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Self-assembling peptides form injectable and biocompatible nanofiber hydrogels; they have proven to be versatile systems for protein delivery ***in vivo****.*

Local delivery of proteins and the use of self-assembling peptides

Vincent F.M. Segers and Richard T. Lee

Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA

Self-assembling peptides are members of a new class of molecules designed for tissue engineering and protein delivery. Upon injection in a physiological environment, self-assembling peptides form stable nanofiber hydrogels. Such biocompatible nanofibers can support three-dimensional growth and differentiation of many cell types. Self-assembling peptides are promising candidates for protein delivery, because they allow non-covalent binding of proteins, tethering of proteins, or incorporation of fusion proteins. Self-assembling peptides can be designed to deliver individual proteins or multiple factors, because the building blocks comprising self-assembling peptides can be designed with great flexibility.

Until relatively recently, a significant proportion of the strategies for treating diseases involved the use of the time-honored approach of antagonism of receptors by small molecule drugs. However, the small molecule antagonism approach is unsuitable for a broad variety of biological processes. For example, activation rather than inhibition of the system may be desirable and this can be difficult using typical drug design strategies. In addition, small molecules are often incapable of generating the effect that a protein can, since the protein often acts over a much larger polar surface area (a typical protein–protein interface covers ~1600 $Å^2$ of protein surface area [1]). Proteins and peptides, natural or engineered, are often excellent candidates for therapeutic agents as millions of years of evolution has represented a far superior SAR campaign, with respect to specificity and potency, than can be achieved in the laboratory. Thus, it is not surprising that biological therapies, in particular antibodies and engineered proteins, are comprising an ever-increasing proportion of approved therapies [2]. A logical next step will be to produce biomedical therapies that are a combination of small molecules, proteins, and even cells, which may allow more flexibility in the design of future therapies.

The advantages of local delivery of proteins

To be effective as a drug, a protein has to reach its site of action without degradation; then it has to remain in the target location sufficiently long to exert its action(s). Oral administration of proteins is generally inefficient because proteins are digested in the gastrointestinal tract [3]. Even intravenous injection is often incapable of delivering functional proteins to the target tissue, as many proteins are degraded in the blood, are captured by receptors and non-specific binding sites, or do not cross the endothelial barrier. Furthermore, to be suitable for systemic delivery, a protein ideally targets a specific cell type, a requirement that only few proteins like erythropoietin or insulin meet. Although growth factors, such as platelet derived growth factor (PDGF) or epidermal growth factor (EGF), may be highly effective and selective *in vitro*, they have a significant number of systemic side effects [4], hence limiting their use as therapies. Moreover, local delivery is frequently the only feasible strategy if locally high concentrations of a protein are necessary. Some proteins, such as chemokines, cannot be delivered systemically because their mode of action requires a concentration gradient; in the case of chemokines, a local shallow gradient may be essential [5]. In summary, local rather than systemic delivery may be the preferred route of protein delivery should (i) the protein not reach the site of action; (ii) the protein acts undesirably on other organs; (iii) high local concentrations be needed; or (iv) local concentration gradients need to be established.

One approach to protein delivery is by gene delivery, through the use of either plasmids or viruses [6]. Transfection of existing or injected cells can result in delivery of the protein for a prolonged

Corresponding author: Lee, R.T. (rlee@partners.org)

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period of time through local production. Since transfection efficiencies are rarely 100%, gene delivery strategies are probably most effective for secreted proteins; in this case, successful transfection of relatively few cells may be able to achieve the desired effect. Although technically it is easier to design and prepare plasmids and viruses for transfection than to design and prepare proteins and delivery systems for therapeutic use, gene delivery suffers from a lack of predictability, with respect to dose and time of expression (and hence half life of action). This unpredictability can limit the utility of gene delivery strategies. Thus, control of the dose with local delivery of proteins may be more feasible than with gene therapy.

Advances in bioengineering of materials for protein delivery have to be balanced by advances in technology of devices for local delivery. Recent progress in catheter-based approaches for intramyocardial delivery [7,8] or advances in minimal invasive surgery [8] make local delivery feasible at sites that were previously hard to reach.

Slow release and tethering of proteins

Growth factors are particularly good candidates for local protein delivery, in part because many growth factors act as agonists by interacting over large surface areas of their cognate receptor — and thus, small molecule agonists for growth factor receptors are unusual. Many studies show the promise of different growth factors in wound healing, angiogenesis, and other target biological processes. Local delivery of bone morphogenetic protein (BMP-2), transforming growth factor-β (TGF-β), and platelet derived growth factor-BB (PDGF-BB) are good candidates for bone and cartilage repair [9–13]. Epidermal growth factor (EGF) [14], fibroblast growth factor (FGF) [14–18], vascular endothelial growth factor (VEGF) [19,20], and hepatocyte growth factor (HGF) [21] stimulate angiogenesis when delivered locally. Nerve growth factor (NGF) [22] has been used for nerve regeneration. In all of these studies, growth factors were delivered locally by systems that allow slow release.

Local delivery of soluble proteins can be hampered by diffusion as well as by the convective losses of the protein into blood and lymph. When injected in highly vascularized tissues, some soluble proteins will be washed away within minutes [23]. Growth factors may need longer to diffuse to the target receptors to exert their effects on cells, and chemokines may need to be present for at least a day to attract a significant number of cells. Two strategies have been developed to prevent rapid diffusion: slow release from degrading or dissolving delivery systems and tethering of proteins to solid substrates. Slow release has been widely applied in local growth factor delivery. For instance, biodegradable acidic gelatin hydrogels have been used for slow release of FGF-2 (basic fibroblast growth factor); FGF-2 was released during biodegradation of the gelatin hydrogel *in vivo* but was not released from the gelatin *in vitro* [17]. Incorporation of heparin in slow-release matrices with different materials can protect heparin-binding proteins from degradation by proteases and at the same time can prevent diffusion [15]. Another well-established approach is the use of biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres incorporated into a porous scaffold (e.g. alginate) for slow release of FGF-2 [24].

Slow-release approaches have been developed for numerous growth factors such as FGF [15,17,24], BMP [12], HGF [21], or VEGF [19] to prevent rapid diffusion and degradation. However, using chemokines instead of growth factors in the same release method is a recent concept in tissue engineering. Chemokines are typically ~10 kDa polypeptides that bind to receptors of the G-protein-coupled receptor family. Chemokines are produced locally and diffuse away to form solid-phase or soluble concentration gradients. Cells expressing the receptor for the chemokine typically migrate up a shallow chemokine gradient. Zhao *et al.* used PLGA microspheres for controlled release of macrophage inflammatory protein-3α (MIP-3α) [25], a chemoattractant for dendritic cells, which plays an important role in primary immune response. They found that MIP-3α, when released from PLGA microspheres, could trigger chemotaxis in dendritic cells millimeters away from the attractant source, elicit directed migration up to 500 μm from the source, and maintain attraction for at least 8 h *in vitro* through a three-dimensional collagen gel model of the extracellular matrix. This strategy may lead to the design of novel immunotherapies and vaccines by inducing specific cell types to concentrate at a local depot of antigen. Microspheres releasing chemokines could be combined with antigen presentation in the same injectable formulation with a stronger immune response as a result.

To elicit a significant local effect, sustained levels of sufficient magnitudes of growth factors have to be present at the cell surface, which is challenged by degradation as well as endocytosis and growth factor-receptor complexes formed after receptor binding. One way to prevent endocytosis and subsequent degradation of ligand and receptor is to tether the factors to a solid substrate as has been shown with EGF [26]. Similarly, tethering of TGF-β1 to a polyethylene glycol hydrogel is more efficient than soluble TGF-1β to induce matrix production in cultured cells [27]. In summary, tethering of factors to solid substrates or insoluble matrices can improve the activity of growth factors on cells, because concentrations are locally high and endocytosis of growth factor–receptor complexes is prevented.

Systems for protein delivery

Materials used for protein delivery have to meet some basic requirements in addition to the obvious necessity of biological compatibility: they have to be non-cytotoxic, non-immunogenic, non-infectious and biodegradable, giving rise to non-toxic products (Box 1) [28]. Biological materials have a higher risk of being infectious or immunogenic, compared to synthetic materials (on condition that synthetic materials are manufactured in a sterile environment). On the contrary, synthetic materials tend to be less biodegradable and more cytotoxic. Materials for protein delivery have to meet some physical criteria as well. They have to provide mechanical strength as well as elasticity, depending on the target organ. For some applications, such as myocardial delivery, the ability to apply by injection is an advantage, since catheter-based approaches are feasible.

In addition, the binding interactions between the protein and the material for protein delivery are crucial. Prolonged protein delivery is likely to be more successful when non-covalent binding interactions between the protein and the material are strong. Materials that can be designed may be preferable because this allows optimization and potentially even the design of covalent binding of proteins if necessary. Materials that protect proteins

BOX 1

Optimal features of materials for protein delivery

Biological properties
- Non-cytotoxic
- Non-immunogenic
- Non-infectious
- Biodegradable with non-toxic products

Physical properties
- Injectable
- Mechanical strength
- Elasticity
- Viscosity

Properties that support protein delivery
- Non-covalent binding interactions
- Engineerable
- Protective against proteases

Technological properties
- Easy production or purification
- Scalable

against local proteases will increase the duration of protein delivery because numerous extracellular, as well as intracellular, proteases are abundant in every tissue. Adding heparin to formulations for FGF-2 delivery is a classic example of successfully decreasing proteolysis of FGF-2 [16] while simultaneously increasing binding interactions. Proteolysis challenges chemokine delivery as well, as the activity of many chemokines is abolished by matrix metalloproteinases and other proteases [29]. For instance, stromal cell derived factor-1 (SDF-1), a stem cell chemoattractant, is inactivated by MMP-2 [30]. Local delivery of those chemokines in inflammatory tissues will result in rapid inactivation unless the protein is protected against proteolysis.

In addition to cleavage by proteases within tissues, proteins can lose their activity by denaturing and deactivation during preparation of protein-carrier systems [31]. Even if proteins are incorporated into drug delivery systems in stable native form, they still might denature or degrade during storage or after injection. Therefore, the maintenance of native protein conformation after incorporation and implantation must be considered for any delivery system. Furthermore, modifications of proteins themselves to modify binding interactions can inactivate the protein. For instance, biotinylation of proteins can be used to tether them to matrices using streptavidin as a linker. However, biotinylation of crucial lysine residues might block protein–receptor interactions if the biotinylation of particular residues is not specific. Generally, construction of fusion proteins allows more control of the region where the modification is inserted. For instance, if the active site of the protein of interest is located at the N-terminus, extra amino acids can be attached at the C-terminus.

Cell attachment and cell growth in the matrix are very important factors to be considered. Materials used for protein delivery should, ideally, support growth and differentiation of cells. For instance, BMP-2 has been approved by the Food and Drug Administration for orthopedic applications. However, large doses are required for healing of bone defects [12], indicating the need for controlled delivery of BMP-2. The ultimate material used for delivery of BMP-2 not only has to deliver large amounts of active BMP-2 over a long time period, but also has to promote migration and growth of cells as well as provide sufficient mechanical strength for generation of new bone tissue.

Both naturally occurring and synthetic materials have been used for protein delivery [28]. Naturally occurring materials include collagen, gelatin, agarose, alginate, amylopectin, fibrin gel, hydroxyapatite, and chitosan. The advantage of naturally occurring materials is their known physiological activities, mechanical properties, and biodegradability. These materials may also be relatively inexpensive. Potential transfer of infectious agents from animals, antigenicity, and unstable material supply are potential disadvantages. Moreover, specific properties of naturally derived materials that are necessary for protein delivery are difficult to design. Synthetic materials can be produced on a large scale, and their structural and mechanical properties can be more easily modified compared with some natural materials. Commonly used materials are polylactide (PLA), glycolide–lactide copolymer (PLGA), poly(vinyl alcohol) (PVA), and poly(ethylene glycol) (PEG) [31]. Most studies on local delivery of proteins have been performed with those polymers as they are degraded into well-tolerated products (lactic and glycolic acid) and their rate of degradation is well known. The physical properties of those polymers can be controlled by varying the lactide:glycolide ratio [32]. A major drawback of synthetic materials is the lack of specific cell-recognition signals. Cells adhere to the extracellular environment via cell surface receptors including integrins; to improve cellular adhesion, cell-adhesion motifs have to be incorporated into biomaterials [33]. Other potential disadvantages of synthetic polymers are a decrease in local pH from acid breakdown products, bulk degradation, inflammatory responses, and poor clearance [11].

Self-assembling peptides

In the past decade, self-assembling peptides have been developed for tissue engineering and protein delivery. Self-assembly is a process that is mediated by non-covalent interactions between molecules *via* ionic bonds, hydrogen bonding, hydrophobic interactions, and van der Waals interactions. In nature, amphiphilic proteins exist with both hydrophilic and hydrophobic regions, and self-assembly of these proteins can occur. The first self-assembling sequence was found in the yeast protein, zuotin, EAK16-II (AEAEAKAKAEAEAKAK, A = alanine, E = glutamate, K = lysine) [34]. Many synthetic self-assembling peptides have been described [34]. Self-assembling peptides typically are 8–16 amino acids long and are composed of alternating hydrophilic and hydrophobic residues (Figure 1). The hydrophilic amino acids in the sequence of self-assembling peptides consist of alternatively repeating units of positively charged lysine or arginine and negatively charged apartate or glutamate. They form stable β-sheets in water, and upon exposure to physiological salt concentration or pH they form a stable hydrogel of flexible nanofibers (7–20 nm in diameter) [34,35], consisting of more than 99% water. The β-sheets formed are stable across a wide range of temperatures, pH, and concentration of denaturing agents urea and guanidine hydrochloride [34]. Sterile self-assembling peptides can be produced if peptide synthesis is performed under sterile conditions. Furthermore, sterile self-

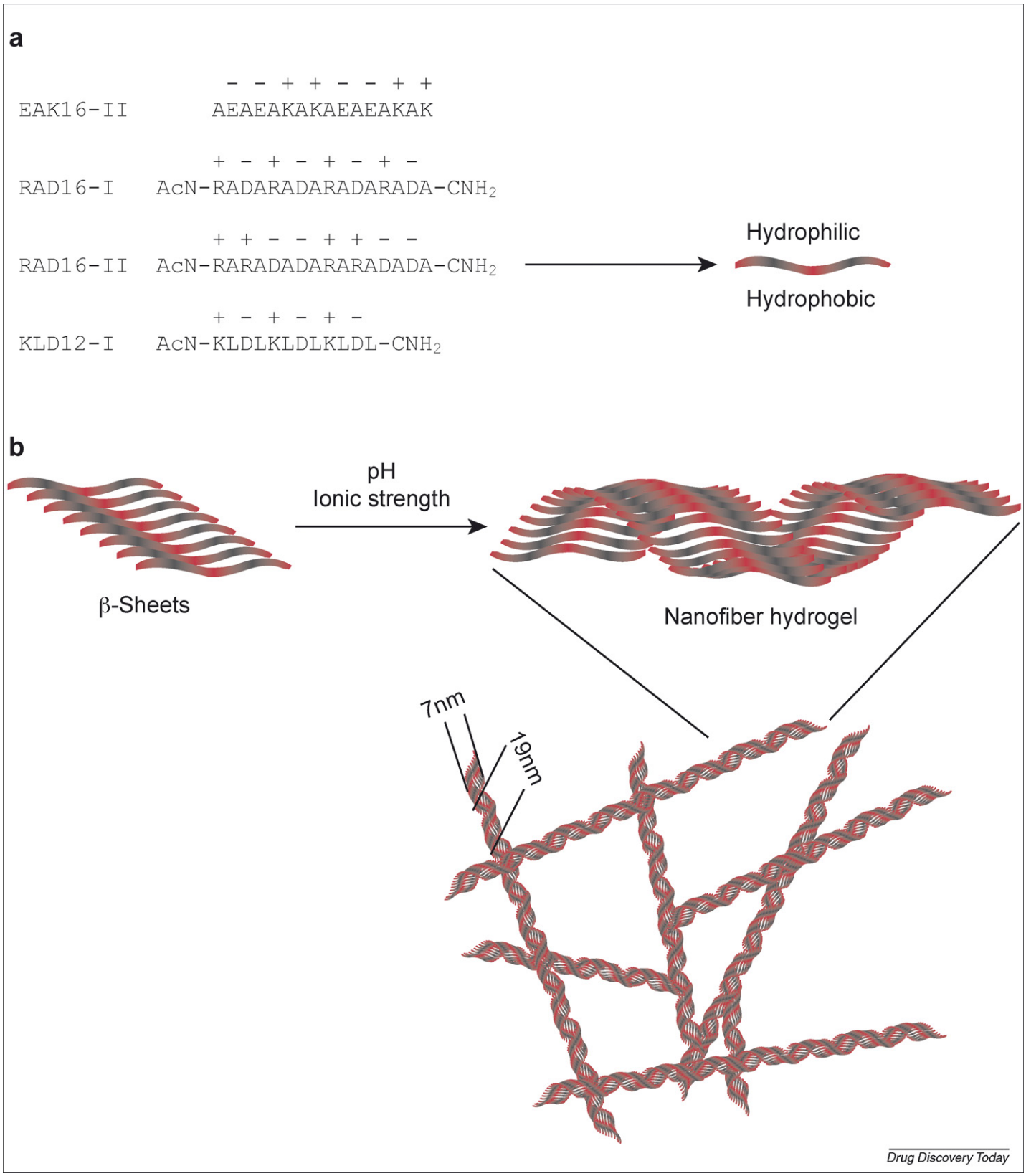


FIGURE 1

Self-assembling peptides form stable nanofiber hydrogels. **(a)** Examples of different sequences of self-assembling peptides. Hydrophobic amino acids (alanine (A) or leucine (L)) alternate with positively (lysine (K) and arginine (R)) and negatively charged amino acids (glutamate (E) and apartate (D)). Hydrophobic amino acids are directed to one side and hydrophilic (positively and negative charged) amino acids to the other side of peptides. **(b)** Self-assembling peptides are arranged in stable β-sheets at low pH and low ionic strength. Upon exposure to physiological pH and ionic strength, they form stable nanofibers with a diameter of ~10 and 50–200 nm pores.

assembling RAD16-I peptides are commercially available (Puramatrix).

Immunohistochemical analysis of self-assembling peptides injected *in vivo* did not show obvious inflammation or immune response [36–38]. Although these results suggest that self-assembling peptides are non-immunogenic, studies testing long-term immunogenity *in vivo* have still to be performed. Furthermore, they support three-dimensional culture of many different cell types. Self-assembling peptides promote growth and capillary organization of endothelial cells [39,40], cell division of chondrocytes and synthesis of glycosaminoglycan by chondrocytes [41,42], osteogenic differentiation of embryonic stem cells [43], neurite outgrowth and active synapse formation *in vitro* [36,44], and differentiation of liver progenitor cells to hepatocytes [45]. The cellular biocompatibility of self-assembling peptides may be related to the size of the fibers. Most biopolymers used for tissue engineering have fiber sizes ranging from 10 to 100 μm, which is similar to the size of most mammalian cells. Essentially, cells grow in these polymers on a curved two-dimensional surface. In contrast, self-assembling peptides form nanofibers of 10–20 nm in diameter, allowing true three-dimensional cell culture more similar to extracellular matrix. Cells can bind to self-assembling peptides with their adhesion molecules, but the size of the nanofibers does not inhibit interaction with other cells in all three dimensions.

One advantage of self-assembling peptides is the extreme flexibility with which they can be designed and modified. For instance, self-assembling peptides can be functionalized by adding adhesion motifs derived from collagen (TAGSCLRKFSTM) or laminin (YIGSR or RYVVLPR) [39]. Those short sequences are present in the basement membrane, can be added during solid phase peptide synthesis, and have been shown to be crucial for endothelial cell migration and morphogenesis [39]. Endothelial cells cultured on self-assembling peptides with adhesion motifs showed enhanced endothelial cell phenotype and higher basement membrane deposition.

Self-assembling peptides and protein delivery

Our laboratory has exploited the design properties of self-assembling peptides for myocardial protein delivery. For example, binding of PDGF-BB to self-assembling peptide nanofibers in a non-covalent manner leads to prolonged delivery of PDGF-BB when injected into infarcted rat myocardium [37,46]. Two weeks after intramyocardial injection, PDGF-BB could still be detected when injected with self-assembling peptides. After myocardial infarction, this protein delivery therapy improved cardiac function, decreased cardiomyocyte apoptosis, and decreased infarct size. On the basis of the PDGF-BB delivery studies [37,46] with self-assembling peptides after myocardial infarction in rats, extrapolations can be made on how much PDGF-BB protein would be necessary in humans. Total myocardial mass of the left ventricle is 200 times higher in humans than in rats. If the concentration of PDGF-BB used is the same (100 ng/ml hydrogel), the volume to be used in humans should be 16 ml (200 times 80 μl). Higher concentrations of PDGF-BB in the nanofiber hydrogel might decrease the volume needed for successful therapy.

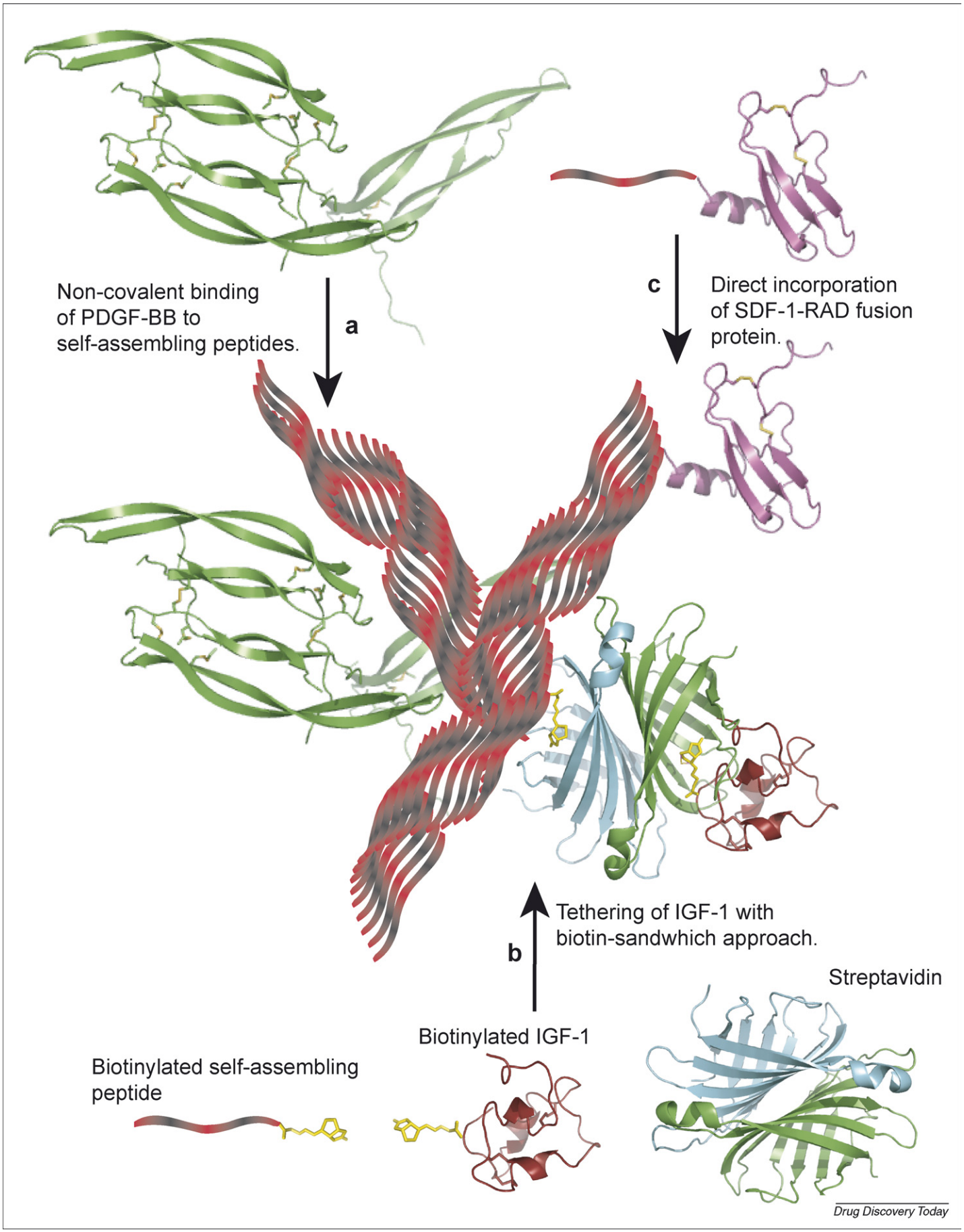
The precise mechanism of the slow release of PDGF-BB has not yet been established. Pore size of self-assembling peptide nanofibers varies between 5 and 200 nm, allowing free diffusion of most nutrients and growth factors. PDGF-BB is a relatively large growth factor, but its largest diameter is less than 8 nm; entrapment of PDGF-BB in the smallest pores is possible, but probably retention is not based on the size of PDGF-BB. Adsorption of PDGF-BB on self-assembling peptides by non-covalent interaction of PDGF-BB with the amphiphilic nanofibers is the most plausible explanation for its slow-release profile (Figure 2a). Because of the small diameter of nanofibers (~10 nm), nanofiber gels have a high surface:volume ratio compared to biopolymers with larger diameter fibers. This high surface ratio permits the adsorption of large quantities of proteins on the amphiphilic surfaces. It has been shown recently that electrostatic interactions determine the release of small dye molecules from self-assembling peptides [47]; acid groups on molecules facilitate electrostatic interactions with RAD16 nanofibers. Moreover, release kinetics can be modified by altering peptide concentrations in the hydrogels: diffusion of molecules is slower in hydrogels with higher peptide concentration [47].

Self-assembling peptides can be modified in different ways, allowing delivery of cell-specific signals or proteins. For example, self-assembling peptides containing an IKVAV epitope (a sequence found in laminin that encourages neurite adhesion and growth) have been synthesized [48]. Self-assembling peptides can be biotinylated, which allows high-affinity binding to any molecule (such as a protein) that can be biotinylated using streptavidin as a linker. This method can be used to deliver biotinylated proteins; for example, biotinylated IGF-1 tethered to biotinylated self-assembling peptides (Figure 2b), *via* injection, into the myocardium and can be detected there for a period of up to 28 days [49]. IGF-1 tethered to self-assembling peptides in this manner improved the survival of cells implanted together with self-assembling peptides into the myocardium.

Self-assembling peptides can also deliver signals by fusion proteins designed with the sequence of self-assembling peptides at the N- or C-terminus to incorporate protein stably in nanofibers (Figure 2c). This technology could be appealing for the creation of chemotactic gradients *in situ*. Fusion proteins of chemokines and self-assembling peptides can be made with a linker sequence susceptible to locally expressed proteases. For instance, matrix metalloproteinase-2 (MMP-2) is activated after myocardial infarction and substrate sequences for MMP-2 are good candidates as linker sequences for this 'smart release' approach. As shown in Figure 3, a gradient in MMP-2 concentration after myocardial infarction could lead to a gradient in the release of chemokine attached to self-assembling peptides.

Self-assembling peptides provide a new technology for protein delivery. However, several issues have to be resolved before they can be implemented into clinical practice. No studies addressed reproducibility and kinetics of protein release by self-assembling peptides yet. It is also unknown to what extent addition of extra amino acids to the peptide sequences alters nanofiber formation. Furthermore, immune response to self-assembling peptides has to be evaluated in a more thorough manner.

Several surfactant-like peptides [50–52] that do not share sequence similarity with peptides described earlier show similar self-assembling properties. They typically consist of a hydrophilic head and a hydrophobic tail, with properties similar to those of phospholipids. Instead of a nanofiber, these peptides form


Non-covalent binding of PDGF-BB to self-assembling peptides.
a
c
Direct incorporation of SDF-1-RAD fusion protein.
Tethering of IGF-1 with biotin-sandwhich approach.
b
Streptavidin
Biotinylated IGF-1
Biotinylated self-assembling peptide
Drug Discovery Today

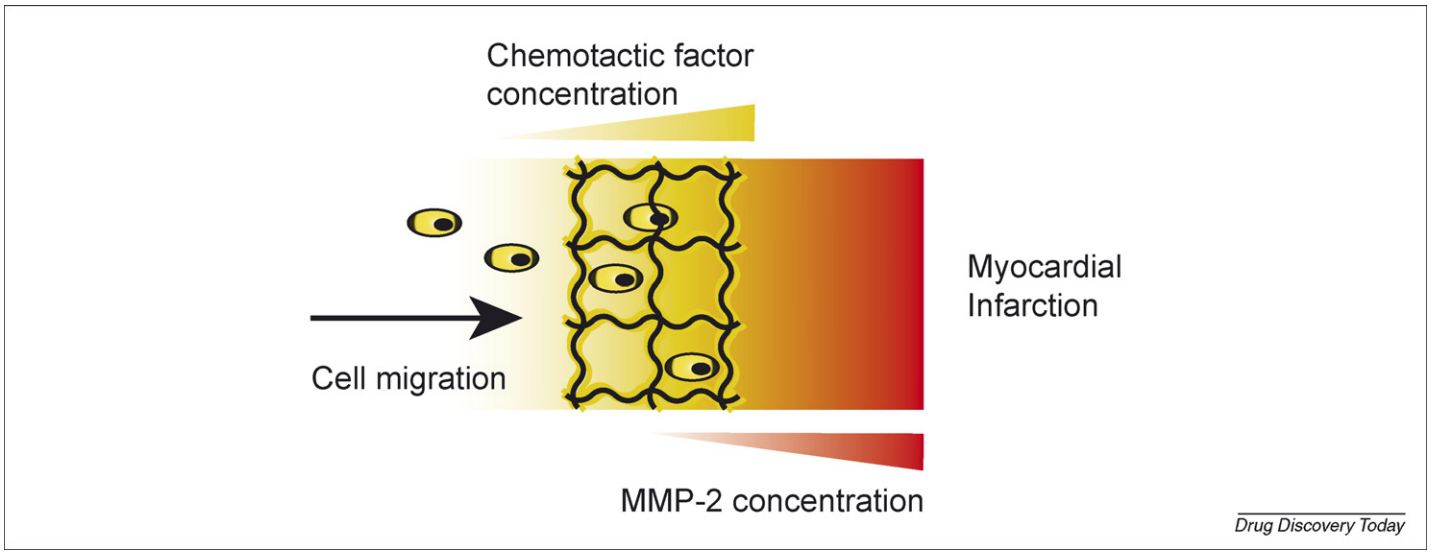


FIGURE 3

Building of chemotactic gradient using self-assembling peptides. Matrix metalloproteinase-2 is upregulated after myocardial infarction. The gradient formed by MMP-2 theoretically induces a gradient of release chemokines if the chemokine is tethered to self-assembling peptides by an MMP-2-susceptible linker. This linker can be incorporated in recombinant fusion proteins of self-assembling peptides and the chemokine to be delivered.

micelles, nanotubes, or nanospheres in physiological solutions. Amphiphilic self-assembling peptides have been used for delivery of FGF-2 *in vitro* and *in vivo* showing release up to 800 h after implantation [18]. FGF-2 delivered subcutaneously using this method increased vascularization of tissue. However, the type of interacting forces between FGF-2 and the self-assembling amphiphilic peptides has yet to be determined. These amphiphilic peptides are theoretically interesting for the delivery of transmembrane proteins. It has been reported that amphiphilic peptides increase stability of transmembrane proteins more than classic detergents do [52]. It has yet to be shown that this leads to increased stability when delivered *in vivo*. As with self-assembling peptides, chemical properties of amphiphilic peptides can be optimized by altering the amino acid sequence: the hydrophilic head can contain anions, cations, or both.

Self-assembling peptides have several advantages: they can be easily produced out of naturally occurring building blocks with high sequence flexibility. They have a high water content (99%) and a small fiber size allowing three-dimensional cell interactions. They are injectable, which allows catheter-based approaches, and form very stable nanofiber hydrogels after injection. They can be biotinylated, or their sequence can be incorporated into fusion proteins for enhanced protein binding. Most disadvantages of self-assembling peptides are related to limited availability of data: although obvious inflammatory responses have not been reported, thorough immunological testing has not been performed. Furthermore, kinetics and *in vivo* reproducibility of protein delivery have not been evaluated yet. Kinetics and extent of *in vivo* degradation are also unknown. Self-assembling peptides are probably good candidates for protein delivery; however, more direct comparisons with other materials will be necessary before one material can be selected above the other.

Conclusions and future directions

Upon injection in a physiological environment, self-assembling peptides form stable nanofiber hydrogels. Those nanofibers are biocompatible because they are non-toxic, non-inflammatory, and degradable. Because they allow non-covalent binding of proteins, they can be used for slow release protein delivery. Release kinetics can be altered by altering charged residues and by self-assembling peptide concentrations in the hydrogel. Proteins can be tethered to self-assembling peptides using avidin–biotin complexes or by construction of fusion proteins. Self-assembling peptides can be engineered by adding extra amino acids during solid-phase synthesis. Because of extremely flexible design capabilities, self-assembling peptides are promising tools for protein delivery and tissue engineering. Self-assembling peptides can not only form a supportive matrix for cell growth but might also be a vehicle for delivery of growth factors to enhance cell survival and differentiation too. Self-assembling peptides also have great potential for attraction of stem cells by building chemotactic gradients. Conditions of tissue injury for which currently no effective therapy exists, such as myocardial infarction or chronic wounds, might benefit from controlled delivery of growth factors or building of chemotactic gradients. In summary, protein delivery by self-assembling peptides is a promising tool for regeneration of injured tissues.

FIGURE 2

Self-assembling peptides allow delivery and tethering of proteins in different manners. **(a)** Self-assembling peptides allow long-term delivery *in vivo* of PDGF-BB. PDGF-BB binds to self-assembling peptides non-covalently by electrostatic interactions. **(b)** Biotinylated variants of self-assembling peptides have been synthesized. They allow binding of every protein that can be biotinylated using streptavidin as a linker. It has been shown that IGF-1 tethered to self-assembling peptides using this methodology improves survival of cardiomyocytes delivered to the myocardium, whereas non-tethered IGF-1 was not effective [49]. **(c)** The sequence of self-assembling peptides can be incorporated at the N- or C-terminus of recombinant proteins, allowing incorporation of proteins in the hydrogel.

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