25th Anniversary Article: Supramolecular Materials for Regenerative Medicine

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In supramolecular materials, molecular building blocks are designed to interact with one another via non-covalent interactions in order to create function. This offers the opportunity to create structures similar to those found in living systems that combine order and dynamics through the reversibility of intermolecular bonds. For regenerative medicine there is a great need to develop materials that signal cells effectively, deliver or bind bioactive agents in vivo at controlled rates, have highly tunable mechanical properties, but at the same time, can biodegrade safely and rapidly after fulfilling their function. These requirements make supramolecular materials a great platform to develop regenerative therapies. This review illustrates the emerging science of these materials and their use in a number of applications for regenerative medicine.

1. Introduction

The objective of regenerative medicine is to create therapies for the repair or replacement of tissues and organs in order to restore impaired function resulting from congenital defects, disease, trauma or aging.[1] Regenerative medicine is highly interdisciplinary and integrates fields of research such as cell and developmental biology, chemistry, and materials science. The materials side of regenerative medicine is focused on designing systems with features that mimic the natural environment of the cells. There has been rapid progress in the field of supramolecular materials over the past 25 years and their unique combination of dynamics and order makes them a great target for regenerative medicine. Supramolecular materials are structures in which the molecular building blocks are designed to interact via non-covalent interactions. These non-covalent interactions can introduce order in these materials but also provide them with interesting dynamic behavior through the reversibility of bonds. This review discusses developments in supramolecular materials and how they can impact regenerative medicine.

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1.1. 25 Years of Supramolecular Materials

In 1987, two years before the first issue of *Advanced Materials*, the field of supramolecular chemistry was recognized with a Nobel Prize in Chemistry to Lehn, Pederson and Cram. Their pioneering work used non-covalent interactions between molecules, such as hydrogen bonding and metal-organic ligand interactions, to create discrete molecular complexes. In the decades that followed, others expanded this concept to larger length scales, thereby creating nanoscale supramolecular architectures[2] and further organization of these nanostructures at macroscopic scales giving rise to functional supramolecular materials.[3] Typical molecular building blocks include amphiphiles,[4] mesogens,[5] conjugated molecules,[6] rod coils (molecules with flexible and rigid parts)[7] and gelators.[8] These building blocks can lead to a vast variety of supramolecular materials such as smart vesicles and other functional nanostructures, responsive gels, self-healing polymers, materials combining strength and liquid crystallinity, and coatings with responsive properties, among others.[9] Over the lifetime of *Advanced Materials*, the field of supramolecular materials has grown dramatically, evolving from serendipity to rational design, as a result of advances in molecular synthesis and a better understanding of intermolecular forces. This progress in tuning the properties of supramolecular materials has been demonstrated by a variety of applications,[10] such as drug delivery vehicles for healthcare,[11,12] liquid crystalline films for displays,[5] organic wires for devices,[13,14] and molecular organic frameworks[15] with a high level of porosity for fuel storage[16] or catalysis.[17] One important area of supramolecular materials is that of functional supramolecular polymers. In supramolecular polymers, the fundamental polymer motif of connecting monomers via covalent bonds is imitated using non-covalent bonds.[6] However, in supramolecular polymers the "monomers" can be designed to have widely varying sizes or possess the functions desired in the material.

The notion of designing structure and function through non-covalent interactions lends itself to the creation of different types of order at varying scales within a single material.[18,19] This approach can generate the hierarchical structures observed in many biological materials like bone, wood and silk.[20] This analogy with natural structures makes hierarchical assemblies attractive as mimics of biological structures for use in materials for healthcare applications. Over the past three

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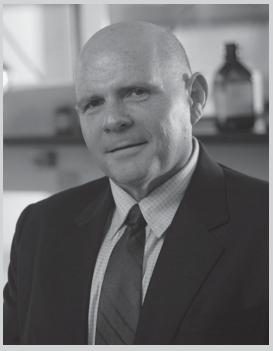
years, hierarchical biomaterials have been reported that could have important functions in regenerative medicine. Some of these structures are formed by the nanoscale assembly of small molecules into nanofibers on a size scale similar to collagen fibers. In one example, these nanofibers form membranes with perpendicularly oriented zones at larger scales when complexed oppositely charged polymers.[21] The resulting architecture bears resemblance to the structure of cartilage extracellular matrix.[22] In two other examples, the nanoscale fibers form liquid crystals on the micron scale[23] or hydrogels with macroscopic alignment.[24]

There are many challenges in bringing the field of supramolecular materials to a level where it can be used in regenerative medicine. Taking advantage of bond reversibility and the opportunity to build highly organized mesostructures, one could consider strategies to create materials that are adaptive, dynamic, or even replicative for self-repair. For these highly advanced properties, one of the strategies to consider dissipative (or dynamic) self-assembly of molecules. Biological dissipative supramolecular architectures are observed ubiquitously,[25] but examples of man-made dissipative supramolecular structure remain limited.[26–28] Dissipative structures are intrinsically unstable and can only be maintained under a constant influx of energy, such as ATP in the case of microtubule self-assembly.[29] Because the microtubules are constantly broken down and rebuilt, they are dynamic, self-healing and adaptive in nature.[30] These dynamics are crucial in biological systems, since they allow rapid remodeling of cell components, cells or entire tissues. Such features in living organisms will inspire researchers to design dynamic or responsive materials for regenerative medicine.

The ideal materials for regenerative medicine will have to be highly dynamic and responsive. Also, it should have physical properties and contain chemical structures programmed to change over time or upon stimulation by cues in the environment. The section below describes some of the known mechanisms of regeneration in biological systems (**Figure 1**a). These mechanisms, ranging from mammalian wound healing to the complex full regeneration of an amphibian limb after amputation[31,32] provide inspiration for materials design.



Job Boekhoven received his MSc in Chemistry in 2008 from the University of Groningen in the Netherlands. In 2012 he got his PhD in Chemistry from Delft University of Technology also in the Netherlands. During his PhD studies in the group of prof. Jan van Esch, he explored the use of dissipative self-assembly and multicomponent self-assembly as a tool to create more complex materials. He pursued his academic career as a Rubicon postdoctoral fellow in the group of prof. Samuel Stupp at Northwestern University. His current research focuses on the use of dynamic materials with properties controlled over space and time and their use in regenerative medicine.



Samuel I. Stupp received his BS in Chemistry in 1972 from the University of California at Los Angeles and his PhD in Materials Science in 1977 from Northwestern University. He remained at Northwestern as a faculty member until 1980 before moving to the University of Illinois at Urbana-Champaign. In 1999 he returned to Northwestern University as a Board of Trustees Professor of Chemistry, Materials Science, and Medicine. He is also the Director of the Institute for BioNanotechnology in Medicine at Northwestern. His research is focused on self-assembly, supramolecular chemistry, and the development of functional materials for medicine and energy.

1.2. Regeneration in Biology

The axolotl, meaning "water monster" in the Mexican language Nahuatl, is a salamander that is capable of regenerating entire lost appendages, jaws, and even the spinal cord without any evidence of scarring or loss of function.[31] In the first hours after amputation, blood clotting prevents the further loss of blood and the wound is covered with epithelial cells. These cells, as well as the damaged vascular walls and nerve terminations, start to secrete molecular signals that recruit extracellular matrix synthesizing cells (e.g., fibroblasts) and immune cells that degrade the old extracellular matrix and cell debris. These processes clean up the wound and create a hospitable environment for new cells with highly proliferative capacity that characterizes the blastema. A recent study has shown that these cells are a heterogeneous collection of restricted progenitor cells that have dedifferentiated from the surrounding tissue.[33] Next, the blastema cells start to proliferate (i.e., multiply) to a sufficient number for the regeneration of the limb. Eventually, the new cells start reorganizing and differentiating to form the new limb. Remarkably, if during this phase the limb blastema is transplanted to another permissive part of the amphibian, such as the eye, a limb can grow from the eye socket.[34] This observation clearly demonstrates that limb regeneration is regulated autonomously by the blastema. The regenerative process from the blastema is only poorly understood, but a recent study found that the highly dynamic extracellular matrix in the blastema is extensively remodeled resulting in a scaffold with a chemical composition that is tightly controlled over time and space.[35] This tight spatiotemporal control over the extracellular matrix and molecular cues instructs cells to perform functions for the successful regeneration of the new limb, such as adhesion or migration and proliferation or differentiation.

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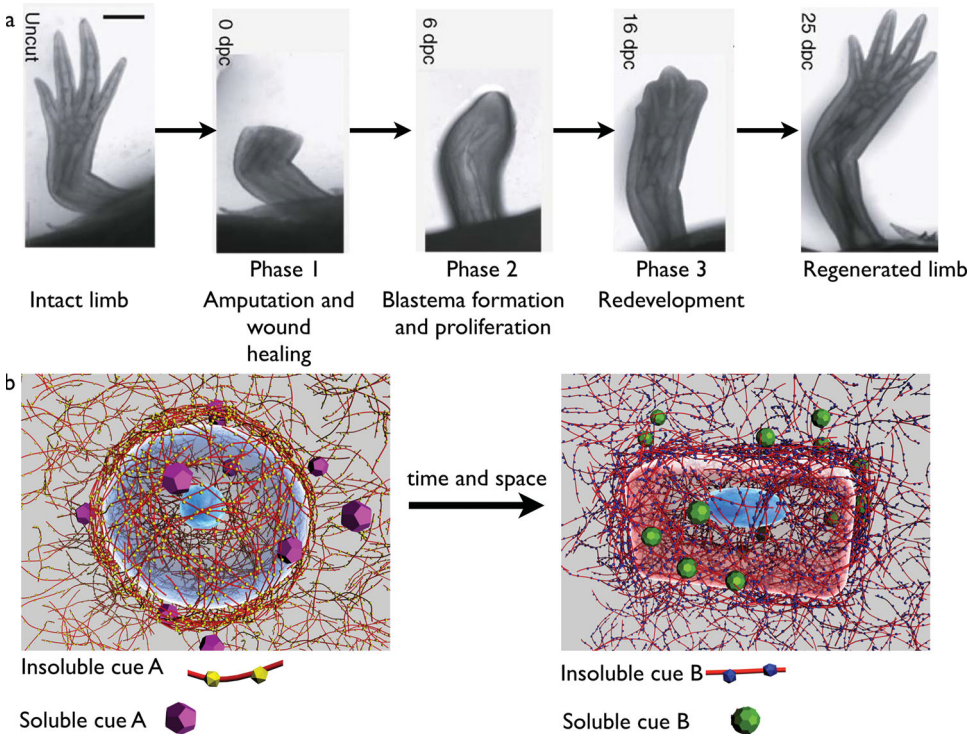

Figure 1. (a) Regeneration of an axolotl limb follows three phases: (1) wound healing, (2) blastema formation and (3) redevelopment. During each of these phases, cells are instructed to perform functions as adhesion, proliferation and differentiation. dpc = days post cut, scale bar = 1 mm, Figure reproduced with permission.[41] Copyright 2010, Elsevier. (b) The regeneration of amphibian limbs can serve as inspiration to design supramolecular materials for regenerative medicine that should be able to similarly instruct cells to adhere, proliferate and differentiate in a dynamic fashion and controlled over space. As is depicted in the scheme, these instructions can come from the surrounding matrix or can be soluble.

Although the regeneration of amphibian limbs might seem extraordinary and not applicable to human regenerative therapies, the signaling pathways that lead to tissue regeneration are well conserved across species[36] and it can serve as an inspiration for regenerative therapies, especially from a materials point of view. In the original tissue engineering strategy,[37] a biodegradable polymer matrix was seeded with cells from a patient and implanted after a certain period in a bioreactor. In this pioneering approach, proposed by Langer and Vacanti, the role of the matrix was to localize the cells and to support them mechanically. In more recent approaches to regenerative medicine, the concept of bioactive materials was introduced by incorporating peptide signals that could interact with receptors or by adding growth factors. These materials could, in principle, activate signaling pathways important in regenerative processes without necessarily using cells. This was first done in polymers[38,39] and the authors' laboratory pioneered the use of this approach in supramolecular self-assembling materials.[40] Recently, the use of hierarchically organized supramolecular materials has been introduced to instruct cells on several length scales. Studies of tissue regeneration and tissue development clearly demonstrate the importance of using matrices that can display signaling molecules, such as growth factors or matrix-bound cues. The challenge, inspired by the amphibian biology described above will be to develop materials that can instruct endogenous or exogenous cells to migrate, dedifferentiate, proliferate, differentiate, and organize with spatiotemporal precision. This will require matrices in which bonds are dynamic to constantly change properties and chemistry to orchestrate regeneration (Figure 1b).

In this review, we discuss some of the essential contributions made so far to create supramolecular materials for regenerative medicine. We will first discuss key work to functionalize self-assembled materials to create niches that can instruct cells either using insoluble or soluble cues. Thereafter we will discuss materials that can instruct cells in a multistep fashion that is with some degree of control over when a bioactive cue is displayed. Finally, we will close with supramolecular self-assembled materials that are ordered on a higher length scale, or hierarchical assemblies, and their use in regenerative medicine.

2. Cell Signaling by Supramolecular Materials

Cells in their natural environment are constantly signaled by surrounding factors to adhere, migrate, proliferate or differentiate. These signaling factors can be divided into two classes: soluble factors, such as growth factors or small molecules, and signals that involve interactions with the extracellular matrix (ECM) or other cells. Instructing cells is a crucial aspect for the field of regenerative medicine and supramolecular materials have been used to mimic natural signaling factors. In this section, we will discuss examples where supramolecular materials

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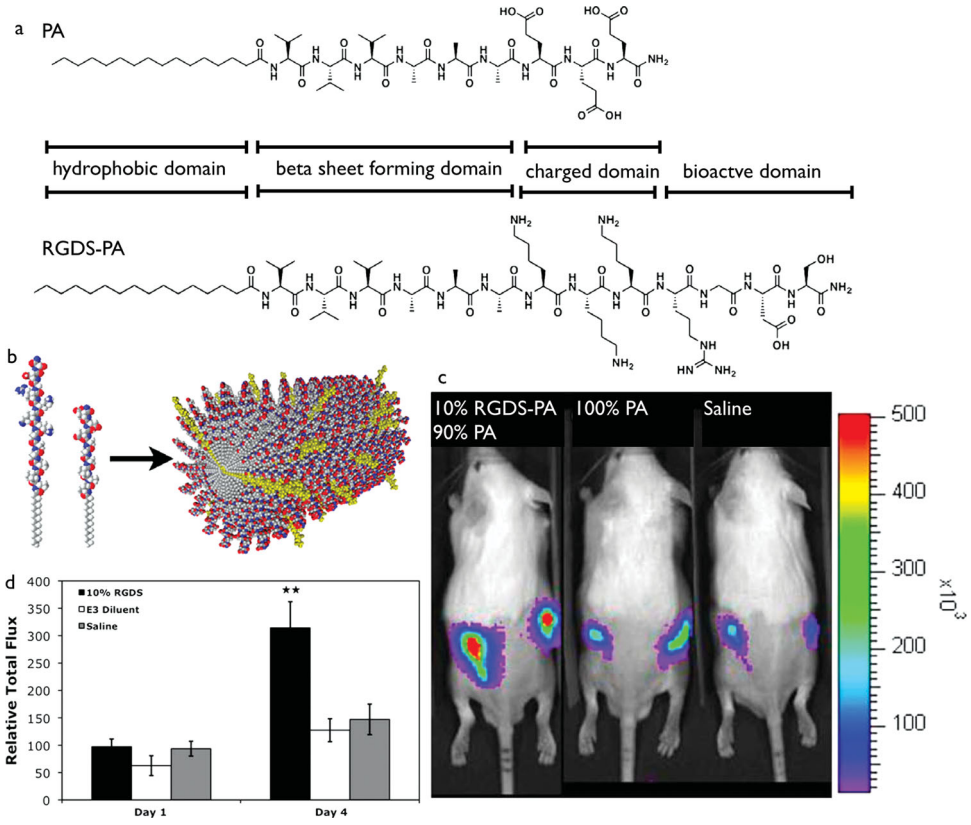

Figure 2. a) The molecular structures of a typical PA and RGDS-PA that consist of 4 domains: a hydrophobic domain, a beta sheet-forming domain, a charged domain and a bioactive domain. b) PA and RGDS-PA coassemble to form fibers expressing the RGDS bioactive cue. c) Bioluminescent imaging of transplanted BMNCs expressing luciferase encapsulated in gels of PA. d) Quantification of the images shown in (c) reveals an increase in bioluminescence after four days for cells encapsulated in gels of 10% RGDS-PA as a result of an increase in cell number. Reproduced with permission.[60] Copyright 2010, Elsevier.

have been designed to instruct cells, either to mimic insoluble ECM cues or to mimic soluble cues such as growth factors.

2.1. Cell Signaling by Insoluble Cues

Cells in their microenvironment are surrounded by an ECM, which is a supramolecular network comprising a complex mixture of fibrous proteins and polysaccharides that provides structural support, offers anchoring points for attachment and expresses signaling cues. The cell's transmembrane proteins recognize specific molecular sequences on the ECM resulting in attachment as well as other processes like proliferation or even differentiation. For instance, the RGD peptide sequence is part of the extracellular glycoprotein fibronectin and can be recognized by cells through their transmembrane integrin proteins. Integrin binding to the RGD sequence initiates an intracellular cascade resulting in the formation of focal adhesions (cell-matrix adhesion points) allowing cells to attach and exert force on the surrounding matrix. Researchers have identified numerous other binding sites on ECM proteins that are responsible for such signaling and attached those to polymeric or supramolecular constructs to mimic the signaling role of the ECM. Here, we will list several key studies that used such supramolecular materials.

The previously mentioned RGD is probably the most commonly used peptide sequence to render supramolecular materials bioactive. For instance, the bioactive RGD or RGDS has been successfully attached to supramolecular materials ranging from self-assembled monolayers,[42–46] small molecule hydrogelators,[47–50] vesicle-forming block copolymers[51,52] and supramolecular polymers.[53] In one example, the RGDS has been utilized to functionalize peptide amphiphile (PA) fibers, which are 1D self-assembled nanostructures structures reminiscent of the ECM.[54,55] **Figure 2**a depicts the molecular structure of a representative structure of a PA, which typically consists of four structural domains resulting in fiber formation.[40,56,57] The terminal domain is used to introduce bioactive sequences such as RGDS. This design has been shown to successfully induce integrin mediated adhesion, spreading or migration of fibroblasts,[58] breast cancer cells,[59] bone marrow mononuclear cells (BMNCs)[60] and rat-derived mesenchymal stem cells (rMSCs)[61] *in vitro*. Not only can RGDS facilitate the adhesion of rMSCs, but it can also induce osteogenesis (differentiation in bone forming cells).[62,63] Such studies demonstrate that supramolecular materials can direct stem cells to a specific lineage *in vitro* and are crucial for the development of materials for regenerative medicine. The RGDS sequence on a PA can also significantly aid in the survival of BMNCs when subcutaneously injected in mice (Figure 2c and d).[60] In another in vivo study, it

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was shown that the RGDS-PA can induce biological formation of tooth enamel,[64,65] a tissue that does not regenerate naturally.

Another system displaying the bioactive RGD was designed by Zhou and coworkers, who described the assembly of fluorenylmethoxy-carbonyl-diphenylalanine (fmoc-FF) coassembled with fmoc-RGD.[66] These peptides, with only two or three amino acids, assemble into 3 nm fibers that subsequently bundle into tapes to form self-supporting hydrogels. Despite this minimalist approach, the resulting structures exhibit bioactivity, as shown by the integrin-mediated adhesion of fibroblasts. Cells in gels comprising fibers with 30% fmoc-RGD showed a significant increase in spreading compared to cells in gels with 30% fmoc-RGE. Moreover, gels that allowed adhesion and spreading of the cells showed an increase in gel contraction as compared to a control that was attributed to the force cells exert on their ECM via their transmembrane integrins.

Besides RGDS, other insoluble cues have been used to instruct cells, including the bioactive peptide sequence IKVAV. This sequence is derived from laminin and is known to promote cell adhesion and induce neurite outgrowth of neural progenitor cells (NPC).[67] IKVAV has been attached to peptide amphiphiles,[68] and these IKVAV-displaying PAs assembled to form hydrogels that are bioactive *in vitro*, promoting neural cell differentiation of NPCs while disfavoring differentiation into astrocytes (**Figure 3**a).[69–71] This selective differentiation is crucial for regeneration of the central nervous system, since neurons are responsible for processing and transmitting signals, whereas inhibiting astrocyte differentiation is believed to be important for the prevention of scar formation.[72] The selective differentiation induced by IKVAV-displaying PAs served as a motivation to use the bioactive gels in vivo for regenerative therapies in a mouse model of spinal cord injury. Treatment with the IKVAV displaying PA after spinal cord injuries reduced astrogliosis (scar formation by astrocytes), reduced cell death, and increased the number of oligodendroglia (a type of brain cells that provide support and insulate the axons of neurons) at the site of injury.[69] Moreover, the treatment promoted regeneration of descending motor fibers and ascending sensory fibers, thereby significantly improving motor function of the mice (Figure 3c, d and e).

Another peptide sequence of interest in the field of regenerative medicine is DGEA which is derived from collagen type-1, the most abundant type of collagen in our bodies mainly found in tendon, skin, ligament and bone.[73] This specific peptide sequence has been found to bind the α2-β1 transmembrane integrin and induce differentiation of bone-marrow stromal cells (BMSCs) into bone-producing osteoblasts.[74] However, the number of studies involving conjugations between supramolecular materials and DGEA remain limited. One interesting example used drop-cast films of M13 bacteriophages (rod-like viruses, 880 nm in length) to display DGEA. The phages were engineered to express DGEA peptides on their surface, and the drop-cast films were shown to induce osteogenesis. The advantages of using phages as supramolecular materials include self-replication and high epitope density. In other work, Jun and coworkers combined the DGEA peptide sequence with a self-assembling peptide amphiphile to induce osteogenesis of human mesenchymal stem cells (hMSCs) cultured in both growth and differentiation media.[75]

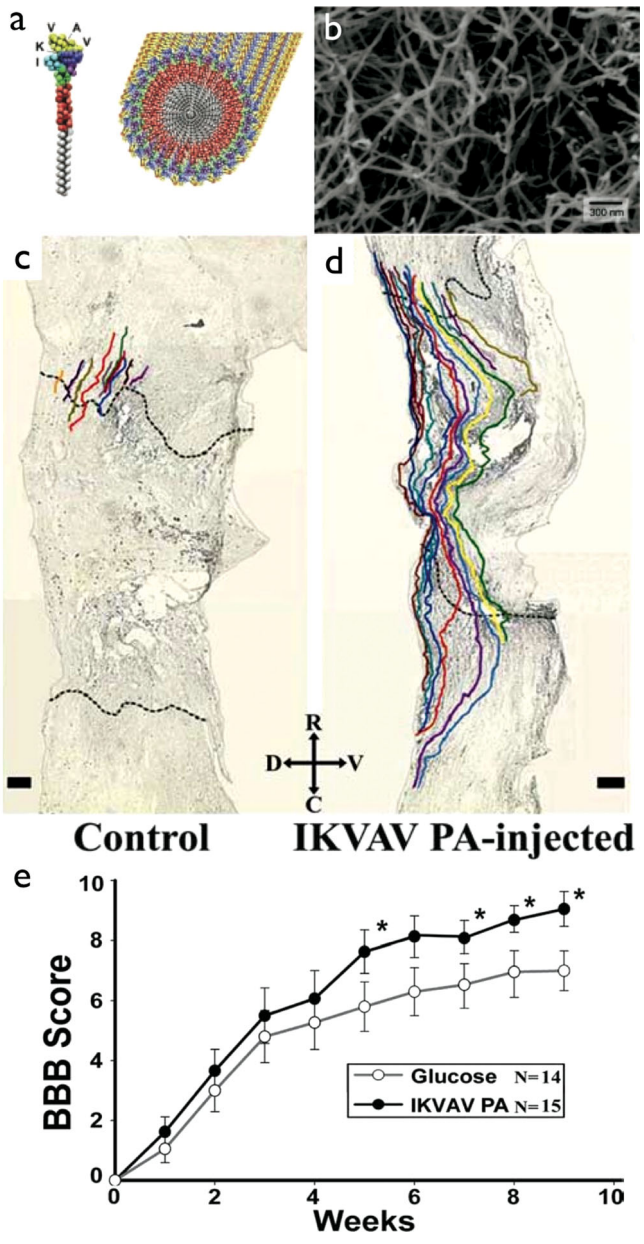

Figure 3. Model of the IKVAV expressing PA and its self-assembly into fibers. (b) Scanning electron microscopy confirms the self-assembly into fibers. (c) Microscopy images of motor fibers of a control group of mice (d) and IKVAV PA injected mice. Dotted line represents the borders of the lesion, scale bar = 100 μm. (e) Basso, Beattie, and Bresnahan (BBB) score as a measure for rat motor function after spinal cord injury. A significant improvement is observed for IKVAV PA injected rats as compared to a control group. Reproduced with permission.[68,70] Copyright 2004, AAAS.

The examples stated above clearly demonstrate that self-assembled supramolecular materials can be rendered bioactive by engrafting them with bioactive sequences derived from ECM proteins. Although this procedure seems straightforward, there are some design considerations to be taken into account regarding the architecture of the supramolecular structure as well as the linkage between the cue and the scaffold. Peptide building blocks are by far the most used components to form

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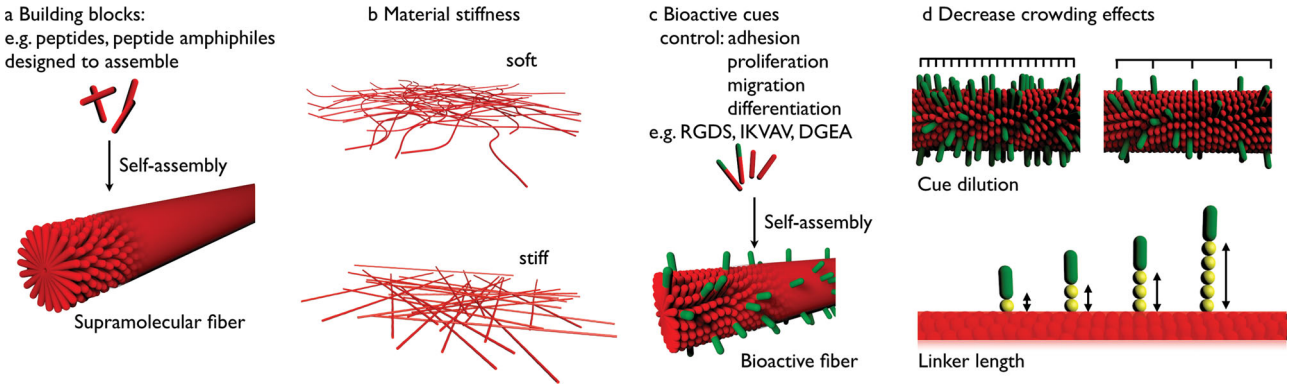

Figure 4. Design elements for supramolecular materials that instruct cells with insoluble cues. a) Supramolecular building blocks are designed to assemble into one-dimensional nanostructures similar to the structural components of the ECM. b) The overall stiffness of the material can influence particular cell responses such as the fate of stem cells. c) Attachment of bioactive cues can instruct cells to adhere, proliferate, migrate or differentiate. d) To avoid crowding by the cues, they can be diluted with non-functionalized (or diluent) building blocks. To avoid crowding by the scaffold, the cues can be attached through a linker.

supramolecular scaffolds that display bioactivity. Provided that an immune response does not occur, the use of natural amino acids in these materials raises the probability of biocompatibility relative to those built with non-natural building blocks. Secondly, the self-assembly of peptides has been sufficiently studied to support the design of materials with specific architectures.[76–78] The design of supramolecular architecture plays an important role in the effective display of bioactive cues, and this was clearly demonstrated for the relatively hydrophobic IKVAV cue attached to fiber-forming PAs. Because of its hydrophobicity, this peripheral cue tends to interdigitate into neighboring fibers resulting in the undesired bundling of the fibers.[71] This bundling decreases the overall surface area of the fibers and with that the availability of the bioactive sequence. By increasing the charge density on the fibers, the bundling of fibers can be reversed, and an increase in bioactivity is observed.

When designing supramolecular scaffolds to display bioactivity, it is essential to consider the nature of the molecular linkage used to attach bioactive cues to materials. For instance, studies have shown that the RGDS cue is displayed more efficiently when separated from its scaffold.[79,80] Lee and coworkers found a tenfold increase in fibroblast spreading for a 20 glycine spacer (roughly ~11 nm long if fully extended) as compared to no spacer at all. Moreover, the authors found that any spacer below four glycine amino acids (~2.2 nm) did not show any significant increase in spreading on the non-adhesive surfaces. It is generally believed that implementing a spacer in the material's design decreases the steric hindrance of the scaffold material to the bulky integrin proteins. This observation does not only hold for RGDS, but similar results have been found for PFSSTKT a motif that stimulates neural progenitor cell adhesion and differentiation.[81] The motif was found to be more efficient in inducing selective differentiation when attached to the $(RADA)_4$ self-assembling peptide via a tetraglycine linker than the peptide attached without a linker.[82]

Steric hindrance by neighboring cues in supramolecular materials can have adverse effects on the bioactivity of cues, as was found by Storrie and coworkers.[59] In this work, RGDS was displayed on the periphery of PA fibers and coassembled with a PA without bioactive cues. Maximum fibroblast attachment was achieved with a mixture of the RGDS PA with 95% of diluent PA. The authors hypothesize that this effect is due to crowding of the RGDS units and saturation of the integrins. Similar effects were observed with bone-marrow mononuclear cells when it is diluted with 90% diluent PA.[60]

Mechanochemical considerations are also important when designing bioactive supramolecular materials. As many bioactive cues are anchoring motifs for cells, they need to able to withstand a certain amount of force exerted on them.[83] A recent study has elegantly shown that the maximum force a single integrin exerts on a RGDS cue during the adhesion process is in the range of 40 piconewton (pN).[84] If the connection between the RGDS cue and the material is disrupted with forces less than this 40 pN, it was shown that cells could not adhere to the scaffold. Equally important, but on a larger length scale is the overall stiffness of a material. *In vitro* studies using polymeric materials have shown that the stiffness of the material drastically affects the differentiation pathways of stem cells.[85] In one example using polyacrylamide gels with different mechanical properties, it was shown that stiff materials resulted in differentiation into bone cells, intermediate stiffness led to muscle cells, while soft scaffolds resulted in fat cells or neurons.[86] Similarly, this effect has also been observed on supramolecular materials with tunable stiffness,[87] illustrating the need to control mechanical properties, as well as, the chemical signals on the materials (**Figure 4**b).

2.2. Supramolecular Materials to Mimic Cell Signaling by Soluble Cues

During tissue regeneration or wound healing, cells are not only instructed by extracellular matrix cues (insoluble cues), but also by soluble signaling molecules such as growth factors. These factors are usually proteins or small molecules that bind to transmembrane receptors thereby inducing a biochemical cascade resulting in cell growth, proliferation, differentiation

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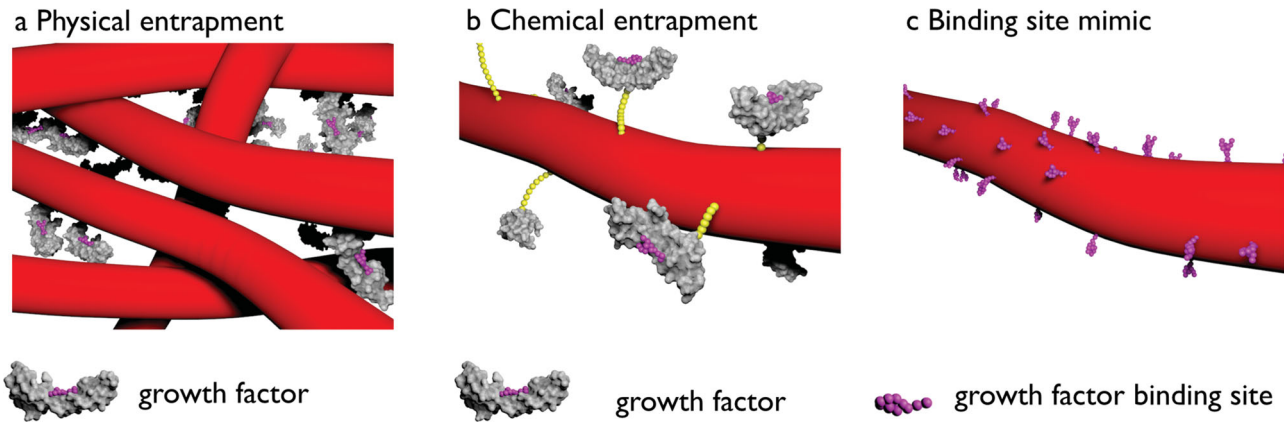

Figure 5. Strategies to overcome the short lifetime of growth factors include physical entrapment and controlled release from a supramolecular scaffold (a), the covalent or non-covalent attachment of growth factors to a scaffold (b) or using peptide sequences that mimic the binding site of growth factors (c).

or migration. All of these cell responses are critical for regenerative therapies. However, because of their relatively short lifetime in vivo, injections of unprotected soluble factors require high and frequent doses, which are not only expensive, but can also induce severe side effects. To reduce the required dose of growth factors, previous investigators have either physically entrapped them in a polymer or supramolecular network or grafted them to supramolecular structures via non-covalent interactions (**Figure 5**), thereby releasing the cues in a sustained and controlled manner. In a third approach, peptides that mimic the active site of growth factors are integrated in the supramolecular structure, eliminating the need for growth factors altogether (Figure 5c). In the last two approaches, the signaling cue is rendered insoluble through its structural integration. In this section, we discuss the use supramolecular materials to instruct cells with cues derived from soluble factors using the three stated strategies.

The first strategy of mimicking cell signaling by soluble cues involves entrapping growth factors in a network and allowing the controlled release (see Figure 5a). This strategy has mostly been applied using polymer networks;[88] however, examples using supramolecular materials do exist. For instance, Kisiday and coworkers have physically entrapped transforming growth factor β1 (TGF-β1) factor in self-assembled peptide $(KLDL)_3$ gels,[89] previously developed in their group.[90] This growth factor is a multifunctional protein that regulates several cell processes including proliferation, ECM metabolism, and differentiation.[91] Since TGF-β1 can induce chondrogenesis (differentiation into cartilage forming cells) of bone marrow stromal cells (BMSCs),[92] it is crucial for cartilage regeneration therapies.[93,94] The entrapped growth factor indeed showed greater sustained release than collagen gels and induced chondrogenesis of BMSCs.[95]

A second strategy to prolong the retention time of growth factors consists of anchoring the proteins to an artificial ECM via covalent or non-covalent bonds (Figure 5b). This strategy has been employed by modifying insulin-like growth factor (IGF-1) with streptavidin and allowing it to bind to a biotinylated self-assembled peptide scaffold via the non-covalent avidin-biotin interaction.[96] This non-covalent construct resulted in a sustained release of IGF-1 in vivo as compared to untethered growth factor, which in turn led to a higher transplanted cell growth when myocytes were co-injected with the scaffold. The delivery system was then tested in a cell-based therapy using a myocardial infarction mouse model. Injection of cardiomyocytes (heart muscle cells) together with IGF-1 tethered to the supramolecular scaffold into the infarct zone showed a significantly improved systolic (contractile) function of the heart, demonstrating that non-covalent entrapment of growth factors can support cell therapies.

The previous strategy requires cumbersome modification of growth factors to allow binding to a scaffold. Several growth factors display ECM binding domains, so such alterations are not always necessary. For instance, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) can bind heparin, an ECM component. The growth factors are not only released in a prolonged manner in the presence of heparin, but also protected from enzymatic degradation[97,98] Researchers have used this feature to increase the retention time of growth factors by functionalizing their self-assembled scaffolds with heparin, a highly sulfated glycosaminoglycan. In a study by Rajangam et al.,[99] a self-assembling PA was functionalized with the peptide sequence LRKKLGKA known to bind to heparin.[100] Gels formed by this PA in the absence of heparin released half of their FGF-2 within 1 day, whereas gels formed with heparin released 50% in 10 days. As FGF-2 is known to induce angiogenesis, the heparin binding PA was screened for its capacity to promote growth of new blood vessels. PA gels with heparin and FGF were injected in the rat cornea and a significant increase of vascularization was observed as compared to collagen gels with heparin and growth factors. A follow-up study revealed that the heparin-binding PA structures showed biocompatibility and retention of at least 30 days in a mouse subcutaneous implantation model.[101] Similarly, PA nanostructures have also been designed to display the binding sequence HSNGLPL for TGF-β1,[102] which plays important roles in the formation of connective tissues.[103] By covalently engrafting PAs with HSNGLPL, the release of TGF-β1 from gels was drastically prolonged as compared to the unmodified PA gels and *in vitro* assays showed that these gels can support

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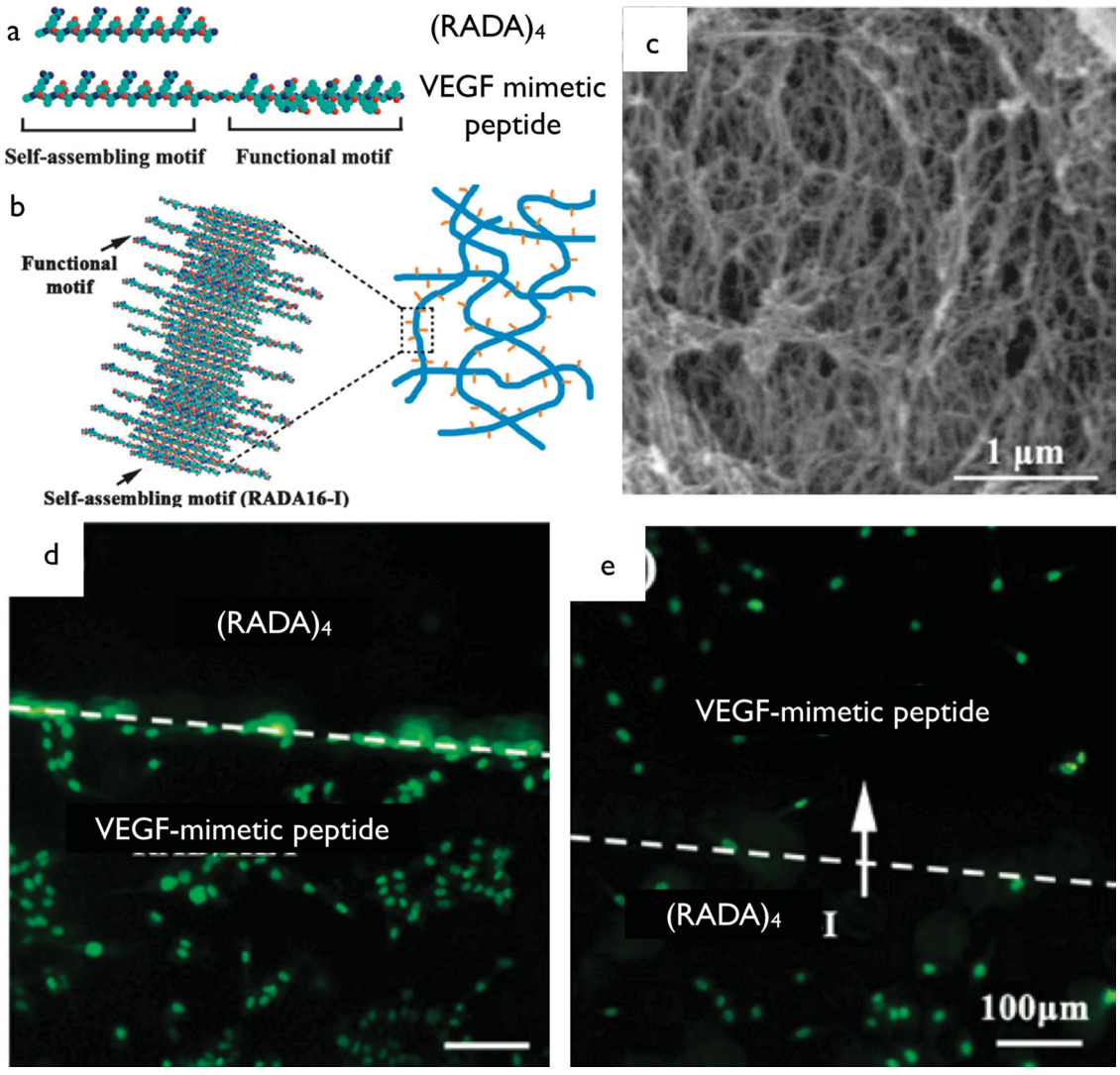

Figure 6. (a,b) Schematic representation of $(RADA)_4$ and VEGF-mimetic peptide self-assembly into a fibrillar network as evidenced by scanning electron microscopy (c). Unidirectional migration of endothelial cells peptide scaffolds: cells migrated from $(RADA)_4$ to VEGF-mimetic peptide. Reproduced with permission.[105,106] Copyright 2012/2008, RSC.

the survival of hMSCs and promote differentiation into chondrocytes, required for cartilage regeneration. Indeed, an in vivo study showed that these materials promote regeneration of cartilage when injected in the damaged cartilage of rabbits. Interestingly, in this study no externally applied growth factors were used.

The previous examples show strategies to control and prolong the release of growth factors, thereby decreasing the amount needed and potentially decreasing the cost of treatment. Another strategy involves identifying the site of a growth factor that binds to a receptor and attaching this peptide sequence to a supramolecular scaffold (see Figure 5c). This approach activates the targeted membrane receptors in proximity of the scaffold and negates the use of growth factors all together. This approach has been applied successfully for VEGF, a growth factor known to stimulate vasculogenesis and angiogenesis. Pedone and coworkers first published the sequence responsible for binding to the transmembrane VEGF receptor: KLTWQELYQLKYKGI-$CONH_2$. This sequence was found by an X-ray structure of VEGF bound to its receptor.[104] In 2008, Wang et al. attached this binding sequence to a self-assembling peptide scaffold based on $(RADA)_4$ via a tetraglycine spacer (**Figure 6**a and b).[105] The self-assembling peptide conjugated to the VEGF mimetic peptide was able to form supramolecular fibers expressing the VEGF mimetic peptide on its surface (Figure 6c). *In vitro* studies showed that the VEGF-mimetic self-assembling peptide significantly enhanced human umbilical vein endothelial cells (HUVECs) survival, proliferation and migration as compared to the native $(RADA)_4$ fibers (Figure 6d and e). In a follow-up study, VEGF-mimetic self-assembling peptide was found to induce angiogenesis in vivo using a chick embryo membrane (CAM) assay.[106] Such materials are promising for the field of regenerative medicine as they decrease the need for expensive growth factors with short half-lives.

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In a similar study, described by Webber and coworkers, the VEGF mimicking peptide was attached to a self-assembling peptide amphiphile to give a VEGF-mimetic peptide amphiphile (**Figure 7**a-c).[107] Similar to the peptide sequence in native VEGF,[104,108] the sequence folded into an alpha-helical conformation. It was found that the VEGF-mimetic peptide was able to bind the VEGF receptors of HUVECs and thereby improved migration, proliferation and survival of the cells. Interestingly, this behavior was not observed for the peptide alone and this difference was attributed to the high local concentration of signaling peptides on the PA fiber. The CAM assay revealed the ability of the VEGF-mimetic PA to induce angiogenesis. Such promising results encouraged further evaluation of the potential of the VEGF-mimetic PA as a therapy for ischemic disease (tissue necrosis as a result of insufficient blood flow) using a mouse hindlimb ischemia model (Figure **7** d-f). Using a mouse model, a significant improvement in tissue salvage was observed after intramuscular injection of supramolecular nanofibers formed by the VEGF-mimetic PA relative to treatment with the bioactive peptide. Most importantly, the treatment with the supramolecular material resulted in enhanced motor function and blood perfusion in the previously ischemic limb over the course of 28 days. Moreover, the VEGF-mimetic nanofibers were retained significantly

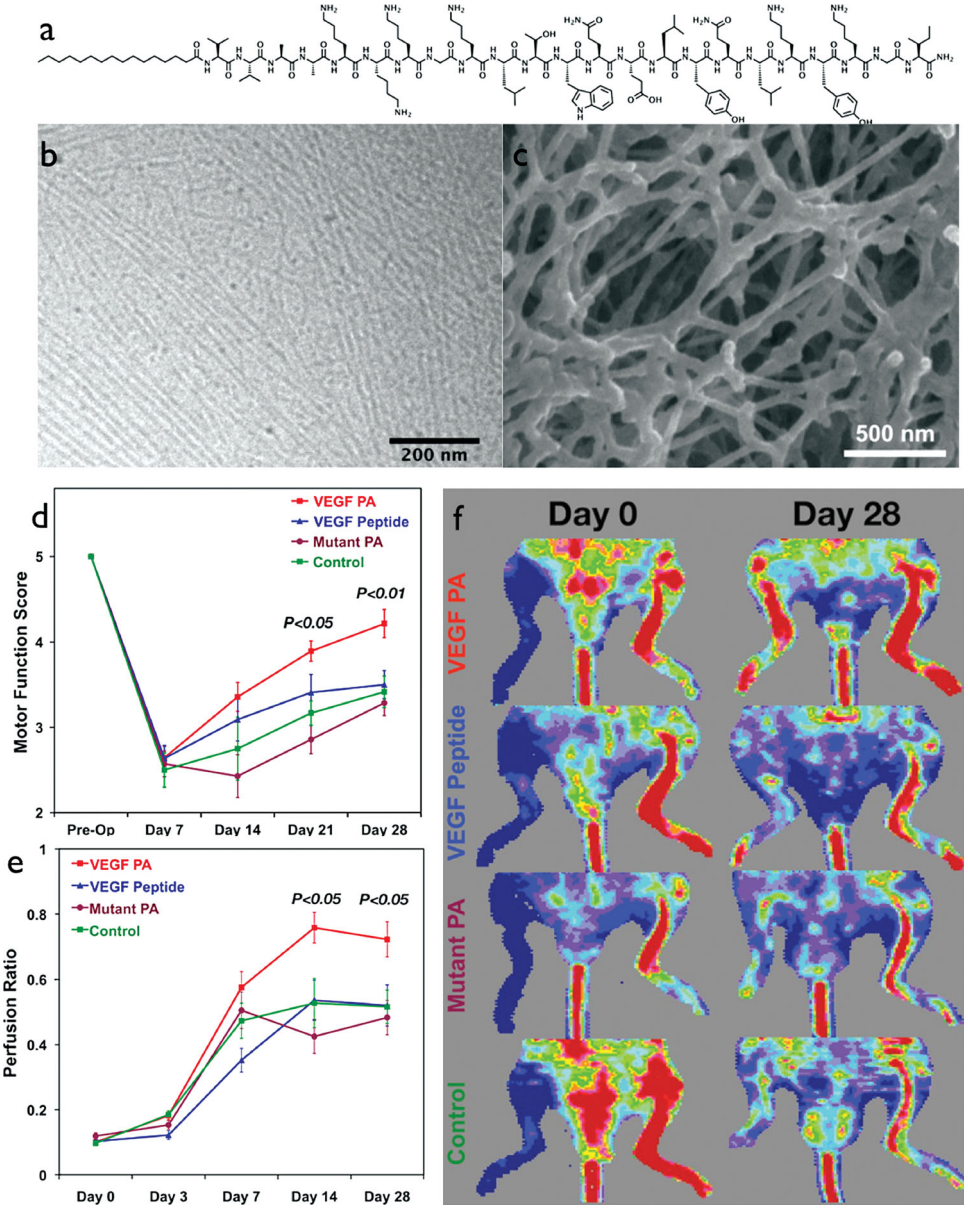

Figure 7. a) Molecular structure of VEGF-mimetic PA that self-assembles into fibers as evidenced by cryo-transmission electron microscopy (b) and scanning electron microscopy (c). d) Motor function scores after the hindlimb ischemia model are significantly higher for the VEGF-mimetic PA than the peptide control. e and f) Laser Doppler Perfusion Imaging shows significant higher perfusion ratios for the VEGF-mimetic PA than controls. Reproduced with permission.[107] Copyright 2012 , PNAS.

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longer in vivo than the corresponding peptide sequence. This enhanced retention is believed to result in the observed therapeutic benefits of the PA compared to the peptide control. Interestingly, the strategy of immobilizing the active sequence of a growth factor is not limited to VEGF, but has also been used to mimic the activity of bone morphogenetic protein-2 (BMP-2).[109–111]

3. Materials Controlled Over Time: Dynamic Materials

Supramolecular materials have the ability to instruct cells and are able to mimic both signaling by soluble and insoluble cues. However, the systems described above can only display a single signaling cue over time, in contrast to the complexity of the native cellular environment which is highly dynamic and changes the displayed bioactive cues depending on the desired function. This dynamic behavior and temporal control over matrix composition is clearly illustrated in the following simplified description of the natural process of wound healing. In a first phase, referred to as hemostasis, damaged collagen fibrils direct platelets to form fibrin clots that minimize blood loss. The clot also serves as provisional matrix for new cells and a reservoir of growth factors including platelet derived growth factor (PDGF). In the next phase of wound healing, the inflammatory phase, immune cells migrate into the wound and, among other activities, start to remove bacteria and cell debris. Again, the ECM plays a crucial role in this phase as monocytes use it to migrate from blood vessels into the wound. The binding of monocytes to the ECM induces their differentiation into tissue macrophages and upregulates the production of growth factors. In the next phase, the migration and proliferation phase, the fibrin matrix starts to incorporate ECM proteins such as fibronectin and vitronectin, which facilitate the migration of fibroblasts and endothelial cells into the wound and towards the released PDGF. The binding of fibroblasts to fibronectin stimulates their production of collagen, proteoglycans and hyaluronic acid. These ECM components aid the further migration of fibroblasts, macrophages and endothelial cells. The attachment of fibroblasts also stimulates the release of matrix metalloproteinases (MMPs) that, by degrading ECM components, facilitate the migration of cells. In the final phase, the remodeling and contraction phase, fibroblasts differentiate into myofibroblasts that bind to the collagen bundles and start to exert forces to contract the wound. Next, macrophages, endothelial cells and epidermal cells release MMPs that break down the ECM. The myofibroblasts, in turn, replace it with the stronger collagen type 1 until the ECM reaches mechanical equilibrium with its surrounding tissue.[112]

From this simplified explanation of wound healing, it becomes clear that tissue regeneration is a multistep process in which each step requires a different composition of the ECM. In biology, these changes in composition naturally arise from the dynamic nature of the ECM, constantly broken down by proteases and remodeled by newly secreted proteins. To attain such dynamic remodeling, the system is constantly consuming and dissipating energy, which drives it far from equilibrium. All of the materials previously described do not share this feature and are self-assembled at a thermodynamic minimum or kinetically trapped. Although some dissipative self-assembled systems have been described, this field of research remains in its infancy and examples of artificial dissipative supramolecular materials are limited.[28] However, there is an alternative to create ECM mimics that display varying bioactivity in a transient fashion, facilitating a multistep process such as wound healing. The recent developments in responsive materials have created a vast variety of materials responsive to light,[113] pH,[114] magnetism,[26] enzymes[115] and many others. Such materials can serve as a scaffold that, upon stimulation, changes its bioactivity. Stimuli responsive systems that allow such multistep display of bioactivity are desired for the next generation of supramolecular materials for regenerative medicine.

Initial work to trigger bioactivity has mostly been focused on inducing the release of a bioactive group. To that end, Sur et al. have recently reported on a PA based matrix that displays the previously discussed RGDS in a triggered fashion.[58] The RGDS sequence is connected to the PA matrix via a photocleavable nitrobenzyl linker (**Figure 8**). The RGDS sequence is recognized by fibroblasts resulting in the formation of focal adhesion points and spreading of the cells. However, upon irradiation with UV-light, the matrix releases its RGDS into solution and loses its bioactivity. Indeed, after irradiation the cells show limited spreading similar to PA fibers without RGDS.

Huskens and coworkers described a dynamic self-assembled monolayer on gold that changes bioactivity toward cells in response to electrochemical cues (**Figure 9**).[116] In this system, host-guest chemistry is used to create a dynamic bond between a gold surface and an RGD peptide sequence. More specifically, viologen is immobilized on a non-fouling surface, this hydrophobic molecule has an affinity for the hydrophobic pocket of macrocyclic cucurbit-[8]-uril (Figure 9a). The cucurbit-[8]-uril can host a second hydrophobic molecule which is the indole ring of tryptophan connected to RGD via a diglycine linker. In other words, cucurbit-[8]-uril is serving as molecular glue between the gold surface and the RGD peptides. Upon formation of the complex the surface displays RGD, rendering the surface bioactive to C2C12 cells and HUVECs (Figure 9b). However, applying a negative potential on the gold surface reduces the viologen molecules thereby dissociating the complex and thus releasing the RGD. The release of RGD deactivates the surface and releases the cells (Figure 9c).

Boekhoven and coworkers reported very recently on a supramolecular construct that uses host-guest chemistry to display bioactivity. In this system, cyclodextrin, a cup-shaped molecule that can host hydrophobic guests, is covalently attached to a surface (**Figure 10**a). Next, the bioactive RGDS sequence, attached via a glycine linker to hydrophobic naphthoic acid, is introduced. The naphthoic acid forms a host-guest complex with the cyclodextrin, and this complex formation renders the surface bioactive. This bioactivity was evidenced by the adhesion and spreading of fibroblasts on the surface (Figure 10b). As the host-guest complex is dynamic, it can be displaced by the introduction of guests with a higher affinity for the cyclodextrin. Indeed, when RGES (a non-active mutated version of RGDS) attached to adamantane was

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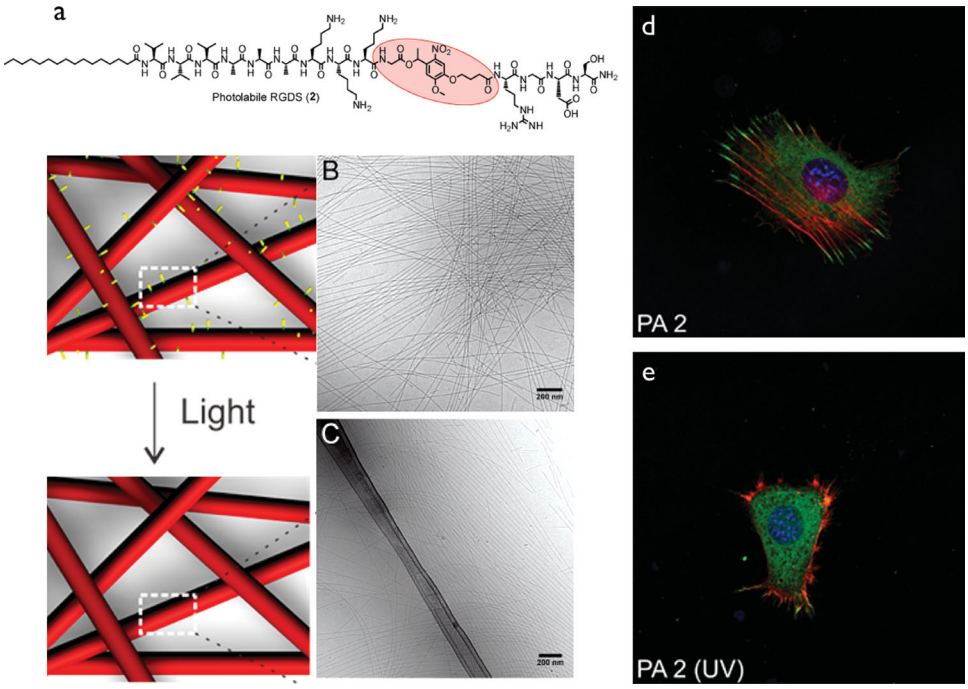

Figure 8. Photochemical control of bioactivity. a) Molecular structure of a self-assembling PA connected to RGDS via a photocleavable linker. b and c) Both the photocleavable PA and the photodegraded product form fibers in solution as evidenced by cryogenic transmission electron microscopy. d and e) Fibroblasts can attach and spread on the RGDS expressing PA but irradiation with light releases the RGDS and renders the surfaces bio-inactive. Reproduced with permission.[58] Copyright 2012, ACS.

introduced, it displaced the RGDS and rendered the surface inactive.[117]

The previous examples all use a trigger to remove the bioactive cues from a scaffold, thereby rendering the supramolecular construct biologically inactive (**Figure 11**a). Although these examples are sophisticated from a materials point of view, they show a mere fraction of the complexity observed in natural ECM. To bring supramolecular materials for regenerative medicine to

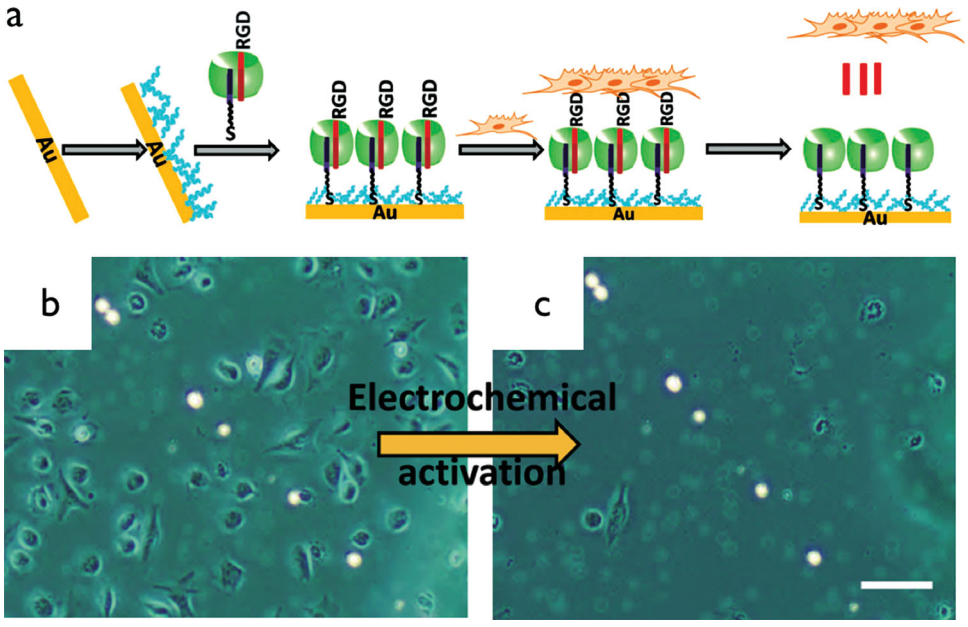

Figure 9. Electrochemical control over bioactivity. a) Scheme of bioactivity that is responsive to reduction potential. A non-fouling gold surface is rendered bioactive by attaching a ternary host-guest complex expressing RGD. By electrochemically reducing the viologen, the complex dissociates and switches the bioactivity. (b and c) C2C12 cells can attach to the bioactive surface but not to the inactivated surfaces. Scale bar represent 100 μM. Reproduced with permission.[116]

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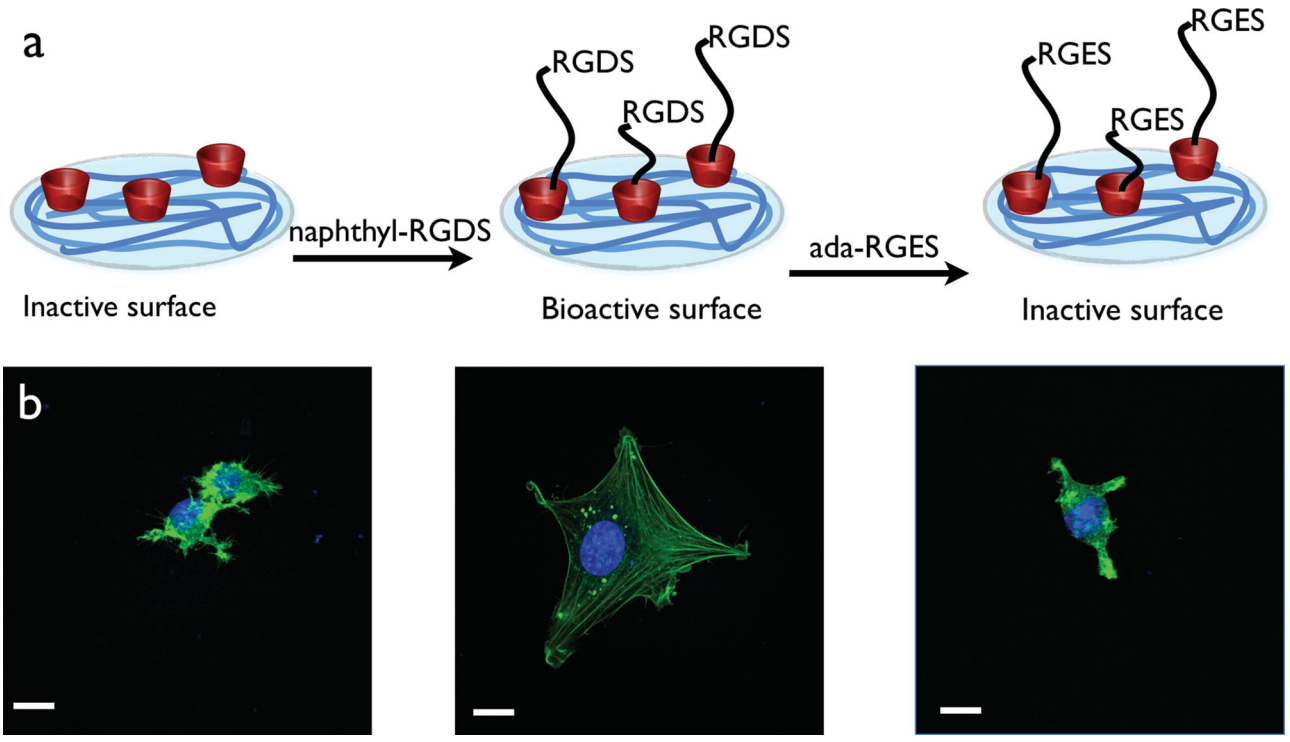

Figure 10. a) Supramolecular host-guest formation between cyclodextrin (red cups) and naphthyl-RGDS renders a surface bioactive. Adamantane-conjugated RGES (ada-RGES) competes with naphthyl-RGDS and makes the surface inactive. b) Confocal microscopy of 3T3 fibroblasts on the inactive surface, the activated surface and the surface with competing ada-RGES present. Scale bars represent 10 μM. Reproduced with permission.[117]

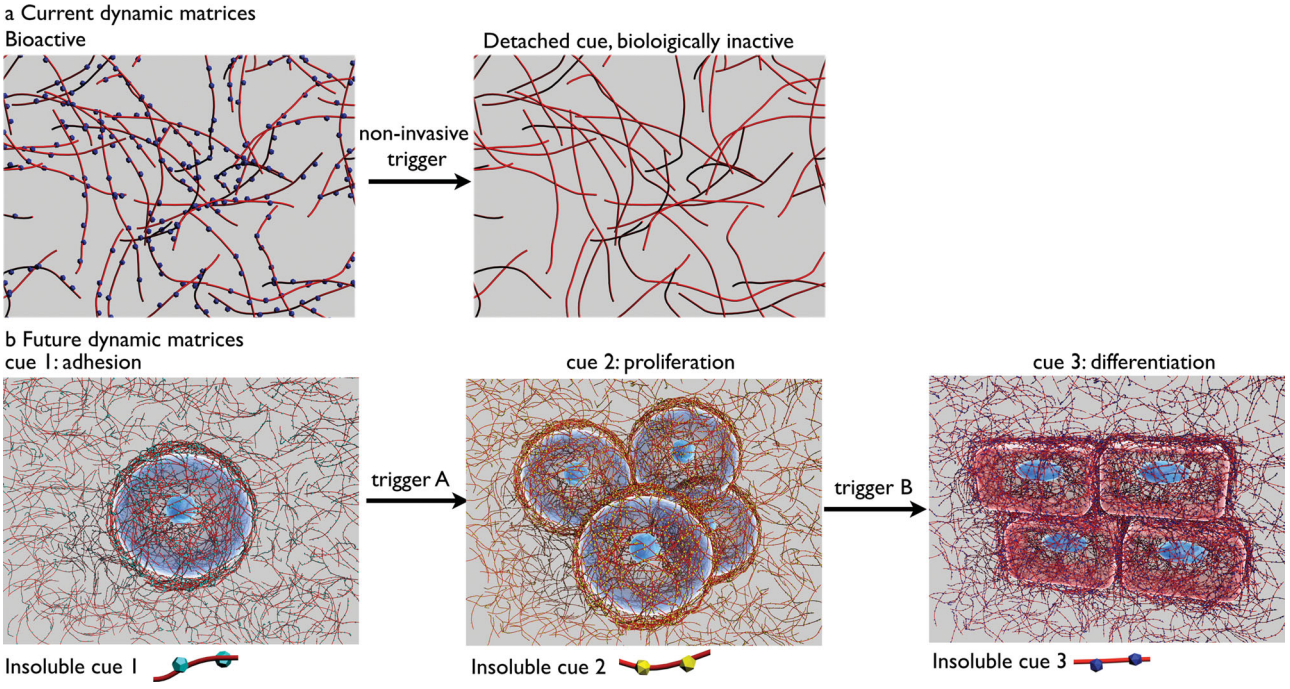

Figure 11. a) A general strategy to display bioactivity by using a responsive material that can release its binding cue upon a trigger. b) To bring the supramolecular materials for regenerative medicine to the next level, we envision a material that can display several cues and can be triggered to show them sequentially.

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the next level, we envision a material that mimics the multistep aspect of the natural ECM using bioactive cues in combination with responsive materials. For instance, an ideal material would display multiple bioactive cues, each responsible for different tasks and responsive to different triggers. Such a material can first instruct its embedded cells to adhere. Upon interaction with the first stimulus, the responsive material can be triggered to express a second cue instructing the cells to proliferate. Finally, when a sufficient cell density is reached, a second trigger could initiate the expression of a third cue instructing the cells to differentiate into desired lineage (Figure 11b).

4. Materials with Order Over Multiple Length Scales: Hierarchical Materials

The nanostructured materials described in the previous sections lack the degree of structural complexity across multiple length scales found in the matrices of living tissues.[25] For instance, skeletal muscles are biological structures that are organized at the nanometer level (actin and myosin proteins assembled into myofibrils) all the way to the centimeter level (fascicles are macroscopic bundles of muscle cells).[118] For the purpose of tissue regeneration, it seems obvious that the materials used as a scaffold should exhibit organization at similar length scales. This requirement constitutes another major challenge for supramolecular materials in regenerative medicine. Examples of hierarchically ordered supramolecular materials are rather limited[119–124] mainly because it is challenging to design building blocks that can assemble in a hierarchical fashion.[125]

Capito and coworkers reported on a hierarchically ordered structure based on a hybrid of PA nanofibers and a biopolymer,[21] and the authors demonstrated the potential of this hybrid construct as a biomaterial. The hierarchically ordered materials form spontaneously over various time scales and the process starts within milliseconds of contact between an aqueous solution of PA nanofibers and a solution of an oppositely charged polyelectrolyte. A diffusion barrier forms instantaneously at the interface as a result of very rapid electrostatic complexation (**Figure 12**a and b). Osmotic pressure

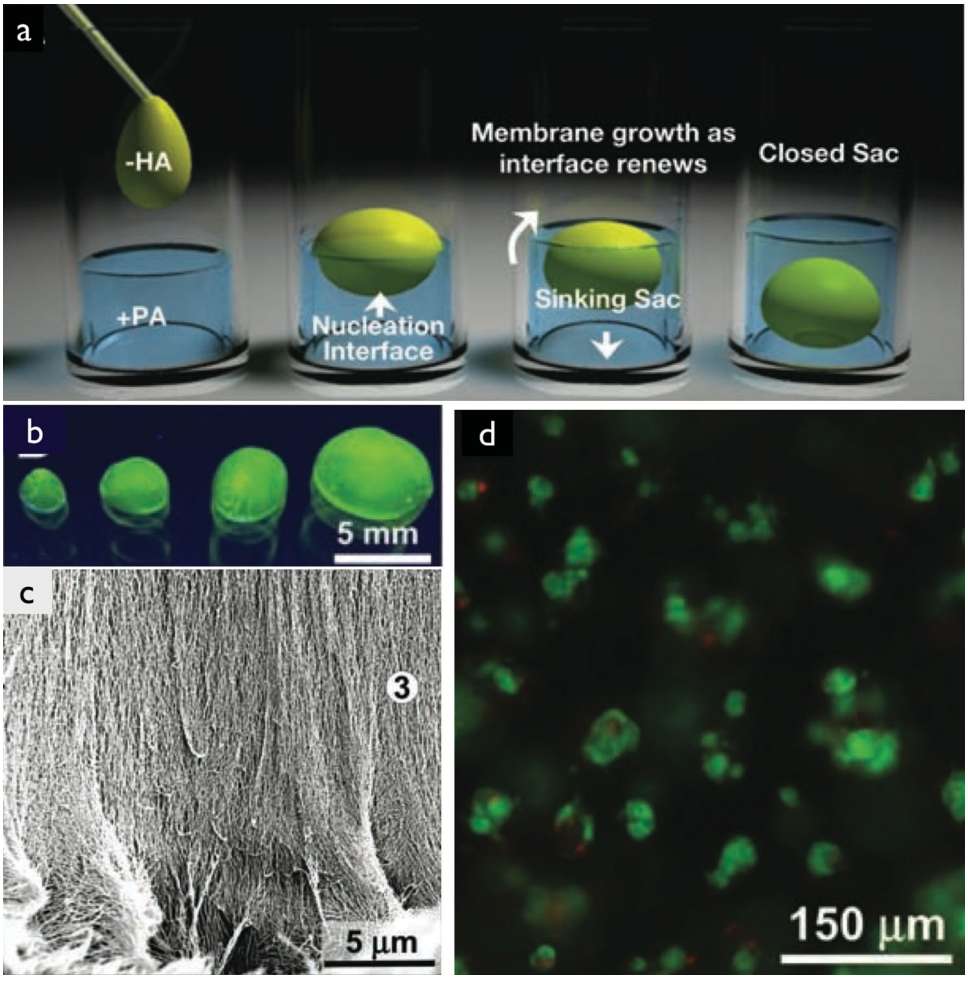

Figure 12. a) Schematic representation of the hierarchically ordered sac. A sample of a negatively charged biopolymer solution is dropped onto a positively charged PA solution. b) Photographs of the resulting hierarchical construct. c) Scanning electron microscopy images as evidence of hierarchically ordered membrane. d) Live/dead assay of hMSCs cultured within the PA gel-filled sacs (green cells are live, red cells are dead) showing that most of the cells remain viable. Figure reproduced with permission.[21]

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drives diffusion of the polyelectrolyte into the PA solution resulting in the assembly and growth of aligned nanofiber bundles perpendicular to the diffusion barrier at the interface of the two solutions (Figure 12c). The resulting membranes or enclosed sacs are on the order of 2–20 μm in thickness and are organized on two hierarchical levels: first PAs are organized into fibers and secondly, those fibers are aligned perpendicular to the membrane.[126] The structure of the membrane shares a resemblance with the hierarchical structure of the ECM of articular cartilage. hMSCs encapsulated in the sacs were viable for 4 weeks in culture and could be differentiated into chondrogenic phenotype when stimulated with chondrogenic media (Figure 12d). A recent study described that the polymer-PA sacs could be miniaturized using an electrospray technique. Moreover, these microsacs as well as the macroscopic structures are permeable to proteins and other macromolecules as a result of their hierarchical structure.[127]

Zhang and coworkers recently described a monodomain gel formed with hierarchical ordering over several length scales to form a string-like gel.[24] The first step of assembly is the aggregation of PA molecules into nanofibers, a phenomenon well described in the literature.[40] Heating these solutions induces dehydration and fusion of the fibers followed by fracture of the fused fibers into bundles through rehydration upon cooling. This process transforms the solution of bundled fibers into a liquid crystal. Dispensing this solution from a pipette by hand over an aqueous solution of calcium chloride results in the formation of a visoelastic string. (**Figure 13**c,d). The shear force and extensional force as the liquid exits the pipette aligns the bundles of nanofibers along the drawing direction over macroscopic length scales. The string gel is now organized hierarchically from the nanoscale to the macroscale (Figure 13a and b). To demonstrate the potential of these hierarchical constructs as supramolecular materials for regenerative medicine, the string gels were prepared with solutions containing hMSCs. The cells survived the process and started to align with the longitudinal axis of the string within 12 hours, throughout the entire scaffold. To demonstrate further biological applications of this construct, a string was prepared in the presence of HL-1 cardiomyocytes, a cell line with spontaneous electrical activity that requires extensive cell-cell contacts to propagate signals. The cells survived the process and proliferated and after 10 days of incubation the cells were spontaneously propagating electrical potentials over the macroscopic length of the string (Figure 13f).

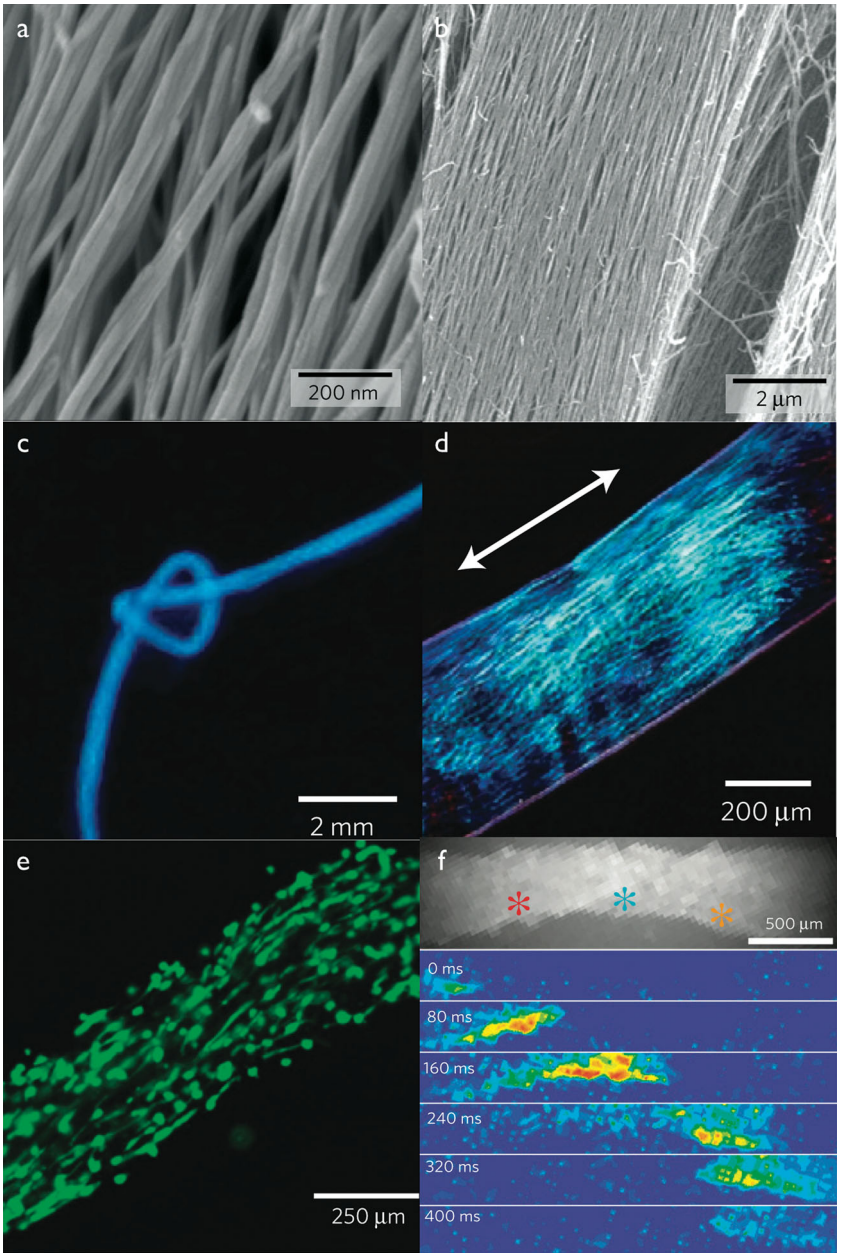

Figure 13. a,b) Scanning electron microscopy images as evidence of alignment of fibers in a string. (c) A knot tied with a PA fiber string. (d) Birefringence of a PA string suggesting the presence of macroscopically aligned domains. (e) Calcein-labeled cells in a PA string show alignment along the axis of the PA string. (f) top: Calcium fluorescence image of cardiomyocytes in a PA string. Below: successive spatial maps of calcium fluorescence intensity traveling at 80-ms intervals, showing the propagation of an electrical signal throughout the entire string and demonstrating a functional cardiac syncytium. Reproduced with permission.[24] Copyright 2010, NPG.

This hierarchical organization has been further explored by McClendon et al. for a scaffold to engineer blood vessels. For this purpose, it has been established that the circumferential alignment of contractile smooth muscles cells (SMCs) is necessary.[128] As the cells follow the alignment of the hierarchical material, a device was engineered that can create PA gel tubes with the fibers aligned with the circumference of the tube

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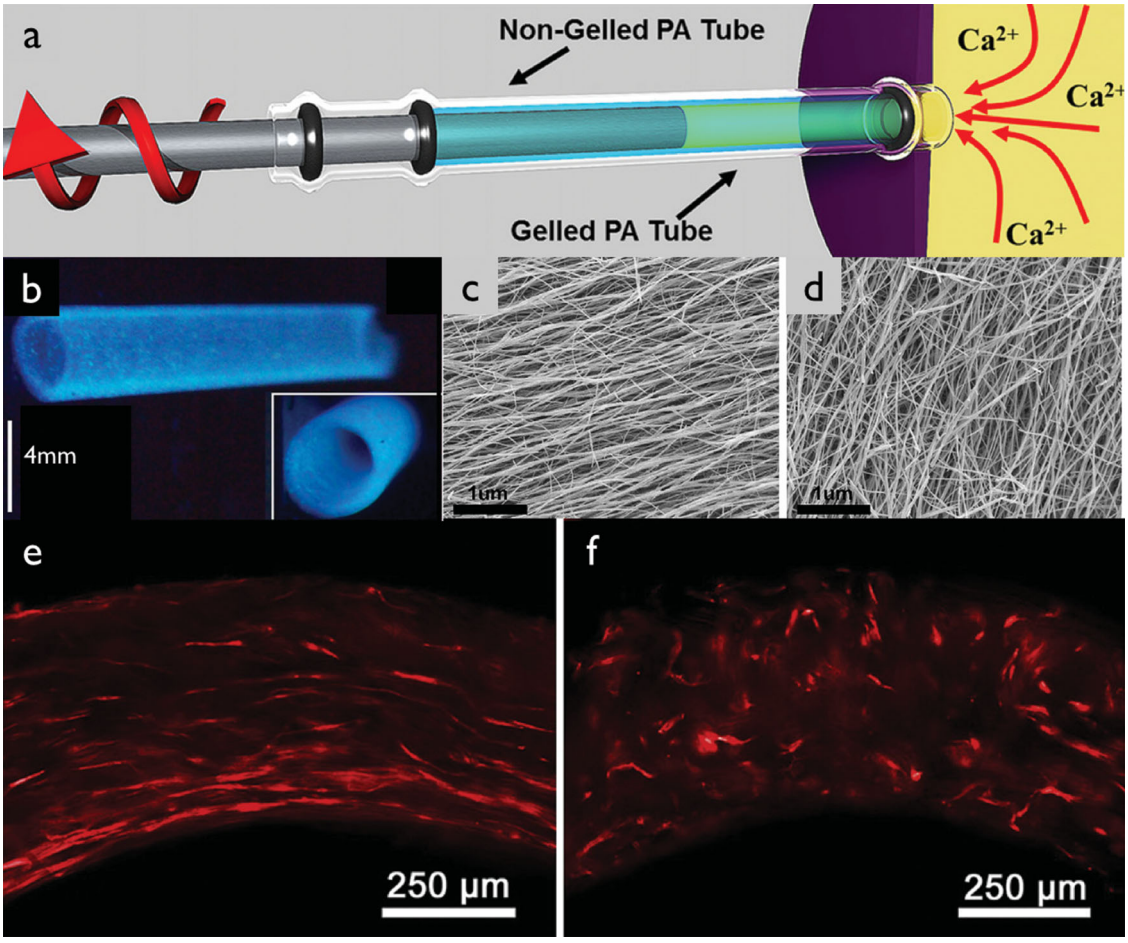

Figure 14. (a) Schematic representation of fabrication of hierarchically ordered artificial blood vessels. A rotating and retracting rod aligns PA fibers circumferentially. Upon retraction, the rod withdraws calcium ions into the tube, thereby crosslinking the PA fibers. (b) Photograph of resulting tubular gels. (c) Scanning electron microscopy images of the inner wall of tubular gel produced with rotation of the inner rod and (d) without rotation of the inner rod. (e) Cellular alignment as observed by fluorescence microscopy for the tube with aligned fibers and (f) non-aligned fibers. Reproduced with permission.[129] Copyright 2012, Elsevier.

(**Figure 14**a-d).[129] SMCs survived the fabrication process and proliferated over the course of 12 days in the construct. Cross sections of the tubular gels showed that, after 4 days, most of the cells had aligned with the circumference of the tube (Figure 14e and f). This finding confirms that the cells can be instructed by a material to align and supports the potential of such hierarchical systems as artificial matrices to direct growth of blood vessels.

5. Conclusion and Outlook

Supramolecular materials have the potential to mimic some of the structural and dynamic features of the cell's natural microenvironment and therefore offer a great platform for regenerative medicine. The use of bioactive components in supramolecular materials can instruct cells to adhere, migrate, proliferate and even differentiate. The ability of supramolecular materials to be highly dynamic due to a high density of non-covalent bonds, present signals with geometrical precision due to their internal order, and biodegrade rapidly due to the absence of high molecular weight backbones could be contributing to their regenerative efficacy in vivo models. However, the supramolecular materials available thus far for regenerative medicine still lack the dynamic complexity found in the biological structures that mediate regeneration. Tissue regeneration is a multistep process that requires the display of bioactive cues with temporal control mediated by the remodeling of the ECM. It is therefore necessary to develop materials that can mimic those properties and act as artificial matrices that change dynamically and are also transient over the necessary time scales. We anticipate that the exciting recent developments in dissipative self-assembly and responsive materials will help develop the next generation of supramolecular materials for regenerative medicine. The materials used for regenerative purposes also lack the structural complexity observed in natural tissue. Biological tissues are organized across several length scales and it seems obvious that materials guiding regeneration should have this property as well. We anticipate that the recent discoveries of hierarchically organized materials will

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help develop the next generation of materials for regenerative medicine.

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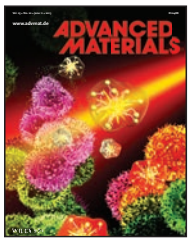

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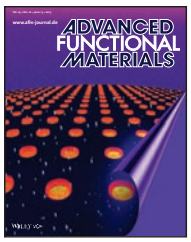

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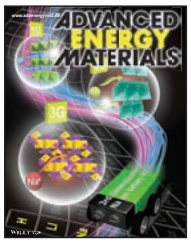

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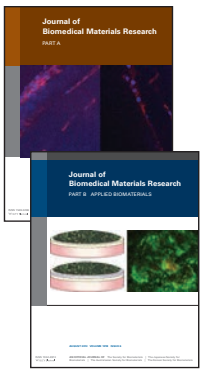

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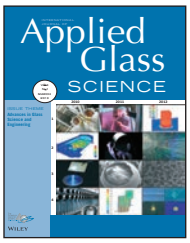

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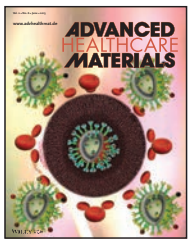

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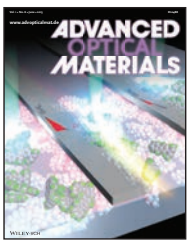

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