distinguish, indicating that the peptide scaffold is a permissible substrate for synapse formation (adapted from [10] © The National Academy of Sciences of the United States of America).

an important issue for the potential use of neuron/matrix cultures for transplantation.

Neurite outgrowth requires the attachment of neurons to a permissive substrate. Several extracellular matrix proteins, such as laminin, fibronectin and collagen, influence neurite outgrowth *in situ* [1]. These extracellular matrix proteins contain specific motifs that are particularly favorable for cell attachment and neurite outgrowth [1,2]. Extracellular matrix molecules and their adhesion domain fragments, either conjugated with polymers or alone, have also been used to coat surfaces (e.g. glass and polystyrene), which would otherwise provide poor support for neurite outgrowth [2]. Other coat materials such as poly-L-lysine and Matrigel™ also provide good support for neurite outgrowth [19]. Several *in vitro* coatings on glass and plastic have been used to examine the interactions between neurons and individual protein-derived materials, as well as their effect on neurite outgrowth [19]. However, such *in vitro* coatings present serious limitations for certain applications such as tissue repair and tissue engineering. Once neurons attach to coated surfaces they can not be readily transported to tissues without the incorporation of non-biological materials such as polymer fibers or glass. Control neurite outgrowth and functional synaptic connections are shown for neurons grown on glass coverslips coated with Matrigel™ (Fig. 3). Nevertheless, cultured hippocampal neurons can form extensive neurites and functional synaptic connections on isobuoyant RAD16-based biomaterial scaffold matrices (Fig. 3b). The neuron-laden culture grown on RAD16-based matrices are readily transportable from one media to another. Thus, neuron and/or peptide-matrix cultures established in tissue culture could be used for transplantation. Preliminary *in vivo* research shows that EAK16 and RAD16 matrices are well tolerated, as shown by peptide injection studies into muscle and brain, followed by histological assays for inflammation [10].

Tissue engineering and repair

Biomaterials have been used extensively for tissue engineering applications. Recent work on biomaterial and cellular interactions has focused on the importance of the physical properties and dimensionality of biomaterials in hollow organ (bladder) and bone tissue engineering. Breakthroughs in these two areas of tissue engineering underline the importance of biomaterials that mimic the naturally occurring geometry of cell–cell and cell–biomaterial scaffold interactions. The availability of cell precursors (e.g. stem cells) for building neo-tissue and an understanding of factors that govern cellular differentiation into desired neo-tissue types also emerge as important factors for successful neo-tissue construction. There are several recent reviews on biomaterials used for bladder and bone and/or cartilage tissue engineering [20,21].

Atala and colleagues have published a *tour de force* that describes their successful transplantation of a tissue-engineered neo-organ bladder in dogs [22]. A neo-organ scaffold was constructed from a mixed-polymer synthetic biomaterial. The biomaterial scaffold was seeded with autologous urothelial and smooth muscle donor cells, which were collected from host animals then separated and expanded in culture into urothelial and smooth muscle cell pools. Biodegradable polyglycolic acid (PGA) coated with the copolymer poly-DL-lactide-co-glycolide (PLGA) was used to construct the neo-organ bladder scaffold. These synthetic biomaterials were chosen on the basis of their biocompatibility and mechanical properties. PGA and PLGA copolymers have been studied more extensively than peptide-based biomaterials, and excellent processing methods have been developed for fabricating structures. The cell-seeded neo-organs were transplanted into sub-total cystectomized dogs (i.e. dogs with partial bladder removal). Control groups included sub-total cystectomized dogs that received either no implant or neo-organ bladder scaffold implants that were not seeded with cells. The dogs that received cell-seeded neo-organ bladder transplants showed normal function for urine retention, urodynamic bladder compliance and histological architecture for up to 11 months after transplantation. The dogs appeared to recover completely, however, the control groups did not show complete recovery for any of these indices.

Bone-tissue engineering presents a different set of requirements than those for hollow organ replacement. Nonetheless, there are several exciting papers in bone tissue-engineering [23,24] that show key elements in common with the previous example. One similarity is the importance of the biomaterial scaffold for maintaining cell–cell geometry that is similar to native tissue. Three-dimensional cell–cell interactions and cellular density appears to be crucial for proper cellular differentiation that leads to the formation of mature bony tissues. Another key element is the careful selection and treatment of the precursor cells, which are used for seeding the biomaterial scaffold. Cartilage regeneration is another promising target for tissue engineering and

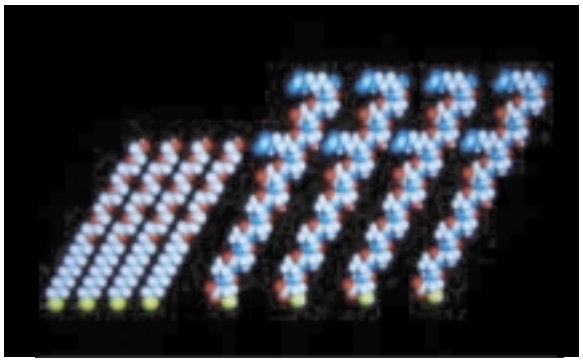

Fig. 4. Self-assembling peptides for patterned surface engineering using microcontact printing: molecular models of the cell attachment peptide RADSRADSAAAAAC (in single letter amino acid code, RADSC-14) and ethylene glycol thiolate (EG_6SH). Both molecules contain terminal sequence homology (SH) groups (indicated in yellow) that covalently bind to gold atoms on the surface. The estimated extended lengths of RADSC-14 and EG_6SH are 5 nm and 4 nm, respectively. (Figure adapted from data shown in [26]).

biomaterials research. Cartilage tends to heal poorly because it is largely avascular. The transplantation of chondrocytes seeded on biomaterial scaffolds shows significant promise for cartilage regeneration [25]. Additional considerations for biomaterial scaffold design and tissue engineering include the use of autologous donor cells to preclude tissue rejection.

Tissue engineering solutions might be more difficult to achieve for tissues composed of cells with poor regenerative capacity. Furthermore, specialized cellular functions, such as axonal regeneration and the ability for damaged neurons to form new functional connections, can be compromised, particularly in adult animals – these are problems associated with central nervous system (CNS) repair. However, recent results suggest a link between poor regenerative capacity and the existence of anti-growth and anti-attachment signals either from matrix (e.g. chondroitin sulfate proteoglycans and certain collagens) or from components of CNS myelin [3]. These encouraging results suggest that the poor capacity of CNS axons to regenerate is not necessarily intrinsic to the neurons themselves. Thus, the masking of such negative signals by a permissive artificial biomaterial matrix scaffold could help to overcome such functional deficits.

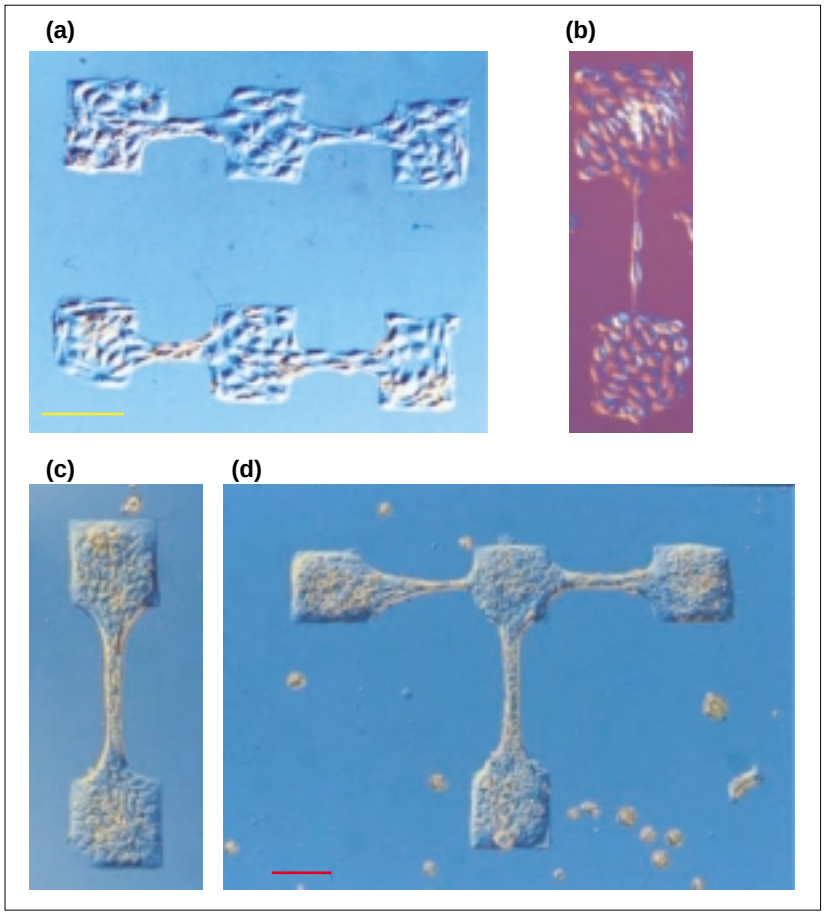

Fig. 5. Patterned cell attachment to RADSC-14. Bovine aortic epithelial cell growth is confined to RADSC-14 coated substrate squares and the coated narrow tracts between squares (a,b). When the RADSC-14 tract between RADSC-14 squares is narrow, only single cells fit. Human epidermal carcinoma cell growth is confined to RADSC-14 substrate I and T patterns (c,d). The cells seen outside of the RADSC-14 coated pattern in (d) are free floating, these cells are not attached to the EG_6SH surface. Yellow and red scale bars (100 μm) in (a) and (d) show the relative size of cells and the RADSC-14-coated patterned surface. The blue background of (a, c and d) and the purple background of (b) correspond to the EG_6SH surface (Figure adapted from data shown in [26]).

Designing better synthetic peptide-based biomaterial scaffolds

Recent studies in diverse areas of tissue engineering underscore the importance of the geometry and physical properties of biomaterial scaffolds for facilitating proper cell differentiation [22–24]. It would be desirable to have a similar degree of control over the fabrication of peptide-based biomaterials as can now be achieved with non peptide-based biomaterials (e.g. PGA and/or PLGA copolymers [22]). Future work on synthetic biomaterial scaffolds will focus on the design of materials with more complex material geometry and improved physical properties, such as greater tensile strength. The ability to incorporate biologically active motifs into peptide-based biomaterials provides strong motivation to develop processes for fabricating these materials into well-defined structures.

In the case of self-assembling peptide scaffolds, it is possible to restrict self-assembling peptides to defined 2D geometries [26]. This approach combines cysteine end-modified RAD oligopeptides and previously developed methods for microcontact printing [27]. Grids are assembled to exhibit alternating patterns of cysteine end-modified RAD oligopeptides or ethylene glycol thiolate (see Fig. 4, adapted from [26]). Both these molecules will form covalent linkages with a gold surface monolayer via their respective SH groups. Cells attach robustly to the RAD–peptide-coated regions of the grid but poorly to the ethylene glycol thiolate-coated surfaces (see Fig. 5, adapted from [26]). It will be interesting to determine whether these methods can be translated into more complex defined 3D geometries of self-assembling peptides.

The specific patterning of cell attachment motifs (e.g. the RGD motif) incorporated in synthetic biomaterial scaffolds could be another way to achieve controlled groupings of different cell types attached to the scaffolds, thus mimicking tissues.

The ultimate goal is to create synthetic biomaterial scaffolds that influence cell adhesion, differentiation and migration of specific cell types to create artificial tissues. Protein–protein interaction motifs, other than cell attachment motifs, could also be engineered into synthetic biomaterial scaffolds. For example, proline-rich ligands that bind to sequence homology 3 (SH3) domains are modular, as shown by chimeric insertion into proteins, thereby conferring SH3 domain interaction with the modified protein [28].

Other future goals for better synthetic biomaterial scaffold design include constructing mixed materials that contain inorganic molecules or metal-binding groups, which could lead to biomaterials with greater tensile strength. Material designers can receive their inspiration from nature. Abalone shell, a natural material with high tensile strength and hardness, contains a mixture of organic and inorganic materials [29]. Biomineralized scaffolds are the physical basis for bone repair and other hard tissue repairs. Along these lines, Hecht and colleagues have constructed self-assembling monolayers that are derived from a combinatorial peptide library [30]. The peptides in this library are selected for their propensity to fold into six-stranded amphiphilic beta-sheets. Monolayer self-assembly of these amphiphilic peptides occurs at air–water interfaces. These exciting results show the possibility of constructing laminated biomaterials composed of sheets of defined organic proteins and inorganic minerals.

Conclusions

The novel peptide-based biomaterial scaffolds described in this article are biology-inspired synthetic materials. These emerging technologies include novel scaffolds for cell-based therapies, tissue engineering and biomineralization for hard tissue repairs. Some of the most promising approaches combine novel biomaterials with stem-cell technology. These biocompatible and biodegradable novel peptide-based biomaterial scaffolds might have a broad range of applications for innovative medicine.

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