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Multi-Component Extracellular Matrices Based on Peptide Self-Assembly†

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Abstract

Extracellular matrices (ECMs) are challenging design targets for materials synthesis because they serve multiple biological roles, and they are composed of multiple molecular constituents. In addition, their composition and activities are dynamic and variable between tissues, and they are difficult to study mechanistically in physiological contexts. Nevertheless, the design of synthetic ECMs is a central consideration in applications such as regenerative medicine and 3D cell culture. In order to produce synthetic matrices having both multi-component construction and high levels of compositional definition, strategies based on molecular self-assembly are receiving increasing interest. These approaches are described here and compared with the structures and processes in native ECMs that serve as their inspiration.

Introduction

The use of non-covalent self-assembly to construct materials has become a prominent strategy in chemistry and materials science, continuing to offer practical routes for the construction of increasingly functional materials in applications ranging from electronics to biotechnology. In this tutorial review, recent progress will be described in applying self-assembling approaches towards a challenging design target: functional extracellular matrices (ECMs). Extracellular matrices are the acellular biological materials that surround cells and provide tissues with much of their mechanical properties. They also play fundamental roles in the morphogenic processes critical for development, regeneration, and healing. Engineered materials capable of functioning as ECMs have received intense attention, particularly in the areas of Tissue Engineering, Biomaterials, Regenerative Medicine, and 3D cell culture. Although a wide range of synthetic or biologically sourced ECMs continue to be introduced, including polymer hydrogels, polysaccharide gels, recombinant proteins, decellularized tissues, or combinations of these, approaches based on the self-assembly of small molecules provide a unique set of advantages. One key advantage is that self-assembly may be exploited to satisfy both the functional needs of complex ECMs as well as practical aspects such as chemical definition, ease of synthesis, modularity, reproducibility, and tunability. Using self-assembly, functionally complex materials can be created from components that are structurally simple. Given the extreme complexity of native ECMs, such a balance between functionality and practicality is an advantage. In this review, native functions of the ECM will be discussed, followed by steps taken recently with self-assembled biomaterials to recapitulate such roles in synthetic matrices. Owing to the brevity

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of the tutorial review format, we unfortunately cannot include exhaustive lists of references and will instead cite only the papers that are most essential to illustrate the points discussed.

ECMs as design targets for self-assembling systems

ECMs are adaptive networks—Extracellular matrices are elusive targets for materials design. They are complex physical networks of proteins, proteoglycans, and non-matrix components such as growth factors, and the summation of all of an ECM's components collectively provide a complex material that can regulate and be regulated by cellular processes.¹ The composition of ECMs varies greatly from tissue to tissue, and within a specific tissue the ECM is adaptive and dynamic, shifting its functionality as its physiological context changes between development, homeostasis, healing, or disease states. In each of these contexts, important roles of ECMs can include: 1) regulating the viscoelasticity of a tissue,²⁻¹⁰ 2) providing mechanisms for cell attachment and subsequent intracellular signaling,¹¹⁻¹⁶ 3) regulating the binding, release, diffusion, and transport of soluble signaling molecules such as growth factors,¹⁶⁻¹⁸ 4) remodeling in specific spatial patterns,¹⁹⁻²³ and 5) influencing the overall morphology and function of a tissue through the combination of roles 1-4, using recursive and bidirectional signaling pathways between the matrix and cells.^{1, 2, 12, 16} The challenge for developing synthetic materials that can function as surrogate ECMs is to produce materials that can likewise serve as adaptive networks and that can perform each of these roles simultaneously. In order to do so, much recent effort in the area of ECM engineering has focused on installing molecular features (peptides, proteins, bio-interactive polymers) that carry out these functions within insoluble scaffolds, whether by self-assembly or through covalent modification of polymer or biopolymer networks.

However, practical considerations limit the number of different molecules that can be combined in engineered matrices. Ideally, functional complexity must be maximized with a minimum of compositional complexity. Issues such as cost-effectiveness, reproducibility, the ability to adjust the material to a particular physiological context, and the ability to secure regulatory approval all factor into the ultimate utility of a particular type of engineered ECM. In this respect, a balance between complexity and practicality is one of the most significant advantages of self-assembling biomaterials. They can be constructed in a modular fashion, allowing the production of tunable, multi-component matrices having many of the ECM's native functions, using a defined set of molecules that can be synthesized and combined relatively easily.² Before discussing strategies in self-assembling biomaterials that are currently being developed for recapitulating the five roles listed above, we will briefly discuss a few examples of the molecules and events underlying each role in native ECMs. Each of these examples is intended to provide a flavor, rather than a comprehensive description, of the ECM's functions. These examples will then be used to illustrate both the rationale for recent strategies in self-assembling ECMs, as well as how far the field needs to advance to truly replicate some of the ECM's exquisitely refined processes.

Matrix viscoelasticity critically influences cell behavior—Providing resistance and responsiveness to the mechanical forces acting on a tissue is one of the earliest appreciated functions of ECMs. To resist tensile forces, fibrillar proteins, usually collagens, are key components. Collagens are assembled into long bundles, in which the primary constituents are the fibril-forming collagens (types I, II, III, V, XI), varying in type and relative abundance depending on the tissue. The shape, lateral association, network formation, and bundling of the supramolecular assemblies produced by these fibril-forming collagens are then tuned through the insertion of additional “fibril-associated” collagens such as types IX, XII, XIV, XVI, XIX, and through collagen-binding proteoglycans such as decorin, biglycan,

fibromodulin, and lumican (Figure 1a). Collectively, the types and relative amounts of the fibril-forming collagens, fibril-associated collagens, and collagen-binding proteoglycans specify the overall architecture of the collagen fibrils and the mechanical properties that their networks possess (Figure 1b). For resisting compressive forces, heavily hydrated proteins and glycosaminoglycans (GAGs) such as hyaluronic acid and heparan sulfate proteoglycans are employed. Again, like collagens, the isoforms and relative concentrations of these glycosaminoglycans and proteoglycans vary widely between tissues. Beyond the presence and concentration of these structural proteins, the covalent connectivity of them is also heavily regulated through cross-linking enzymes such as lysyl oxidase and transglutaminases (Figure 1c). As many as hundreds of different proteins and proteoglycans may be hierarchically assembled in mammalian ECMs, but using this discrete toolbox of co-assembling molecules, they can be constructed with a wide range of viscoelastic properties (Figure 1b),^{3,4} with elastic moduli ranging from tens of Pa in the softest tissues such as fat to the MPa range for articular cartilage.³ Recent studies on the relationship between ECM stiffness and cell differentiation have shown that stem cell fate, spreading, and proliferation can be controlled by substrate stiffness.^{5,6} Further, in breast tumorigenesis, the ECM can be stiffened through collagen cross-linking, which is associated with increased malignancy.⁷ Observations such as these have in part recently drawn considerable attention to the rapidly expanding field of mechanobiology, and mechanical factors are becoming central considerations in the design of self-assembling biomaterials as well.⁸⁻¹⁰ In addition to the biological role of matrix viscoelasticity, some degree of strength and stiffness is also practically important to enable the consistent manufacture, handling, and manipulation of self-assembled gels. To achieve such mechanical control in self-assembled biomaterials, the ECM's native modular design has been a source of inspiration. Several recent self-assembling approaches described below follow the concept of utilizing a primary network-forming component (analogous to collagens in native ECMs), modulating lateral assembly through the addition of fibril-modifying components (roles served by decorin, fibromodulin, or fibril-associated collagens in native ECMs), or covalently cross-linking the network to varying degrees (roles served by transglutaminases or lysyl oxidase in native tissues).

Cell-matrix binding is context-dependent and arises from a confluence of physical and biological factors—

Cell attachment to ECMs is dynamic, spatially precise, and regulated bi-directionally between cells and the matrix. Integrins are the major class of receptors through which cell-matrix interactions occur, and they can bind with a range of specificities to many different ligands in matrix proteins such as fibronectin, laminins, collagens, fibrinogen, and others. The integrins are a large family of α/β heterodimers, and cells can express many different integrins at the same time, the relative number and activation state of them being highly dependent on cell type and physiological context. They are differentially expressed depending on disease state, developmental stage, or whether a healing process is ongoing. Beyond their role as mediators of cell attachment, integrins are at the same time signal transduction receptors, capable of initiating intracellular signaling events upon ligand binding that can in turn influence growth, migration, proliferation or differentiation. Further, within a single cell type or even within a single cell adhesion structure, different integrins can be organized in precise spatial patterns. For a recent review of such structures see Berrier and Yamada.¹¹ The challenge to biomaterials developers is to engage the appropriate combination of integrins in the correct spatial arrangement at the correct time, and to evolve these binding interactions through a desired time course, as occurs in natural healing or regenerative processes. The RGD amino acid sequence found in fibronectin, collagens, and other ECM proteins has provided biomaterials developers with a simple and chemically defined tool that has been heavily investigated as a means for improving cell attachment to synthetic surfaces.¹² However, RGD can bind at least a dozen integrins, including $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha IIb\beta 3$, and $\alpha 3\beta 1$, creating a situation which

may be broadly advantageous for universal cell adhesion but a liability when cell-specific or context-specific cell behaviors are desired. As a result, it has become increasingly apparent that it is necessary to develop materials with significantly more information content than is provided by short RGD peptides alone. As will be discussed in the sections below dealing with specific self-assemblies, non-covalent methods can be relatively simple ways of achieving such co-presentation of multiple signals.

An additional challenge in designing biomaterials with predetermined engagement of specific integrins is that integrin-ECM binding is modulated by many matrix characteristics beyond the mere identity and concentration of particular ligands; for a recent review, see Carson and Barker.¹² In the native ECM, the spatial arrangement of ligands, the stiffness of the matrix on which the ligands are displayed, and the time-resolved revealing of cryptic ligands by mechanical or proteolytic processes all ultimately determine which integrins are engaged at what times. One example of the relationship between cell binding and mechanical forces is the behavior of fibronectin (FN), one of the central matrix proteins in cell-ECM attachment. It is susceptible to being unfolded by force, especially within its type III (FNIII) domains, which contain many of its cell binding sites.¹³ Small forces, in the range of tens of pN, are capable of partially unfolding the FNIII domains and changing the distance between the RGD sequence found in the 10th FNIII domain and the PHSRN “synergy” sequence found in the 9th FNIII domain (Figure 2a). Based on this conformational change, in modeling studies it has been hypothesized that a switch in integrin specificity can occur with only very small forces, with $\alpha 5 \beta 1$ binding favored in the unstretched state and $\alpha v \beta 3$ binding favored in the stretched state.^{13,14} Additionally, mutations or splice variants that alter the conformational stability of FN may also affect its susceptibility to forced unfolding.^{15,12} These examples illustrate how precisely cell binding is regulated in the ECM by a confluence of physical and biological interactions. More examples are likely to be uncovered as research in mechanobiology progresses,¹² illustrating the complexity of ECMs and how challenging a design target they are for synthetic biomaterials. Nevertheless, some guidance can be taken from the native construction of ECMs by viewing them as co-assemblies of multiple discrete proteins whose identities, spatial arrangements, and mechanical properties collectively drive cell behavior.

ECMs modulate the transport, availability, presentation, and activity of

soluble factors—The ECM's regulation of growth factor activity operates through several modes of action, in concert with the ECM's roles in cell binding and mechanical regulation. Glycosaminoglycans (GAGs) such as heparin and heparan sulfate, which are present in the ECM as part of proteoglycans, can bind several different types of growth factors, including vascular endothelial growth factors (VEGFs) fibroblast growth factors (FGFs), and platelet derived growth factors (PDGFs).¹⁶ In this way, ECM proteoglycans can modulate the local concentrations of growth factors or establish gradients of them, to be released at a later time when the matrix is degraded by cell-secreted proteases. This concept has played a large part in the development of growth factor-releasing biomaterials, including those based on self-assembly.¹⁷ In recent years, the concepts of growth factor-matrix interactions have become increasingly nuanced, providing additional information for biomaterials development. For example, in some cases, GAG chains co-bind with a growth factor to its receptor, illustrating that GAG chains can play more complex roles than simply retaining or releasing growth factors at a particular ECM site.¹⁶ Also, some growth factors can bind directly to ECM proteins, as exhibited by VEGF binding to fibronectin, which forms a molecular complex that promotes proliferation.¹⁸ It has been found that the synergy between FN and VEGF in promoting proliferation is dependent on having the integrin-binding and VEGF-binding regions of FN physically connected,¹⁸ implying that control over cellular responses to engineered matrices may benefit from the co-assembly of both integrin-binding and growth factor-binding features within the same supramolecular structure. One of the most

thoroughly documented mechanisms of matrix-growth factor synergy is that of transforming growth factor- β (TGF- β).¹⁶ This growth factor is secreted by cells in an inactive form and bound within a complex of multiple ECM proteins, including fibronectin, fibrillin, and latent TGF- β -binding proteins (LTBPs). Combinations of proteolysis or mechanical strain, and in some cases mechanical strain alone, can then act to switch the growth factor into an active form (Figure 2b-c). In these instances, the mechanical strain is brought about by cytoskeletal contractility, which applies force to the ECM through the binding of multiple different integrins including $\alpha v\beta 6$ and $\alpha 5\beta 1$. For a more detailed review of this process, see Hynes.¹⁶ The implication for biomaterials is that to faithfully reproduce the effects of growth factors in engineered systems, their binding, release, presentation, and activity must be considered in the context of both cell binding and mechanical factors, and these interactions must be arranged precisely in space at nanometer resolution. Moreover, progress in the development of synthetic ECMs, whether by self-assembly or not, requires both an increased understanding in the basic mechanisms at work in native ECMs, as well as the efficient, cost-effective recapitulation of those roles in engineered systems.

ECMs are deposited, degraded, and remodeled in spatially resolved patterns

—An active area of recent biomaterials research has focused on patterning synthetic substrates with ECM proteins using microfabrication techniques. This work has been aimed at producing surfaces that modulate cell behavior through the micron- or nanometer-scale positioning of ligands.¹⁹⁻²¹ Such engineered surfaces have clearly demonstrated that spatial patterns of ECM proteins can strongly influence cell migration, proliferation, growth, apoptosis, and differentiation in cell cultures.²¹ Interestingly, although analogous processes must exist in native tissues, experimental demonstration of them *in vivo* has proven somewhat elusive, largely owing to a lack of suitable techniques. In particular, there has been relatively little information available regarding the native micron scale or nanoscale patterns of ECM proteins that cells deposit during normal processes. Recently however, progress has been made in this regard through cell culture experiments investigating cell-secreted patterns of laminins, which are a family of basement membrane matrix proteins that play central roles in tissue homeostasis, morphogenesis, and regeneration.²² In processes such as wound healing, laminins are degraded and deposited in spatially organized and time-dependent processes.^{12, 22} Recently, Jones and coworkers have found that cultured keratinocytes can deposit a wide variety of micron-scale laminin patterns, including rosette-like patterns, long trails, punctate focal adhesion-like patterns, or fibrillar arrays, in part depending on their motile behavior (Figure 3a-d).²² These cell-deposited laminin patterns, if decellularized, can also function as templates for the migration of freshly seeded cells, in a manner similar to substrates patterned via microfabrication (Figure 3e-f). Additional knowledge gained through these and similar *in vitro* studies, in conjunction with better *in vivo* imaging techniques, will continue to shed light on the types of patterns that exist in natural ECMs, thus providing suggestions for spatial arrangements useful within self-assembled biomaterials. Patterning techniques such as those recently reported based on diacetylene-containing self-assembling peptide-amphiphiles (Figure 3e-f) may then prove to be useful ways of realizing such arrangements synthetically.²³

Although the spatial organization of ECM proteins is clearly critical, it is also dynamic, and unique bioactivities arise both during matrix degradation and deposition. Cryptic cell-binding ligands can be exposed by proteolysis, soluble ECM fragments can be liberated to induce signaling, and one isoform of a protein can be replaced with another having different activities.¹² For example, laminin-332 can have different integrin specificities depending on whether it has been proteolytically processed. In its final processed form, it binds preferentially to $\alpha 6\beta 4$ integrins, but in its unprocessed form, it binds more readily to $\alpha 3\beta 1$. The $\alpha 6\beta 4$ integrin is normally found in stable hemidesmosomes of non-migrating epithelia, whereas $\alpha 3\beta 1$ integrins are more associated with cell motility, and so the processing of

LN-332 appears to be involved in the switching between these two states. For review of this and similar processes, see Carson and Barker, and references therein.¹² Within synthetic ECMs, it may be important to provide for similar dynamics in integrin specificity, especially for applications in regenerative medicine.

ECM mimicry with self-assembling biomaterials—Synthetic self-assembled ECMs have advanced significantly in the past 15 years, largely owing to their increasing ability to take on the native roles of the ECM described above. To date several of the more basic functions of the ECM have been recapitulated, particularly the ability to support cell attachment, to be tuned mechanically, and to control the diffusion of small molecules. In this section, these strategies will be outlined. More complex functions exhibited by native ECMs such as feedback between mechanics and cell surface receptor binding, concerted interactions with multiple different ligands, synergistic relationships between growth factor activation and cell binding, and temporally controlled unmasking of cryptic sites have not yet been significantly addressed in self-assemblies. However, given the modular construction of self-assembled ECMs and the establishment of several basic strategies for co-assembling different functional biomolecules into integrated networks, it may be possible to achieve these more complex roles in the coming years.

Matrices based on β -sheet-rich structures: β -sheet peptides, β -hairpins, aromatic short peptide derivatives, and peptide amphiphiles—Work in the area of self-assembling synthetic ECMs has focused significantly on peptides or peptide derivatives, though recombinant proteins have also been investigated and are continuing to be developed as improved expression systems are designed.²⁴ Many approaches based on peptides share a similar overall strategy, in which a simple folding motif is propagated extensively to form fibrillar networks. Ligands appended to such motifs are then displayed on the surfaces of the self-assemblies. In this regard, β -sheet-rich fibrils have been produced from a variety of different classes of molecules including peptides, β -hairpins, aromatic short peptide derivatives, and peptide amphiphiles. Collectively, they have proven to be versatile assemblies that can be used as a basis for the incorporation of many different biological or structural features relevant to their functions as synthetic ECMs.^{1,2, 8-10, 17, 23, 25-47} For underivatized peptides, although it is generally challenging to predict the extent to which any given amino acid sequence will form β -sheets, certain patterns of amino acids are known to favor β -sheet fibril formation. Commonly, alternating polar/non-polar amino acid sequences form β -sheets, owing to the placement of all hydrophobic residues on one face of the sheet and all hydrophilic residues on the opposite face. This amphiphilicity then drives further assembly of the β -sheets into tertiary structures such as β -sandwiches or fibrils. In 1993, Zhang and coworkers reported the use of such alternating peptides for producing hydrogels.²⁵ In subsequent years these materials have been continually refined as 3D culture materials, including the commercially available PuraMatrix™, and many extensions of this initial concept have been built from this early work. Hinting at the versatility of β -sheet fibrillizing peptides as building blocks for functionalized biomaterials, β -sheet fibrils are remarkably consistent in their morphology, even when they are composed of peptides or proteins with widely differing amino acid sequences or lengths. With relatively few exceptions, β -sheet fibrils are typically around 8-15 nm in diameter, with their peptide backbones running perpendicular to the fibril axis. They tend to be relatively unbranched and are prone to significant lateral association, forming bundles and tangles that at high enough concentration produce physical cross-links and viscoelastic hydrogels (Figure 4). Although general design rules continue to be refined, there are many proteins and peptides of significantly divergent sequences, lengths, and hydrophobic patterning that can fibrillize via β -sheet assembly. As an example, transthyretin, the SH3 domain, and lysozyme have little similarity in terms of primary

structure, and none of them possess strictly alternating primary structures. Yet they all form similar β -sheet fibrils.²⁶ Fibrillizing peptide materials have been reviewed recently by Jung and co-workers² and Ulijn and co-workers,^{27, 28} wherein additional examples of different β -sheet-rich fibril-forming molecules are discussed. These include β -hairpins such as MAX-1 and MAX-8,²⁹ fiber-forming peptide amphiphiles,^{17, 23, 30, 31} short peptides with aromatic modifications,³² and unmodified L-amino acid peptides with a variety of different primary structures.^{2, 25, 28, 33, 34, 35, 36} The existence of so many peptides and peptide derivatives forming β -sheet-rich fibrils suggests that they can generally tolerate a wide variety of modifications, which can be exploited to endow specific bioactivities to their assemblies, as has been achieved in recent years.

One of the most straightforward ways of installing a specific biological activity within a molecule forming β -sheet-rich structures is by extending the self-assembling domain with a short peptide ligand of interest (Figure 4a). Early pioneers of this concept were Hartgerink, Beniash, and Stupp, who designed fiber-forming peptide-amphiphiles that contained terminal functionalities including a calcium-binding phosphoserine residue and an RGD sequence.³¹ Since then, the concept of extending a fibrillizing component with a ligand of interest to produce ligand-decorated fibrils has been applied towards a number of different peptides, peptide amphiphiles, and aromatic short peptide derivatives. Stupp and colleagues have built extensively upon their initial concept to produce, among others, fibers displaying heparin-binding peptides,¹⁷ the laminin-derived ligand IKVAV,³⁷ growth factor-binding peptides,³⁸ other ligands,³⁷ and light-triggerable components.³⁹ Zhang, Semino, and colleagues have produced ligand-appended β -fibrillizing peptides based on RAD-16-type peptides.⁴⁰ Collier and co-workers have investigated co-assemblies of different ligand-bearing peptides based on the peptide Q11,^{33, 41} and aromatic short peptide assemblies displaying RGD ligands have been designed by Ulijn and coworkers.³² Similar ligand-bearing β -sheet-rich fibrillizing peptides, peptide-amphiphiles, or short aromatic peptide derivatives have also been investigated by the groups of Hartgerink,⁴² Gras,⁴³ Nomizu,⁴⁴ and Jun.⁴⁵ Now that a number of ligand-bearing self-assemblies have been reported, fibrils of multiple co-assembled molecules are within reach, and have already been reported.^{30, 33, 46} As discussed earlier, the relative availability of multiple different integrin ligands can influence integrin specificity and subsequent cell behaviors. It is reasonable to hope that by co-fibrillizing precise combinations of ligands, improved integrin specificity could be approached, though this has yet to be proven experimentally. In such efforts, means for controlling the distances between ligands would of course be extremely advantageous. In addition, given the increasing numbers of available ligand-displaying self-assembled fibril systems, it may prove useful to mix not just cell-binding ligands, but other functional components as well. Proteolytically susceptible self-assembling peptides, for example, provide the opportunity to control and investigate synergies between integrin binding and ECM degradation,^{35, 42} and growth factor-binding self-assemblies offer similar opportunities.³⁹

Given the increasing awareness of viscoelasticity's importance in determining cellular responses to ECMs,^{5, 8} recent approaches have sought to modulate and target specific mechanical properties of β -sheet fibrillar self-assemblies. Like within the native ECM, viscoelasticity-modifying strategies have tended to fall into one of three categories: modulating the stiffness of individual fibrils, controlling the non-covalent lateral association of those fibrils, or establishing specific covalent cross-links between self-assembled components. In some cases, these strategies have additionally been designed to be time-dependent or spatially resolved, in order to enable triggered transformations such as in situ gelation, or to produce patterned materials.²³ From the outset, an important aspect of controlling mechanics is maintaining consistent mechanical properties despite the inclusion of bioactive components. In this regard, the most popular strategy has been to dope ligand-

bearing peptides or peptide-amphiphiles in relatively small amounts into backgrounds of unmodified fibrillizing peptides so as to minimally perturb gel mechanics (Figure 4a).^{30, 32, 33} Gross control over the stiffness of such materials may then be achieved by changing the concentration of total peptide, presumably owing to an increase in physical crosslinking points between fibrils. Next, the viscoelasticity of hydrogels can be adjusted by controlling the lateral aggregation of peptide fibrils, which in turn can be modulated by controlling the rate of folding and self-assembly. For example, amino acid substitutions in the in MAX-1 β -hairpin peptide that produced faster gelation kinetics also led to significant increases in gel stiffness.^{9, 47} In another example of controlling lateral aggregation, a synthetic peptide-GAG mimicking the collagen-binding and aggregation-modulating activity of decorin was designed.⁴⁸ This synthetic decorin delayed collagen fibrillogenesis, inhibited lateral aggregation of collagen fibrils, and significantly increased gel stiffness. Although initially applied to modulate the assembly of full-length collagen, similar strategies could be applied to self-assembling short peptides. Viscoelasticity of hydrogels can also be modulated by ion complexation, as has been achieved by stiffening MAX1 networks through borate complexation⁹ or by stiffening multidomain peptides with phosphate or magnesium ions.¹⁰

Covalent cross-linking has been one of the most effective ways of stiffening self-assemblies. With native chemical ligation or disulfide bond formation, the storage modulus of self-assembled peptide hydrogels can be significantly enhanced without disrupting the gels' fibrillar structure.^{8, 10} In previous work, it was not conclusively determined the extent to which such covalent capture strategies operated within fibrils (intrafibril cross-linking) or between discrete fibrils (inter-fibril crosslinking), as it is challenging to determine the relative contributions of both types of covalent bond formation. However, both would be expected to increase the stiffness of non-covalent self-assemblies. Covalent cross-linking or polymerization, if light-initiated, can also be employed to produce microfabricated self-assemblies, as shown by recent work with patterned diacetylene-containing peptide amphiphiles (Figure 3e-f).²³ As more specific details are uncovered regarding native ECM patterns *in vivo* and *in vitro* (Figure 3a-d), such approaches may be useful for reproducing them within synthetic self-assemblies.

Matrices based on β -helical folding—Alpha-helical coiled coils are found in a wide range of proteins, including ECM proteins such as fibrinogen and laminins, suggesting their natural utility in constructing fibrillar networks. Owing to their relative simplicity compared to other protein folds, coiled coils have received significant attention for the design of nanostructured materials, and recent advancements have been made in the ability to link them into extended structures, thus enabling their use as building elements for synthetic ECMs. The primary structure of coiled coils is characterized by the heptad repeat, denoted $(abcdefg)_n$, where *a* and *d* positions tend to be occupied by non-polar residues. These residues are aligned along a hydrophobic stripe that arises from the 3.6-residue pitch of the helix, and burial of the hydrophobic stripe drives the assembly of multiple helices. Packing of the *a* and *d* residues within the hydrophobic core can influence the coiled coil's stability, as well as its multimerization state (e.g. dimer, trimer, tetramer, etc.). In addition to these hydrophobic interactions, residues in the *e* and *g* positions also contribute to coiled coil stability and topology by providing charge-charge interactions between adjacent helices. Residues in the *b*, *c*, and *f* positions are solvent-exposed and tend to be either polar or charged. Elucidated design rules for directing coiled coil materials assembly also include several additional levels of strategies that can be combined to dictate oligomerization behavior, including specifying the packing of hydrophobic residues, controlling the number of heptad repeats, placing buried polar residues in the hydrophobic core, providing for “sticky-ended” coiled coil bundles, and managing electrostatic interactions between helices

via specification of **b**, **c**, and **f** residues. A detailed account of these strategies is beyond the scope of this tutorial review, but can be found in several other recent reviews.⁴⁹⁻⁵¹

For constructing gels and ECMs based on helical structures, approaches can be categorized into several styles, including expressed proteins that oligomerize via coiled coils, peptide-polymer conjugates that assemble through coiled coils, and short peptides that form long, rope-like coiled coil fibers. In high enough concentrations, all of these systems form gels (Figure 5). Foundational strategies that established coiled coils as useful building blocks for gels or synthetic ECMs included proteins designed by the Tirrell group and peptide-polymers designed by the Kopeček group. Tirrell's proteins contained terminal helical peptide sequences flanking a central hydrophilic and disordered sequence, and oligomerization of the helices into coiled coils produced a network hydrogel.⁵² Kopeček's materials employed hydrophilic polymers with helical peptides attached as side chains to form 'hybrid' materials.⁵³ Oligomerization of the hybrid materials' helical peptides into coiled coils induced gelation, and physical properties such as temperature responsiveness, swelling behavior, and viscoelasticity could be tuned based on peptide design and the number of peptide grafts on each polymer chain (Reviewed recently by Kopeček).⁵⁴ Since their introduction, both protein-based and hybrid coiled coil biomaterials have been progressively improved.⁵³ Recent advances have included strategies for minimizing intramolecular association or the formation of short loops, neither of which contribute meaningfully to the gels' mechanical properties.⁵⁴ In addition, the establishment of covalent inter-helix bonds has been used to prevent dynamic rearrangements that could lead to dissolution.⁵⁵ These strategies have enabled the formation of stable coatings recently, which may enable the translation of these materials into the biomedical device arena.⁵⁵ Also, gels employing coiled coil peptides presenting the solvent-exposed **b**, **c**, and **f** residues of native human ECM proteins have been developed in order to mimic epitopes expected to be found in normal tissues.⁵⁶ Modular versions of self-assembling coiled coil proteins containing RGD motifs have been designed,⁵⁷ and the production of additional variants may provide an excellent platform for producing multi-component matrices, analogous to the strategies discussed above for β -sheet fibrillizing systems.

Although most gel-forming materials based on the coiled coil have included disordered or polymeric segments for connecting coiled coils into extended networks, gels have also been fabricated from peptides that are formed *entirely* from coiled coils. These gel-forming helical peptides have primarily come about through the progressive refinement of 'sticky-ended' coiled coil peptide assemblies (Figure 5b-e, reviewed recently by Woolfson),⁵¹ though it has also been reported recently that coiled coils with aligned, or 'blunt' ends are also capable of forming gels.⁵⁸ In the sticky-ended approach, electrostatic interactions between **e** and **g** residues, buried polar residues in **a** positions, and charged N- and C-termini were combined to produce coiled coil dimers with single-helix overhangs, which oligomerized axially to form long fibers (Figure 5b).⁵⁹ A critical step that has allowed hydrogelation of these peptides was recently achieved by limiting the lateral association or coarsening of the formed fibers through the minimization of inter-fiber electrostatic interactions.⁶⁰ Rat pheochromocytoma (PC12) cells were cultured in this matrix, establishing its applicability for cell culture. Now that a strategy for forming hydrogels has been developed based on coiled coil peptides, the time may be right for decorating these or similar systems with some of the functional components previously investigated within β -sheet-rich assemblies. Such strategies for functionalization may build upon previously established techniques for installing branches, kinks, or crosses within coiled coil fibers.⁵¹ Further, employing coiled coils may provide some advantages in comparison with β -sheet fibrillizing materials, in that coiled coils' oligomerization may be more precisely controlled.

Future challenges and trends

Self-assembling biomaterials from proteins—Despite the advantages of using short peptides for self-assembled ECMs, peptide synthesis tends to rely on techniques with relatively small batch sizes. Peptides are also limited to short amino acid sequences. Although large-scale production of synthetic peptides is possible, and processes such as chemical ligation are available for synthesizing longer polypeptides or proteins, self-assembling biomaterials may also benefit from being expressed recombinantly. Expressed self-assembling systems have mostly been accomplished for materials that oligomerize via coiled coil formation,^{52, 55, 57} but recently there have been successes for β -sheet peptide expression as well. For example, self-assembling peptides were recently expressed in the form of several tandem repeats, which after expression were cleaved into short individual fibrillizing peptides.^{24, 61} In this study, the self-assembling recombinant peptides behaved similarly to their synthetic counterparts and were able to form pH-responsive hydrogels. Continuing advances in the production of these and similar materials may significantly enable the cost-effective scale-up of self-assembling peptide technologies.

The more complex limitation for self-assembled materials based on short peptides is that they do not present well-folded proteins to cells. Given the importance of domain conformation and co-presentation of multiple cell binding sites on cell behavior (Figure 2), necessary levels of integrin specificity may not be achievable unless self-assembled matrices present well-folded proteins. As the field progresses, strategies for attaching expressed proteins to peptide matrices post-assembly may prove useful. Most early work for conjugating proteins to surfaces has been conducted on hard, two-dimensional surfaces, which would need to be translated to soft, self-assembled networks. In order to achieve precisely oriented presentation of proteins on surfaces, protein tags with increasingly refined specificity have been developed, from the well known glutathione S-transferase and oligohistidine tags to more specific strategies such as immobilization via cutinase capture⁶² or coiled coil oligomerization.⁶³ Further, recombinant strategies may bypass the need for attaching domains onto fibrillized matrices altogether, instead producing proteins that assemble into a matrix themselves.⁶⁴ Expressed proteins described by Heilshorn and coworkers that self-assemble into gels via specific binding between WW domains and proline-rich peptides are examples of recent progress in this area.⁶⁵

Immunological considerations—The multivalent and highly oligomerized protein arrays of the native ECM are inert to the immune system, notwithstanding certain autoimmune disorders. At the same time, aggregation or oligomerization of exogenous proteins, even those with completely human amino acid sequences, can lead to immunogenicity and can complicate the development of protein therapeutics.⁶⁶ Given this disparity, along with the fact that immunological considerations are a centrally important factor in the clinical development of self-assembled biomaterials, the immunogenicity of self-assembled peptide biomaterials based on β -sheet fibrillization was investigated recently.⁴¹ Previously, a few studies had found minimal or immeasurable inflammatory or immune responses to fibrillized peptides or peptide-amphiphiles,^{33, 67} but they had investigated either fibrillizing peptides without ligands or peptides containing ligands with native peptide sequences (e.g. RGD), leaving the possibility that strong epitopes had simply been avoided over the course of these materials' development. To determine if this previously observed minimal immunogenicity of self-assembled peptide fibrils was a general property, Q11 derivatives with N-terminal epitope sequences from the protein ovalbumin (OVA) containing known T cell and B cell determinants (Figure 6) were investigated. The self-assembling OVA epitope peptide (O-Q11) formed fibrils (Figure 6c) and was surprisingly immunogenic in mice (Figure 6b). IgG responses to fibrillized O-Q11 were as high as those elicited by the soluble OVA peptide delivered in complete Freund's

adjuvant (CFA), one of the strongest known adjuvants.⁴¹ These strong antibody responses were abolished when the OVA and Q11 sequences were not contiguous (Figure 6b), and no antibodies were detected against unmodified Q11, even when it was delivered in CFA. What do these findings mean for the development of self-assembling biomaterials? Although only a few immunological studies have been conducted to date, one picture that may be emerging is that the presence or lack of a relevant immune epitope on the surface of the fibrils can render a self-assembling peptide biomaterial either extremely immunogenic, and therefore useful in immunotherapies, or entirely non-immunogenic, and therefore useful as scaffolds for regenerative medicine. If this holds true for additional epitopes, additional fibrillizing systems, and additional species beyond the mouse, self-assembling peptide biomaterials may be able to move beyond their traditional area of regenerative medicine and into other areas such as immunotherapies or bioreagent production in animals. Self-assemblies of proteins may also prove to be useful in this regard, especially for more complex or conformation-dependent epitopes. For regenerative medicine applications, it is encouraging that the Q11 peptide by itself was not immunogenic, even when delivered in adjuvant or with the soluble OVA peptide. This may indicate that Q11 or other fibrillizing peptide biomaterials could be injected with other proteins and still retain their non-immunogenic character. Nevertheless, the mechanism of the immune response to self-assembled biomaterials would benefit from further investigation.

Concluding remarks

ECMs are robust, overspecified systems. They have evolved to be robust in order to carry out their essential roles. By regulating viscoelasticity, providing for cell attachment, regulating growth factor activity and availability, and being remodeled precisely, ECMs are capable of directing complex biological processes. During these processes, many factors (physical, chemical, biological) act in parallel to drive cell behaviors. The key for designing synthetic ECMs is to understand the dominant factors in these relationships so that clinically meaningful biological processes such as regeneration can be effected through specification of a limited set of manageable parameters. Cell attachment peptides have been shown to be among these important parameters; however, in isolation, short peptides such as RGD lack an ability to provide for specificity in integrin engagement or to participate synergistically with other processes such as growth factor activation or mechanotransduction. Increased understanding of these interrelated processes and simple strategies for translating them into synthetic matrices will likely be powerful contributions to the fields of regenerative medicine and 3D cell culture in the coming years. Given the expanding ability of self-assembled materials for incorporating multiple different functional components into defined matrices, it appears that they will serve a useful role in such developments.

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Figure 1.

The stiffness of native ECMs is highly variable, in part owing to the ECM's modular construction. Collagens, which significantly determine the mechanical properties of a tissue, are constructed in a modular, hierarchical fashion (a). The axial and lateral oligomerization of the fibril-forming collagens (blue) is modulated via co-assembly with fibril-associated collagens (orange) and proteoglycans such as decorin (green) and fibromodulin (red). Elastic moduli of native soft tissues range from tens of Pa in the softest tissues to the MPa range for the stiffest (b). Note that stiffnesses are reported as ranges owing to the significant dependence of stiffness on species, tissue site, strain rate, testing method, and whether Young's modulus (E) or storage modulus (G') is measured. Values in the chart are reported in Levental et al., and references therein.³ Covalent processing can occur within assembled ECMs to stiffen them further, for example by transglutaminases and lysyl oxidase (c).

Figure 2.

Cell behaviors arising from interactions with the ECM, including those dependent on integrin binding or growth factor signaling, are regulated by multiple interrelated chemical, physical, and biological factors. As an example of an interaction between cell binding and mechanics, cell binding to fibronectin is dependent on the spatial positioning of the RGD and PHSRN loops in the 9th and 10th FNIII domains, and modeling studies have suggested that forces in the tens of pN are sufficient to modulate integrin specificity by altering the distance between these two loops (a), adapted with permission from Krammer et al., *Matrix Biol.*, 2002, **21**, 139-147.¹⁴ As an example of an interaction between cell binding, mechanics, and growth factor signaling, TGF- β is secreted as an inactive complex and bound to the ECM (b); force applied across the ECM, originating from cytoskeletal contractility, is sufficient to activate this bound TGF- β (c).¹⁶

Figure 3.

In 2D culture, cells deposit diverse ECM patterns, depending in part on the cells' motile behavior. Non-migrating keratinocytes produced the laminin $\alpha 3$ subunit in rosette patterns (a), while alveolar epithelial cells produced the same subunit as fibrillar arrays (b). Immortalized endothelial cells deposited the $\alpha 4$ laminin subunit (c, green) in focal adhesion-like structures, which co-localized with an antibody against αv integrin (d). Scale bars in (a-d) are 20 μm ; reproduced with permission from Hamill et al., *J. Cell Sci.*, 2009, **122**, 4409-4417.²² Using self-assembled biomaterials, similar control over patterning has been achieved using photopolymerizable diacetylene-containing peptide amphiphiles (e, diacetylene indicated), shown here dictating the patterning of human mesenchymal stem cells (f, blue=nuclei; red=actin). Reproduced with permission from Mata et al., *Soft Matter*, 2009, **5**, 1228-1236.²³

Figure 4.

Self-assembling biomaterials based on β -sheet fibrillizing peptides. Co-assembling peptides may be mixed to form multi-peptide matrices (a). Self-supporting gels are formed when aqueous solutions of β -sheet fibrillizing peptides are incubated under a layer of neutrally buffered saline (b, 30 mM Q11 in water; c, same sample after overnight incubation under a layer of PBS). d-f: TEM images of ligand-bearing fibrils with their respective ligands labeled by gold nanoparticles. Recently reported systems include those based on Q11 (d, mixed fibrils of 10% biotinylated RGD-bearing Q11 and 90% Q11, reproduced with permission from Jung et al., *Biomaterials*, 2009, **30**, 2400-10);³³ transthyretin-derived peptide TTR1 (e, mixed fibrils of 1% dansylated RGD-bearing TTR1 and 99% TTR1, reproduced with permission from Gras et al., *Biomaterials*, 2008, **29**, 1553-1562);⁴³ and peptide-amphiphiles displaying a heparin-binding peptide (f), reproduced with permission from Rajangam et al., *Nano Lett.*, 2006, **6**, 2086-2090.¹⁷ Co-assembled peptide-amphiphile fibrils retain their biological activity *in vivo*, as shown by enhanced bioluminescence of

fluorescent bone marrow mononuclear cells delivered in a binary mixture of 10% RGD-bearing/90% unfunctionalized peptide amphiphile (g), compared with 100% unfunctionalized peptide amphiphile (h) or saline control (i). Reproduced with permission from Webber et al., *Acta Biomater.*, 2010, **6**, 3-11.³⁰

Figure 5.

Coiled coil formation drives the gelation of hybrid peptide-polymer-peptide triblock molecules (a, reproduced with permission from Jing et al., *Biomacromolecules*, 2008, **9**, 2438-2446).⁵⁶ Peptides that form fibrils via sticky-ended assembly have been utilized to form gels (b, reproduced with permission from Papapostolou et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2007, 104, 10853-8).⁵⁹ Portions of the peptides with positively charged residues in *e* and *g* positions of the $(abcdefg)_n$ heptad (blue) align with negatively charged *e* and *g* residues on a complementary peptide (red), a registration that is reinforced with a pair of buried asparagine residues (stars). Fiber thickening is driven by additional charges on the solvent-exposed residues (black plusses and minuses on the peptides' surfaces). To form 3D ECMs, peptides having extensive charge-charge interactions in the *b* and *c* positions that were prone to fiber thickening (c) were re-designed to have weaker, more general interactions in these positions (d), producing smaller, more flexible fibers that entangled to form hydrogels (e, shown encapsulating rat PC12 cells; c-e are reproduced with permission from Banwell et al., *Nat Mater*, 2009, **8**, 596-600).⁶⁰

Figure 6.

Self-assembling peptides can be well tolerated immunologically or highly immunogenic, depending on the epitope peptide attached to them. The peptide Q11 is non-immunogenic by itself or when conjugated to RGD peptides.³³ However, when it is placed in tandem with a peptide from the protein ovalbumin (OVA) containing known T and B cell epitopes (O-Q11, a), it elicits the production of high titers of specific antibodies in mice without the co-administration of any adjuvant (b). Total IgG titers determined by ELISA; groups correspond to peptides shown in (a) delivered to mice either in PBS or in complete Freund's adjuvant (CFA), as indicated. The OVA+Q11 group corresponds to mice that received a mixture of soluble OVA peptide and unmodified Q11 in PBS. O-Q11 formed uniform fibrils by TEM (c) that appeared morphologically similar to those of Q11 (d). Adapted with permission from Rudra et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2010, 107, 622-627.⁴¹