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Self-assembled peptide-based nanostructures: Smart nanomaterials toward targeted drug delivery

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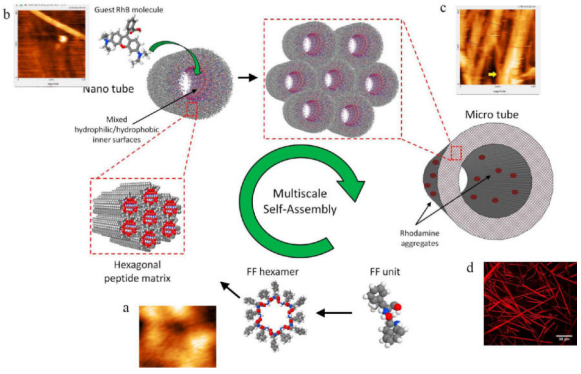
Abstract

Self-assembly of peptides can yield an array of well-defined nanostructures that are highly attractive nanomaterials for many biomedical applications such as drug delivery. Some of the advantages of self-assembled peptide nanostructures over other delivery platforms include their chemical diversity, biocompatibility, high loading capacity for both hydrophobic and hydrophilic drugs, and their ability to target molecular recognition sites. Furthermore, these self-assembled nanostructures could be designed with novel peptide motifs, making them stimuli-responsive and achieving triggered drug delivery at disease sites. The goal of this work is to present a comprehensive review of the most recent studies on self-assembled peptides with a focus on their "smart" activity for formation of targeted and responsive drug-delivery carriers.

Graphical Abstract



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Keywords

Self-assembled; Peptide; Nanostructure; Drug delivery; Smart nanomaterials

1. Introduction

Molecular self-assembly is the spontaneous formation of ordered structures. These processes occur under thermodynamic and kinetic conditions that are a consequence of specific and local molecular interactions [1]. Hydrogen bonding, hydrophobic interactions, electrostatic interactions, and van der Waals forces combine to maintain molecules at a stable low-energy state. Self-association to form hierarchical structures at both the nano and/or microscales occurs in order to achieve these energy minima [2].

Self-assembly occurs spontaneously in nature during protein folding, DNA double-helix formation, and the formation of cell membranes [3]. Self-assembling nanostructures fabricated from natural biomolecular building blocks such as amino acids are highly preferable to their synthetic self-assembled monolayer (SAMs) alternatives [4] due to their biocompatibility and ease of “bottom-up” fabrication [5]. Although several self-assembly platforms have been introduced for biomedical applications, self-assembling peptides remain the most attractive soft biomaterial option for several reasons:

- **I.** Peptides are easily synthesized using solid-phase methods, which allows for sequence-specific modifications at the molecular level [6].
- **II.** Additional peptide functionalization can easily be performed by introducing compounds such as antibodies, enzymes, magnetic particles, or fluorescent compounds to the peptide structure [7].
- **III.** Custom supramolecular structures can be designed through engineering of self-assembled peptide building blocks [6].
- **IV.** Naturally occurring self-assembly motifs present in proteins such as α-helices, β-sheets, and coiled-coils can be used to drive the self-assembly process [8].
- **V.** Peptides are the most attractive biomaterials for regenerative scaffolds, since the main “signaling language” in the extracellular matrix (ECM) is mediated via peptide epitopes [9].
- **VI.** Self-assembly is important in cell-penetrating peptide (CPP) mechanisms, which play a major role in introducing drugs inside cell membranes and translocating genes inside a nucleus [10].

Though there has been several reviews on peptides and their self-assembling properties, most are focused on tissue engineering rather than drug-delivery applications [11-13]. Therefore, there is a lack of comprehensive reviews on the use of self-assembled peptides as “smart” drug-delivery platforms that are capable of specific tissue or cellular targeting, and release of therapeutic components in response to environmental cues. The focus of this review is on factors that govern self-assembled peptide (SAP) targeting activity, and controlled-release properties. We aim to provide insight into how SAPs can be engineered

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into smart drug-delivery platforms that exhibit enhanced biological functions, such as intracellular and targeting uptake, controlled release, and reversible enzymatic hydrogel formation. Finally, we also cover a broad range of self-assembled peptides and peptide derivatives. We believe that this review highlights the importance of self-assembled peptide nanostructures for nanomedicine applications and can facilitate further knowledge and understanding of these nanosystems towards clinical translation of such therapeutic materials.

2. Self-assembling peptides

Self-assembled peptides were categorized according to their building blocks: into peptides and peptide derivatives. In the section on peptides, naturally occurring peptide motifs (α-helical, β-sheets, β-hairpins, etc.,) are discussed and in the section on peptide derivatives, self-assembled peptides such as peptides amphiphiles (PAs) with alkyl chains are introduced. Relevant examples are presented to highlight the importance of these structures as basic building blocks for targeted drug-delivery carriers.

2.1 Peptides

2.1.1 α-helical peptide /Coiled coil—For decades it has been known that physical and biological properties can promote the formation of helical structures. However, with the advent of material design, only recently have key molecules been discovered in order to incorporate these helical structures into biomaterials.

The α-helical structure results from hydrogen bonding between backbone amides that form right-handed α-helices with a periodicity of 3.6 residues per turn. Interaction with other helices is possible through the side chains of the amino acids involved, as they protrude outwards from the helix. However, it is challenging to produce these structures in practice, in part because longer lengths (20-30 amino acid) are usually required to establish stable α-helical interactions.

Coiled-coil structures are formed through the assembly of α-helices into higher ordered structures. These architectures form due to the repeated pattern of hydrophobic and charged amino acid residues. In 2009 Smith *et al.* reported a self-assembled hydrogel [14]. This novel design consisted of two complementary leucine-zipper peptides (SAF-p1 and SAF-p2), which co-assembled into a sticky-ended dimer with complementary overhanging ends. Multiple weak interactions between the fibers prompted the formation of the hydrogel, which was shown to support PC12 cell adhesion and proliferation into neurons [14].

2.1.2 β-sheet—β-sheets are the most common natural motifs that can be used in driving the self-assembly of peptides. β-sheets consist of sequences that possess alternating hydrophobic and hydrophilic amino acids, providing the peptide backbone an amphiphilic property that directs formation of β-sheets. Fishwick *et al.* proposed that the P11-II peptide sequence QQRFQWQFEQQ and its derivatives form twisted β-sheet tapes, naturally reinforced by the amphiphilic nature of the sequence. These β-sheet tapes are triggered to form hydrogels by screening the charges between fibers [15].

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2.1.3 β-Hairpin—Orientation of two β-sheets in anti-parallel directions results in formation of β-Hairpins in proteins. Shneider *et al.* [16] proposed a β-Hairpin structure design with the sequence VKVKVKVKVDPPTKVKVKV, where DP is an enantiomer of proline. This peptide possesses an alternating hydrophilic-hydrophobic motif and intermittent tetrapeptide (-VDPPT-) intended to form a type II′ turn structure. This design allows intra-molecular folding and β-sheet formation, yielding a "hairpin" structure that can subsequently associate into higher-order fibers and self-supporting hydrogels when the pH is raised. These structural designs were used to form responsive hydrogels, linking pH intra-molecular folding to self-assembly.

The β-hairpin MAX1 presented by Schneider *et al.* exhibited inherent antibacterial activity against both Gram-positive and -negative bacteria, likely due to the material disruption of the bacterial cell membrane [17]. Analogously, most cationic peptides that form α-helices or β-sheet-like structures could insert into and thus disintegrate negatively charged bacterial cell membranes [18].

2.1.4 Triple helical peptides (Collagen mimetic peptides)—Collagen is the most abundant protein in the human extracellular matrix (ECM), interacting with cell-surface integrins for adhesion [6]. Collagen isolated from animals has been extensively used for engineering numerous tissue-mimicking scaffolds in vitro. However, due to the complex characteristics of collagen fabrication, creating hybrid scaffolds that combine collagen with other biomaterials is difficult. Other challenges including immunogenicity, poor mechanical properties, and the lack of tissue-specific adhesion ultimately limit the utilization of purified collagen for making tissue-engineered scaffolds. To recapitulate native tissue architecture and chemical composition, studies have been carried out using a "bottom-up" approach to mimic the structure and functionality of collagen proteins with shorter peptide sequences [6].

Earlier studies of collagen mimetic peptides (CMPs) based on the sequence –(Pro–Hyp–Gly)x– or –(Pro–Pro–Gly)x– (where Hyp=hydroxyproline) have used models exploring the structure and stability of collagen triple helices [19]. A collagen fiber is constructed when three peptide chains come together to form a triple helix. Building new biomaterials is possible once the subunits assemble into larger fibers that form crosslinks, which generate 3D scaffolds. To construct 3D scaffolds, CMPs have been incorporated in hydrogels such as photo-polymerizable poly (ethylene oxide) diacrylate PEODA or PA molecules [20]. Hartgerink *et al.* was the first to develop hydrogels based purely on peptide interactions. They initially developed these structures using trimers with sticky ends capable of directing the assembly of larger fibers into scaffolds (Fig. 1.) [21]. These gels had mechanical properties similar to those of native collagen gels, and could even be degraded by collagenase enzymes. These mimetic peptides have tremendous applications in drug-delivery systems, since they can be combined with therapeutic drugs or cells and then injected into tissues, where they can self-assemble into stable gels capable of delivering therapeutic components.

2.1.5 Di-phenylalanine (FF)—Di-phenylalanine (FF), with the structure L-Phe-L-Phe, is a peptide building block associated with the pathogenesis of Alzheimer's disease. It was

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identified from Alzheimer's β-amyloid polypeptide studies, where it was proposed as the core recognition motif able to guide self-assembly [22]. Since Reches *et al.* reported that di-phenylalanine produces nanotubes [23], many studies have been carried out to form a variety of functional nanostructures from FF-based building blocks such as nanotubes, spherical vesicles, nanofibrils, nanowires, and ordered molecular chains (Fig. 2). The application of FFs includes bioimaging, biosensors, guest encapsulation, nanofabrication as well as drug delivery.

FF is an aromatic dipeptide that displays interesting features as a building block for tubular nanostructures. In its self-assembly, cyclic hexamers are formed with six FF units (Fig 3). Subsequently, stacking of hexamers produces narrow channels, which leads to the formation of self-assembled sheets due to hexagonal packing. The coiling of the sheets produces nanoscale tubes with hydrophobic external walls. Finally, these nanotubes can be self-assembled on even larger scales, forming bundles. The self-assembly processes are illustrated in Fig 3. The backbone hydrogen bonds and π-π bond interactions from the aromatic peptide side-chains hold the self-assembled FF structures together. The interactions between the side-chain aromatic rings create well-ordered tubular structures of significant length (100 μm) [5, 24].

FF nanotubes have been transformed into spherical vesicle structures by diluting the solution of FF in pH 7. It should be emphasized that different morphology can be created by simple modification of the solution's parameters, such as the solvent used and at level of concentration. In fact, concentration plays a critical role in defining the final nanostructure morphology. The transition from FF nanotubes to vesicles is reversible and dependent on FF concentration. This is attributable to the sufficient free energy association gained by intermolecular interaction at high concentrations of FF (100 mg/ml), while decreased concentrations disassemble the organized arrangement (Fig. 4) [25]. Yan *et al.* has described, in more detail, the various nanostructures derived from FF, their self- assembly mechanisms, and their potential applications [5]. Despite the tremendous advantages of FFs as basic building blocks, research on FFs as drug-delivery systems has been scarce.

In 2013, Silva *et al.* reported utilizing FF peptide nanotubes for loading rhodamine. This platform was intended as a model system conjugating a hydrophilic drug to self-assembled peptide arrays [24]. The aim was to obtain data related to the location of the drug (based on its hydrophobic characteristics) on peptide arrays. There was clear formation of microtubular needles with average diameter of 2.2 μm (Fig. 5a). The homogeneous distribution of RhB fluorescence across the needles suggests uniformity of drug conjugation with the peptide structures (Fig 5a). For detailed analysis, colocalization was performed by labeling the nanotubes with both ZcPc (a highly hydrophobic reagent colored green) and rhodamine (a hydrophilic drug colored red). Typical drug distribution images are displayed in Fig 5 b, c. The surface of the nanotubes was covered with the non-polar dye (green) and was co-located with RhB at various points. These results suggest that the RhB was conjugated not only to the external surfaces of the arrays but also to the inside of the structures. Atomic force microscopy (AFM) results showed FF microtubes (FFMTs) carrying cargo in either of two conformations: either as small aggregates located on the surface or homogeneously distributed within the structure (Fig. 5 e, f). These results are attributed to the arrangement of

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polar and non-polar groups in the organization of nanotube arrays. The external and internal walls of the FFMTs are highly hydrophobic. The walls are composed of aromatic rings that make up each of the two FF side chains. Alternatively, in the peptide matrix, additional sites could host hydrophilic moieties. Therefore, both hydrophilic and hydrophobic compounds could be hosted in these FF nanotubes, giving them an advantage over other platforms [24]. However, FF peptides are still better overall carriers for hydrophobic components due to their large number of hydrophobic aromatic functional groups [24].

2.2 Peptide derivatives

2.2.1 PAs with alkyl tail—The most well-known PAs are molecules with hydrophilic peptide sequences linked to a hydrophobic tail. According to a definition proposed by Kunitake in 1992, a typical PA consists of a hydrophobic tail, a linker, a spacer, and a hydrophilic head [26]. These kinds of amphiphilic peptide molecules have four regions: 1) amino acids (charged residue) or hydrophilic peptides; linked to 2) hydrophobic alkyl chains or lipid tail; 3) β-sheet sequence; and 4) biological epitope for desired function (Fig. 6) [6].

Once a PA with an alkyl chain is exposed to an aqueous solution, the hydrophobic tail of the peptide shields itself from water to induce and/or stabilize a three-dimensional structure of a peptide head group, driving the self-assembly process to form protein-like molecular architectures. This process is similar to spontaneous protein folding. As a result of this self-assembly process, various types of structures such as nanofibers [8], micelles and vesicles [27], nanotapes [28], nanotubes, and ribbons are constructed to reduce the unfavorable interactions of their hydrophobic components with their surroundings. For example, introducing β-sheet sequences into the structure of PAs often results in the generation of cylindrical nanofibers with diameters ranging between 6-10 nm and length up to several micrometers.

One of the first PAs was described by Berndt *et al.* in 1995 [29]. The design consisted of a peptide sequence derived from collagen (a1(IV) 1263–1277 (called IV-H1), conjugated onto C12, C14, and C16 mono- and dialkyl tails to form amphiphilic collagen-like peptides. These structures are usually generated as a monolayer at the air–water interface.

The morphology, functionality, and surface characteristics of PAs can be altered simply by changing the structural elements of the amphiphilic molecules. Stupp's group played a significant role in pioneering various PA molecules based on their understanding of the self-assembly mechanism of nanofibers for tissue-regeneration applications [30]. In 2001 Hartgerink *et al.* developed PAs that allowed mineralization with hydroxyapatite, which mimics the bone ECM to create bone-interfacing biomaterials [21]. The PA molecules consist of five regions: 1) alkyl tail; 2) cysteine residue for polymerization; 3) glycine residue for the hydrophilic head group; 4) phosphorylated serine residues to interact with the calcium of hydroxyapatite; and finally 5) a cell-adhesion motif consisting of Arg-Gly-Asp RGD. The design of the PA allows formation of nanofibers with reversible cross-linked activity. In this case, the phosphorylated residue of the PA provided the possibility of interaction with hydroxyapatite. Therefore, the fibers were able to guide mineralization of hydroxyapatite. This alignment was shown to be similar to interactions between

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hydroxyapatite crystals and collagen fibrils in natural bone, demonstrating the potential of PA molecules for mimicking the architecture of ECM fibers [21].

In 2010 Zhang *et al.* discovered another remarkable application of PA, which allows cell encapsulation for applications such as blood-vessel engineering [31]. The PA molecules in their study were made from a C16 alkyl tail and a V3A3E3 (CO2H) peptide sequence and were able to self-assemble and form a gel. When sheared gently (e.g., as by the force of pipetting), these PA solutions formed aligned monodomains (Fig. 7a); and when divalent cations like calcium were added, the fibers formed string-like “noodle” gels with fiber alignment over macroscopic distances (Fig. 7c). The strings of aligned nanofiber gels were used to direct the orientation and alignment of mesenchymal stem cells (hMSCs) and encapsulate them in 3D culture (Fig. 7b). The process of string formation did not affect cell viability, and within 12 hours the cells started to elongate along the string axis. Thus, these PAs could eventually support advanced therapies that require guided cell growth, migration, or spatial cell interconnections in numerous tissues that include the spinal cord, heart, and brain.

The utility of PA for drug delivery has also been investigated by Fernandez-Carneado *et al.* from Giralt's group. They were able to combine a proline-rich sequence (i.e., a family of cell-penetrating peptides or CPPs) with fatty acid moieties for internalization in HeLa cells [32]. Flow cytometry and confocal laser scanning microscopy indicated that including fatty acids into the CPP structure significantly improved internalization. Studying the interaction of these peptides with model dioleoylphosphatidylcholine (DOPC) membranes demonstrated that the identity and length of the fatty acyl moieties are crucial for cell membrane penetration. Therefore, using C6 fatty acids did not lead to any improvement in internalization. This highlights the importance of the structural design of PAs for their targeted drug delivery [32].

Sardan *et al.* designed a cell-penetrating arginine-rich peptide with a lauryl structure (C12) - PPPPRRRR-NH2, integrated non-covalently into liposomal structures to improve bilayer penetration of MCF7 cancer cells. The amphipathic properties of the peptide eliminated the need for crosslinking methods used for integration into liposome formulations [33]. The effectiveness of peptide integrated liposomal system as drug delivery platforms was investigated by using the anticancer drugs doxorubicin-HCl and paclitaxel. Compared to free drugs, peptide-functionalized liposomes had enhanced cell uptake.

In 2012 Matson *et al.* designed four PAs with hydrazone groups linked to ketone-containing model drugs such as prodan, via hydrazine linkages [34]. The peptide-based C16-V2A2E2 sequence self-assembled into hydrogels. For example, by controlling hydrazone hydrolysis, sustained drug-release profiles could be attained in an aqueous medium at physiological pH. However, based on their results it was concluded that the packing density of the assembled nanofibers of PAs was the most significant factor controlling drug-release kinetics. Furthermore, as the hydrophobic PA core and β-sheet activity and order decreased, the release rates increased.

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In 2008 Cui *et al.* demonstrated an interesting morphologic transformation of PAs consisting of a 2-nitrobenzyl group, a palmitoyl tail, and an oligopeptide GV3A3E3. The N-terminal amide of the glycine residue was covalently attached to the 2-nitrobenzyl group and was susceptible to cleavage by 350 nm irradiation. Interestingly, the quadruple helical structure was transformed to a typical cylindrical nanofiber upon irradiation at 350 nm, indicating its potential use as a responsive system for a broad range of targeted delivery medicine [13].

2.2.2 PAs with only amino-acids (self-complementarity)—PAs composed solely of amino acids contain both hydrophilic and hydrophobic domains organized in an amphiphatic sequence. In PAs with an alkyl chain, the self-assembly process is governed by helices, while ionic interactions play a secondary role. In these types of peptides, however, electrostatic interaction plays a major role. The structure of these peptides is based on the self-complementary nature of ionic bonds within alternative repeating units of negatively charged amino acids (i.e. Glu and Asp) and positively charged amino acids (i.e., Lys and Arg) [35] Once exposed to aqueous solutions, the hydrophobic part shields itself from water, driving the process of self-assembly. This approach was first demonstrated by Zhang *et al.* in 1993 with the sequence (AEAEAKAK)2 derived from the yeast protein zuotin [36]. The alternating hydrophobic region and charged amino acid region forms a β-sheet structure together with ionic bonds between positively charged K and negatively charged A side chains. Later, a new structure was introduced termed (RARADADA)2 or RADA16, which replaced the E residues with D and the K residues with R and was used widely to create hydrogel scaffolds for controlled release of proteins and small molecules.

2.2.3 Small-molecule peptides with aromatic tail—In 2009, Xu proposed formation of supramolecular hydrogelators from small molecules. Through several non-covalent interactions that include π-π stacking, hydrophobic interactions, hydrogen bonding and van der Waals forces, small organic molecules can form gels. These are referred to as molecular hydrogelators since they can interact with each other to produce gels [37]. Recently, several groups have started to develop “self-delivery” systems based on hydrogelator principles, where the goal is to use therapeutic agents [31, 38] to form gels with superior pharmacokinetic profiles. Currently, one of these hydrogelator systems was even approved for clinical use by the FDA [39].

In addition to commercial drug formulations, several therapeutic agents that include drug candidates such as lanreotide, Ganirelix, Biotin, and F-moc L-lysin have been used to form hydrogelators [37]. Some of these candidates can by themselves undergo spontaneous self-assembly into supramolecular hydrogels, while others require additional functionalization or structural modifications. Using enzymes such as β-lactamases and phosphatase/kinases as models, Yang *et al.* illustrated the formation of supramolecular hydrogels. The design and tuning of enzyme-catalyzed hydrogelators could offer novel strategies for screening enzyme inhibitors and developing drug-delivery systems. Yang *et al.* introduced the concept of producing anti-bacterial effects from within the cell itself. They accomplished this through enzyme-regulated self-assembly of small molecules inside cells [40].

Similarly, using Fmoc-FF and Fmoc-RGD, co-assembly of these aromatic peptide amphiphiles can generate bioactive hydrogels. These small molecule–based hydrogels can

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be used to mimic extracellular matrix. Ultimately, when combined with cells such as dermal fibroblasts, these hydrogels can form dense fibrous networks through the robust secretion of ECM from cells even within these simple Fmoc-FF/RGD scaffolds [41].

2.3 Peptide Synthesis Routes

For more than a century, peptide synthesis has had a significant impact in several fields including pure organic synthesis and protein chemistry. The formation of peptides usually occurs via repetitive amidation reactions. The N-terminal amino group and the carboxylic group of an incoming amino acid are coupled to produce the peptide bond. Bruce Merrifield was one of the pioneers of utilizing insoluble resins as support during the synthesis of peptides that could then be cleaved off at the end of process to isolate the final peptide in solution [42, 43]. Quickly these strategies were adopted in solid-phase peptide synthesis. However, remaining challenges include matrix swelling, limited selection of linker groups, and the necessity of using protecting groups for some amino acids. Over the last 15 years, advances in the peptide industry have lowered the cost of raw materials and improved the chromatographic methods used for peptide purification. It should be mentioned that the number of animal-derived peptides on the market is decreasing implying that the solid phase methods are more economics. In addition, the use of enzymatic procedures (reversed proteolysis) to synthesize specific sequences is gaining renewed interest [44].

3. Biomedical applications of self-assembling peptide-based nanostructures

3.1 Controlled and triggered-release from stimuli-responsive hydrogels

Bioactive peptide molecules capable of self-assembly into ordered supramolecular structures are ideal building blocks to form hydrogels, scaffolds, and nanofiber gels for use in tissue engineering, regenerative medicine [45], and injectable drug formulations [46] [47]. The interest in utilizing self-assembled systems stems from their advantages over other polymers in forming hydrogels.

Peptide-based hydrogels are fully biocompatible and capable of incorporating functional molecules into their scaffolds [48]. Manipulating these structures at the molecular scale offers the remarkable capability of tuning some features such as chemical material, viscoelastic and mechanical properties. Incorporating bio-functionality into the material ligand and metal recognition of metal and ligand, biodegradability and biocompatibility is now possible through employing peptide-based molecular building blocks for self-assembly [49].

This technology also allows specific sequence modifications at the molecular level that eventually affects the bulk properties of the hydrogel. Thus small amino-acid sequence variations can be used to tune material properties, and comparatively easily, through solid-phase methodology. Thoughtful design of individual molecules used in preparation of biological scaffolds via self-assembly makes it possible to determine the properties of the larger material [47].

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Preparation of self-assembled hydrogels from short peptides relies completely on self-assembly, which takes advantage of the intra-molecular folding of specific peptides. This process eliminates the need for the toxic cross-linker reagents commonly necessary for building hydrogels from high-molecular-weight polymers thorough chemical crosslinking techniques.

Importantly, these types of peptides are designed such that release properties are responsive to intramolecular folding events. Advanced drug carriers need to be biocompatible, robust, capable of slow release at specific times through the entire path of delivery, and must have triggered release based on the requirements of the therapy. In this regard, increased therapeutic efficacy could be achieved by designing peptides that are stimuli responsive and capable of controlled release [50].

Self-assembling peptide hydrogels are an important class of hydrogels. They are potentially good choices for preparing a strong drug delivery system able to respond to external stimuli under variable physiological conditions of temperature and pH [50]. Discussed here are the capabilities of self-assembling peptide molecule designs amenable to tailoring for the specific needs of therapy.

3.1.1 Stimuli-responsive hydrogels with physically encapsulated drugs—The KLD12 peptide (Ac-KLDLKLDLKLDL) is known for its alternating hydrophobic and ionic hydrophilic amino acids, which can form established β-sheet hydrogel structures in aqueous solutions [51]. Based on this, Law *et al.* designed a peptide-based hydrogel KLD12 that is sensitive to protease and contained a protease-cleavable region in the self-assembling motif [52]. The peptide hydrogel, when exposed to enzymatic treatment, released its drug via a model therapeutic pro-apoptotic peptide. Further use of self-assembled peptide sequences could allow release of therapeutics upon explicit interaction with proteases associated with disease.

Yishay-Safranchik *et al.* examined the design of a peptide hydrogel formed from self-assembling KLD motifs with two separate β-sheets linked with three or four glycine spacers to prolong the release of doxorubicin (DOX) and Smac (second mitochondria-derived activator of caspases)-derived pro-apoptotic peptide (SDPP) [47]. The two separate β-sheets act as a cross-linker for the hydrogel, which prolongs the release rate, while a penetrating sequence (harboring Lys, KRRMKWKKK (FITC)) was added to the C terminus to increase uptake of hydrogel by cells. The hydrogel could be injected in situ, advancing both the safety and effectiveness of cancer chemotherapy.

A novel hydrogel scaffold formed from self-assembling RADA16 has been reported by Zhang *et al.* [35]. RADA16 is characterized by a stable β-sheet structure consisting of systematic repeating units of positively charged (arginine or lysine) and negatively charged (glutamate or aspartate) residues disjointed by hydrophobic residues (alanine or leucine) (Fig. 8a). The scaffold is formed once it is exposed to physiological solutions and consists of more than 99% water (Fig. 8d.). The scaffold remains fully biocompatible due to fact that no chemical or physical treatments are required. Controlled release of small molecules such as bromophenol blue, phenol red, 8-hydroxypyrene, and Coomassie Brilliant Blue G-250

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(CBBG) from RADA hydrogel scaffolds was studied using diffusion models. The release kinetics correlated to the chemical structure of the compound and the concentration of gel provide an alternative means of controlling release kinetics. The results indicated that release profiles can be tailored by controlling nanofiber-diffusing molecular-level interactions [50].

A variety of proteins, such as lysozymes, trypsin inhibitors, BSA, and IgG were also encapsulated within the Ac-(RADA)4-CONH2 peptide hydrogel and released slowly [53]. The peptide solution that is combined with proteins with therapeutic properties may also be applied to a certain tissue and release its load locally over time.

Elastin is a highly elastic protein in connective tissue that helps many tissues resume their shape after extending or contracting. Recombinant techniques allow one to create protein-based materials that exhibit some feature found in natural proteins together with other properties of technological/medical interest. One of the most interesting protein-based materials is the family of elastin-like recombinamers (ELRs), which can form a variety of structures such as nanoparticles, nanofibers, films, and hydrogels.

Alicia *et al.* designed a silk-elastin-based injectable multiblock corecombinamer that spontaneously forms a physically stable nanofibrillar hydrogel under physiological conditions. The idea was to create a system as the basis for developing injectable fibrillar biomaterial platforms toward a fully functional, biomimetic, artificial extracellular matrix, and cell niches [54].

Machado *et al.* investigated the use of ELRs for the transplantation of autologous retinal pigment epithelial (RPE) cells for treatment of age-related macular degeneration, since they were shown to maintain the functional features of (RPE) cells [55].

Inspired by the cells' structure, Torre *et al.* [56] presented compartmentalized capsules responsive to magnetic field and temperature. The microcapsules were coated with a temperature-responsive chitosan/ ELR nanostructured shell. These engineered systems can be applied for transportation of bioactive agents and cells.

The effects of pH on intramolecular folding of β-hairpin peptide self-assembled hydrogels were studied by Schneider *et al.* [49]. Infrared spectroscopy and circular dichroism showed that at low pH, individual peptides in the hydrogel were unstructured, resulting in a low viscosity and aqueous solution. Thus, varying the intramolecular folding of small individual peptides on the nanoscale can alter macroscopic properties [49]. β-hairpin self-assembled hydrogels were also used to encapsulate curcumin; an anti-inflammatory antioxidant and anti-tumorigenic polyphenol derived from a plant, whose therapeutic use is limited by poor solubility. The peptide hydrogel has been proven to be an effective carrier for the localized, consistent delivery of curcumin over time [57].

One challenge to the controlled-release use of self-assembled nanofibers is their extreme resistance to degradation by protease. Collier *et al.* designed self-assembling depsipeptides that contain ester bonds within the peptide backbone; these degraded in a period of days to weeks by ester hydrolysis [58].

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3.1.2 stimuli-responsive hydrogels with chemically conjugated drugs—In the past decade, supramolecular hydrogels, with networks of nanofibers formed through the self-assembly of small molecules (i.e., hydrogelators), have proven to be promising biomaterials. Peptide-based hydrogelators have proven their effectiveness due to their ability to produce a large set of various functional molecules from a small array of residues. The combination of enzymatic reactions with self-assembly of small molecules offers an effective method to form nanofiber networks, which results in hydrogels under a variety of conditions.

Studies have been undertaken by several groups including Xu`s and Yang`s, who have been investigating development of supramolecular hydrogels from self-assembly of peptides with chemically conjugated drugs [59] [40] [60] [61].

Li *et al.* connected Taxol covalently with a self-assembly motif and also group cleavable by an enzyme. A precursor has also been designed for developing a Taxol hydrogel without compromising the activity of the Taxol [59]. This approach holds promise as a general method to generate nanofibers of therapeutic molecules with a dual role: delivery carrier as well as drug.

In another study, tripeptide derivatives containing a naphthyl group, two phenylalanines, and one modified lysine residue were conjugated to an olsalazine moiety (substrate of azo reductase), which self-assembled into hydrogel under mildly acidic conditions. The reduction of olsalazine not only led to a transition from gel to solid phase, but also released 5-aminosalicylic acid, which is anti-inflammatory agent. This approach will eventually yield new biomaterials for site-specific drug delivery.

One of The first molecular hydrogelator system was reported by Mao, *et al.* in 2011; it was made of two anti-cancer drugs that complement each other. In this study Dex-FFFK(Taxol/HCPT)E-ss-EE was designed and synthesized as a precursor of molecular hydrogelators. The principle of this novel design included Dexamethasone regularly applied to drive self-assembly (also an anti-inflammatory and immunosuppressant used after chemotherapy); dipeptide and tripeptide of phenylalanine (F) to form molecular hydrogelators; and finally the disulfide bond, acting as a cleavable linker connecting the hydrophilic parts and molecular hydrogelators. This structures could be applied in the cavity after surgical tumor removal for the long-term release of anti-cancer drugs [60]. This injectable self-delivery system contains a high weight percentage of anti-cancer drugs and can prolong their release [62]. Further, Wang *et al.* found that even the conjugates of Taxol with amino acids containing hydrophilic side chains such as serine, arginine, and glutamic acid were also efficient candidates for formation of molecular hydrogelators [61].

Ultra-short peptides with the ability to form hydrogels were functionalized with oxaliplatin (a platinum-based anticancer therapeutic). The oxaliplatin-peptide conjugate was tested for localized breast cancer therapy, and the injectable gel showed significant tumor growth inhibition [63].

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3.2 Enhanced cellular uptake with targeting activity

One of the most desirable properties of a drug carrier is to bind specifically with the target site [9], whether cell membranes [64] or molecules of interest. To this regard, peptides represent the most important biological recognition motifs. As an example, NTFR and RGD PAs have been widely used by researchers to bind fractalkine and $a_5\beta_1$ integrins. Self-assembled peptides are used to enhance the functionality of common motifs and integrins. Cell-penetrating peptides (CPP) play an important role in introducing drugs inside cell membranes and translocation of genes inside the nucleus. Self-assembly is key to CPP penetration mechanisms. Furthermore, the self-assembly process can produce various structures suitable for specific delivery and loading of a vast array of drugs. In this regard, PAs (which contain an alkyl chain) mimic the capabilities of lipid systems, thus providing improved drug-delivery through cell membranes. Self-assembled peptides have also been used to functionalize conventional drug delivery carriers, such as liposomes, for enhanced cellular uptake and targeting [65]. In addition, folate-targeting functions have been investigated for anticancer drug delivery [66]. Functional groups of self-assembled structures act as a template for the alignment of recognition molecules [67]. Self-assembled peptide motifs play a large role in increasing targeting activities of drug carriers.

3.2.1 Targeting function to improve uptake—Drug delivery carriers designed with peptide motifs have been extensively studied for targeted delivery. PAs have been designed to enhance the targeting activities of common recognition motifs. For example, researchers have initially applied RGD (the primary recognition site for α5β1) peptide-based techniques to target the integrin α5β1 (significantly up-regulated in tumor vasculature). However this process has limitations, since RGD recognizes several integrins, making specific targeting difficult.

Garg *et al.* designed a novel peptide-amphiphile sequence namely PR_b that closely mimics the cell adhesion domain of fibronectin [65]. The design structures included a C16 dialkyl ester tail, a glutamic acid (Glu) tail connector, a – (CH2)2– tail spacer, and a peptide headgroup. The peptide headgroup contained a spacer KSS, PHSRN (the synergy site for α5β1), a linker (SG)5, and RGDSP (the primary recognition site for α5β1) (Fig. 9.). The KSS spacer was used to keep the bioactive region further away from the interface so the availability of the peptide could enhance the binding affinities to the targets [9, 68]. The PR_B peptide-amphiphiles were incorporated on stealth liposomes in order to target integrin $\alpha_5\beta_1$, which is expressed in colon cancer cells. Functionalized liposomes were covered with polyethylene glycol (PEG). The role of PEG is to allow the liposomes to circulate in the blood, while the modified PR_b peptide specifically recognizes and binds to cells expressing α5β1. Functionalizing stealth PEG-liposomes decorated with PR_b peptide-amphiphile [69] were found to function better than RGD targeting, providing better binding and greater uptake of liposomes by colon cancer cells. The ability to design a peptide by sequence-specific modifications at the molecular level allows control of the activities of common motifs for desired applications [9].

3.2.2 Membrane penetration function to improve uptake—Cell-penetrating amphiphilic peptides such as HIV-Tat based peptides, oligoarginines, and chimeric cell-

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penetrating peptides have also been applied alone or in combination with other self-assembled peptides for the delivery of therapeutic cargos to their targets [70].

CPPs have been used as drug delivery vehicles, because they are able to translocate the cell membrane [71]. CPPs, which are cationic short peptides of fewer than 30 amino acids, and oligoarginine-based CPPs the length of 8-10 arginine residues have shown the most efficient membrane penetration [72].

From the classic mechanism [72] the membrane penetration of CPPs is not associated with their self-assembly. Membrane penetration is based on the hydrogen bonding interaction between the guanidinium groups of arginine residues and the carboxyl, phosphoryl, or sulfuryl groups of the carbohydrates and phospholipids on the cell surface (Fig. 10). Initially the pathway of CPP translocation through membranes was defined via receptor- and endocytosis-independent mechanisms, but now, a novel CPP internalization mechanism has been demonstrated. The role of self-assembly in these peptides and their ability to traverse cell membranes was discussed by Pjulas *et al.* [10].

A third helix peptide (Antennapedia Homeodomain) was reported to translocate biological membranes. It was found that after internalization into E15 rat embryos, the peptides were recovered as a multimeric complex. The results suggest that CPP could internalize through cell membranes either in monomers or self-assembled defined aggregates. Moreover, some important studies demonstrated that the retaining of their conformation by CPPs after self-assembly is the key to their membrane-penetrating abilities.

The effect of self-assembled aggregates on DNA encapsulation and delivery has also been studied. Peptides such as 46Δ8 (LARLLARLLARLLARL) and Hel 11-7 (KLLKLLLKLWKKLLKLLK) form aggregates with DNA and transfer DNA. However, transfection efficiency was shown to be poor in the case of 46Δ12, when the peptide was not able to form aggregates. Aggregate formation also prevents DNA degradation.

Tirrell *et al.* used an amphiphilic peptide (Gly-Pro_Hyp)4-[IV-H1] to modify PEG-coated liposomes in order to target mouse fibroblast cells. After incorporation of IV-H1 peptide-amphiphile, they maintained their native triple-helical structure and enhanced uptake of the IV-H1 modified liposomes over non-targeted liposomes [73].

Dendrigraft poly-L-lysines (DGLs), a new kind of self-assembled peptide containing lysine, was recently modified by a peptide (sequence: EPRNEEK) derived from _Streptococcus pneumoniae_, a pathogen that causes meningitis. The modified peptide was used for targeted gene delivery to a glioma (brain tumor). The aim was to take advantage of the process by which certain pathogens can cross the blood-brain barrier (BBB) in a transcellular or paracellular manner. In vivo imaging results and cellular uptake indicated that EPRNEEK peptide-modified NPs showed enhanced brain tumor–targeting activity compared to a pentapeptide derived from endogenous laminin [33, 74].

Arginine-rich peptides have also been designed and used for enhanced cellular uptake. Sardan *et al.* designed an arginine-rich cell-penetrating amphiphilic peptide (lauryl–PPPPRRRR–Am), which was integrated into a liposome formulation (DOPG) in order to

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target the activity of anticancer agents (doxorubicin-HCl and paclitaxel) inside MCF7 breast cancer cells. Arginine PA integrated on liposomes enhanced the uptake of model reagents by MCF7 breast cancer cells compared to native liposomes and free reagents [33]. Both hydrophilic (i.e., rhodamine and doxorubicin-HCl) and hydrophobic (i.e. paclitaxel) anticancer agents have been loaded in arginine-rich peptide liposomes in order to improve the uptake of both drugs inside cancer cells [33].

Stimuli-responsive supramolecular peptide-amphiphiles (SPAs, dendritic poly (L-lysine) non-covalently linked poly (L-leucine)) were synthesized with the ability to reassemble in lower pH for intracellular drug delivery. SPAS are shown to encapsulate guest molecules at pH 7.4 and release them at pH 6.2. In this way, SPAS can act as a smart platform to deliver their target to tumor cells with intracellular pH as a trigger. The nanovehicle was loaded with DOX and delivered inside HepG2 cancer cells. Confocal scanning microscope results (CLSM) indicated NVs can considerably enhance the endocytosis of DOX into HepG2 cells and deliver DOX to their nuclei [75].

3.3 Biomimetic membranes for model studies of cellular phenomena

Cell interaction studies are usually performed with secondary molecules such as fluorescent probes. A biomimetic membrane allows one to study cell membrane signals and interactions with ligands directly [9]. Self-assembled peptides play a significant role in fabrication of model biomimetic membranes. Short peptide sequences are a substitution for ECM proteins. They mimic cell adhesion and eliminate the complexity of ECM structural effects on cells [73]. In this regard peptide ligands have been incorporated into biomaterials that can be used as biomimetic membranes. Examples are polyethylene glycol hydrogels, biodegradable copolymers, and SAMs [76].

PAs, which contain an alkyl chain linked to a head group, also represent an attractive alternative to biomimetic membranes. The alkyl chain of PAs has the capability to self-assemble and mimic cell membranes, while the head group sequence binds specifically with cell receptors. The long alkyl chains of peptide-amphiphiles allow self-assembly and interaction with other hydrophobic biomaterials. Active binding sites are available, since the orientation and composition of molecules on the surface are well controlled. Furthermore, these long chains can also improve the stability and activity of the peptide relative to the parent protein by protecting the secondary structure [9, 77].

Earlier studies on stimulation of ion channel membranes were carried out using self-assembled β-sheet cylindrical structures. Cyclic peptide structures formed by an even number of alternating D and L amino acids can adopt a flat ring shape to establish a connecting hollow tubular structure. An assembly of 8 to 10 subunits with a distance of 7-5 Å could serve as an artificial ion channel membrane. The results demonstrated that the effective channel mediated ion transport at the rate of 10^{7} ions/s.

Further studies have used model LB membranes composed of peptide-amphiphiles and lipidated polyethylene glycol [68] to study the fundamental effects of pH, electrostatic interactions, hydrogen bonding on bioadhesion, ligand availability, cell spreading, and migration availability.

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The conformation presentation, organization, and accessibility of peptide ligands in LB films could be controlled, offering valuable insights into cell adhesion. LB membranes were mixed with lipidated polyethylene glycol and used to study the effect of ligand accessibility on cell adhesion. The results showed that ligand accessibility plays a major role in cell adhesion to biomimetic membranes and ligand-receptor interaction. [78]. Studies on LB membranes of triple-helical collagen mimetic peptides showed that various head groups of PEG lipidated molecules with different length could affect ligand accessibility and eventually control adhesion and spreading of melanoma cells [79].

The effect of conformation and orientation of RGD PA on cellular response was studied by Pakalns *et al.* [80]. The results revealed that looped RGD could support adhesion and spreading of M14#5 human melanoma cells. RGD peptide motif and the synergy site PHSRN were placed in LB membranes, and the effect of their secondary structure on cell adhesion, cell spreading, and integrin binding affinity was studied by Johnson, Kendall, Roberts (JKR) [81] and AFM collective studies. [78] [69]. Dillow *et al. [82]* employed RGD (primary binding site for $\alpha_5\beta_1$ integrin) and PHSRN (the synergy binding site for $\alpha_5\beta_1$ integrin) in peptide amphiphile membranes and investigated specific adhesive interactions between fibronectin-derived peptide-amphiphiles. The result demonstrated that adhesion was dependent on membrane composition, density, and temperature, which affect peptide mobility.

Most recently, self-assembling peptides have been promising tools for handling structural and functional studies of membrane-bound proteins such as G-protein-coupled receptors (GPCRs). It is worth noting that abundant membrane proteins are vital for the functioning of biological cells. Almost 30% of the human genome codes for membrane proteins, which are principal targets for nearly 50% of medical drugs [83]. However, understanding their functionality remains a challenge, and only a limited number of high-resolution structural studies have been carried out so far [84]. In 2012, the Nobel prize in Chemistry was awarded to Brian Kobilka and Robert Leowitz for their effort in understanding several protein complexes from the important class of G-protein coupled receptors 3 (GPCRs). New biomimetic materials such as self-assembling proteins allow membrane proteins to organize in a 3D membrane lattice, opening new possibilities for their crystallization, biochemical studies, and integration in nanodevices [84]. Apoliprotein A1 (ApoA1)-based lipoprotein particles have been recently recognized as a new platform to study membrane proteins. In combination with phospholipids, they can self-assemble to form discoidal-shaped particles that can stabilize membrane proteins [85]. ApoA1 is the main constituent in high-density lipoproteins (HDLs) and is known to form discoidal particles when associated with lipids. Midtgaard *et al.* examined an ApoA1 mimetic peptide referred to as A18, and its solution structure [86]. A18 is an 18-amino-acid amphipathic a-helical peptide of class A, assembled synthetically into bicelles to mimic the in vivo properties of ApoA1 (Fig. 11.). Two approaches for incorporation of membrane proteins are nanodisc and bicelles. Bicelles are two types of phospholipids that separate locally to make discoidal bilayer systems. Although nanodiscs are shown to be more effective in the stabilization of membrane proteins, using bicelles is easier when handling proteins.

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Therefore, the focus of work by Midtgaard *et al.* [86] was to investigate whether A18 could provide the flexibility of bicelles along with the native membrane-like environment of nanodiscs. They observed that membrane proteins could be easily incorporated in 18A discs and maintain their stability.

Recent studies also showed that self-assembling peptides could interact with proteins or protein assemblies in cells and could be selectively formed in certain cells, thus providing novel strategies to manipulate the fate of cells. Wang *et al.* developed self-assembling peptides with L- and D-amino acids (NBD-DFDFDYGLKKTETQ) and studied their biological functions in cells. Interestingly, the result demonstrated that the utilization of D-amino acids in self-assembling peptides boosts their cellular membrane localization [87]. D-amino acids were specially enriched in cellular membranes at self-assembled stages. However they were distributed uniformly in the cytoplasma of cells at unassembled stages. Wang *et al.* [87] demonstrated the possibility of using such D-amino acid-based peptides to engineer cell surfaces.

Membrane interaction of a model β-sheet self-assembled peptide, P11-2 (CH3CO-Gln-Gln-Arg-Phe-Gln-Trp-Gln-Phe-Glu-Gln-Gln-NH2), was studied by Salay *et al.* in order to mimic the toxicity and structural behavior of β-amyloid peptide (Aβ) [88]. In Alzheimer's disease β-amyloid peptide (Aβ) is a main protein component of plaques. However its role in neurotoxicity is not fully understood. Model peptides could help evaluate the possible mechanisms of toxicity, which is common among self-assembling peptides associated with disease. The results confirmed that transitional oligomers had higher toxicity against cells than monomeric forms and higher aggregates of the peptide. Thus a rationally designed yet simple model β-sheet peptide recapitulates a variety of the important features of Aβ peptide function and structure. This supports the theory that toxicity arises from a common mode of action on membranes regardless of specific aspects of the sequence of amino acids.

3.4 Anticancer drug delivery

Cancer is among the most widespread causes of death, and chemotherapy is still the most common treatment. However, conventional chemotherapy agents have tremendous disadvantages, as they can easily distribute non-specifically in body tissue, causing damage to normal as well as tumor cells. This induces nonspecific drug distribution in the body with severe systemic side effects, and therefore chemotherapy often results in an unsatisfactory curative effect due to the side effects of the drugs. Nanoparticles have been extensively used for delivery of anticancer drugs, as they can increase the efficacy of treatment and reduce undesirable side effects. Nanosized carriers offer advantages such as high drug-loading ability, improved stability by avoiding fast clearance by the reticuloendothelial and renal systems, and minimized drug loss during blood circulation [89].

Among various nanosized carriers, self-assembled peptide nanostructures have gained attention for the delivery of anticancer agents and seem to be a promising approach for cancer treatment. Self-assembly mechanisms allow formation of various types of nanoparticles such as tubes, vesicles, and hydrogels, each suitable for delivery of specific types of anticancer agents. For example, injectable hydrogel formulations can be placed in direct contact with tumor tissues and therefore improve the safety and efficacy of cancer

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treatment [47]. Nanotubes also have been applied to load doxorubicin with high efficiency in their inner core [24]. Amphiphilic peptide nanovesicles have been used for loading hydrophobic drugs in their alkyl core, while the shell is modified to improve intracellular delivery [66]. Moreover, modifications have been applied to increase targeting activity and intracellular delivery efficacy [33]. Several self-assembled peptide structures have been used for loading anticancer agents, such as doxorubicin, paclitaxel, curcumin, and fluorouracil and studied in preclinical and clinical trials, owing to their excellent biodegradability and biocompatibility. There have been several recent advances in anticancer treatment using self-assembled peptides.

Standley *et al.* designed a PA with highly cationic membrane lytic (Lysin) sequence $(KLAKLAK)_2$ conjugated to lauryl acid (C16-A4G3(KLAKLAK)2) capable of forming cylindrical nanofibers and disrupting cell membranes [90]. The results demonstrated that PA is more cytotoxic against the transformed cell membranes of cancer cells due to increased negative charge on these surfaces [91]. Moreover it was revealed that in addition to passive permeation, KALA epitope mediated uptake targeting [92]. A limitation associated with these kinds of PAs, however, is rapid degradation of peptide after systematic delivery [93]. One tactic to enhance the therapeutic-delivery vehicle is to functionalize the surface with polymers such as PEG, which can protect from proteolysis, improve circulation time, and lessen immune response [94]. Furthermore, in 2012, Toft *et al.* introduced the use of cytotoxic KLAK PA and a co-assembled combination of pegylated PA (PEG PA) to make an effective antitumor therapy [95]. Addition of the pegylated PA to the nanostructure limits degradation of the cytolytic PA by the protease trypsin. Systemic administration of cytotoxic pegylated PAs to an orthotopic mouse xenograft model of breast cancer significantly reduced tumor cell proliferation and overall tumor growth, thus suggesting its effectiveness and versatility for cancer treatment.

A study carried out in 2012 proposed molecular, electrostatic interaction-based binding between EAK, a self-complementary peptide and hydrophobic anticancer drug ellipticine (EPT). EAK was able to increase the stability of EPT in aqueous solution [96-97] at a concentration much higher than the drug's solubility in water (~0.62 μM at neutral pH) [98]. Furthermore a fluorescence technique that differentiates the two molecular states (protonated and crystalline EPT in situ) was used to evaluate different molecular states of EPT in its anticancer efficacy. An in vitro cytotoxicity assay has been applied to analyze the efficacy of the two molecular states of EPT, indicating that complexes with protonated EPT had higher cytotoxicity than those with crystalline EPT [99].

Researchers have also utilized micro- and nanoparticles containing self-assembled peptides conjugated to doxorubicin as an effective cancer treatment. In 2015, Rubert Pe Rez *et al.* proposed hierarchical assembly of PA nanofibers with alginate to shape micro-particles, in which doxorubicin was conjugated to an alginate core, and the shell was constructed of peptide amphiphile nanofibers functionalized to target the folate receptor. Folate groups were covalently linked to C16-V3A3K3 via a lysine linker to target the doxorubicin-containing particles to cells overexpressing the folate receptor. The PA shell increased the surface-over-volume ratio to its ideal amount for targeting [6].

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Furthermore, hybrid NPs were made of low-molecular-weight polylactide (PLA) [100] and self-assembling V6K2 peptides (VVVVVVKK) [101], which self-assembled into spherical nanoparticles with 100 nm as an average diameter. PLA-V6K2 were used to host doxorubicin and paclitaxel for cancer treatments, and their toxicity, release kinetics, and cellular uptake were studied. The results revealed that PLA-V6K2 NPs had much higher tumor cell uptake than PLA NPs that were stabilized with PEG (PLA-EG). This was due to the electrostatic interactions between the lysine groups of the peptide. The results indicated the greater efficacy of nanoparticles functionalized with self-assembled peptides for cancer treatments [102].

Furthermore, different self-assembled dipeptide NPs with a non-protein amino acid, α, β dehydrophenylalanine were used for entrapment of curcumin. The di-peptide curcumin Nps could be assembled in a mixture of aqueous and organic phase and enhanced the biological activity of curcumin [103].

Li *et al.* investigated dendrimer peptides with the structure of glycyl-phenylalanyl-leucyl - glycine tetra-peptide spacer (Gly-Phe-Leu-Gly, GFLG) to host anti-cancer drugs as a suitable substrate for protease cathepsin B, which is an enzyme overexpressed in many tumor and tumor endothelial cells. GFLG peptide could be used to design smart enzyme-responsive drug-delivery vehicles. Fluorescence imaging studies demonstrated that the nanoparticles can concentrate in tumors and be retained for a long period [104]. Furthermore, multifunctional delivery platforms such as dual-functional liposomes with active targeting hyaluronic acid (HA) and pH-responsive cell-penetrating peptide (CPP) were fabricated for enhancing the efficacy of tumor-targeted cancer treatments [105].

Perhaps the most interesting application of self-assembled peptides for cancer treatment, however, is the injectable gel formulation, in which chemotherapeutic agents (unlike traditional chemotherapy) can be located next to or into target tissues at higher local concentrations. Due to the slow rate of release, these formulations provide more prolonged and sustained cytotoxic action than conventional chemotherapeutic agents, thus enhancing therapeutic efficacy. With this in mind, Yishay-Safranchik *et al.* verified the possible use of in situ-forming hydrogels made of self-assembled KLD motifs to tune or extend the release of the conventional cytotoxic drug (doxorubicin [DOX]) [47].

3.5 Gene delivery

Development of non-viral vehicles for efficient delivery of genes is still requiring major improvements. Gene carriers have to be well organized to overcome the limitations of vector gene delivery, such as poor uptake into cells, toxicity and immunogenic response, and short-lived transgene expression [106]. Research behind the designing of advanced gene delivery vehicles has focused on improving characteristic of efficient loading, enhanced cellular delivery, and specific delivery. Cationic nanoparticles have been manipulated and applied extensively due to their high loading capacity for nucleic acids, their ability to transfer through the cell membrane, and their ability to protect nucleic acids from degradation. However, cationic formulations can have problems in some applications in vivo, as they deal with proteins in biological fluids and nonspecifically with cells to shape aggregates, which can lead to toxicity as well as poor bio-distribution and immune responses [107].

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Peptide-based self-assembled nanostructures also represent a promising approach for efficient gene delivery. Solid-phase methods enable precise control over peptide fabrication at the molecular scale. Oligonucleotides can be delivered to the interior of cells through endocytosis following the transition of self-assembled nanotubes into vesicles by changing the concentration of building blocks [108]. Thus, recent developments in bio-inspired gene delivery vehicles use a combination of viral genomes and self-assembled peptides [109]. This framework highlights some of the advances in the development of viral vectors with self-assembly of peptide-engineered nanostructure.

For efficient delivery the vector must pass four criteria. The vector must target specific cell-surface receptors, efficiently protect DNA from degradation, deliver the DNA cargo to the nucleus, and disrupt the endosomal membrane [110]. For this, cationic peptides could condense with DNA through electrostatic interactions. The most famous DNA-condensing cationic peptide is L-lysine, which interacts with the negative charge of DNA phosphate. Poly-L-Lysine is a synthetic peptide containing repeated lysine and a large number of surface amines. It is capable of electrostatic interaction with polyanions. The length of the peptide has a strong effect on its properties, such as the size and stability of DNA condensate. However, high-molecular-weight PLLs exhibit high cytotoxicity, and PEGylation is known to be useful to increase circulation half-life of the complex and avoid plasma protein binding.

CPPs enable cellular uptake of various molecular cargos. CPPs are known to be powerful tools in overcoming limitations associated with gene delivery vehicles and are able to transfer genes with endosomal escape.

In 1997, Morris *et al.* [111] used MPG-designed peptides to deliver oligonucleotides. MPG is a synthesized 27-residue peptide vector that contains both a hydrophobic domain, derived from the fusion sequence of HIV gp41 (residues 1–17: G-A-L-F-L-G-FL-G-A-A-G-S-T-M-G-A)K-R-K-V', and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen (residues 21–27: P-K-S-K-R-K-V). The hydrophobic domain is known to be essential both for its membrane fusion activity and structural stabilization, and the hydrophilic domain is useful for improving permeation of the nucleus. An acetyl group at the N-terminus and a cysteamide group at the C-terminus were added to enhance the ability to cross the cell membrane.

When MPG was combined with oligonucleotides in solution, they quickly shaped into a complex with tight non-covalent interactions. This implies that binding between oligonucleotides and MPG occurs through electrostatic interactions. Efficient delivery of this complex into the nucleus of mammalian cells happens in about an hour. MPG shows high attraction for single- and double-stranded oligonucleotides. Further studies were carried out to understand the mechanism of MPG peptides in gene delivery to mammalian cells [112]. The result revealed that a mutation in the nuclear localization sequence (NLS) of the SV40 antigen domain prevents the carrier from entering the nucleus, making it a highly useful tool for robust siRNA delivery into cell cytoplasm, which does not require nuclear entry. Targeting cyclin β1 in mouse tumor models validated the potential therapeutic utility of MPG for siRNA delivery in cancer treatment, and it was shown to inhibit tumor growth

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[113]. Cyclin B1 together with Cdk1 kinase forms the ‘mitosis-promoting factor’. This is essential for entry into and progression through mitosis.

New gene delivery carriers have been designed with an N-terminal stearylated (STR) nuclear localization signal (NLS), PKKKRKV. The aim was to overcome cell membrane and nuclear pores, and to improve gene delivery [114]. The inclusion of arginine facilitates DNA binding, histidine is used for endosomal escape, and hydrophobic residues enhance cellular uptake [115].

To achieve high transfection efficiency, non-viral lipopeptide transfection agents were designed with four different sections, including an alkyl chain, one cysteine, 1 to 4 histidines, and 1 to 3 lysine residues. The lipopeptides were designed to facilitate dimerization through the cysteine residues, DNA binding at neutral pH through charged lysine residues, and endosomal escape through weakly basic histidine residues [116].

Furthermore, a new generation of CPPs was introduced by Crombez *et al.,* namely CADY, based on a secondary amphipathic peptide that was capable of improving delivery into challenging suspensions and primary cell lines and forming stable complexes with siRNA. CADY is a 20-residue amphiphatic peptide with the structure Ac-GLWRALWRLLRSLWRLLWRA-cysteamide, which forms a helical structure. CADY was able to efficiently introduce siRNA into cells and lower the expression of GAPDH at both mRNA and protein levels [113]. Try and Arg were replaced in the structure of CADY, since Arg has been shown to enhance cellular entry of CPPs and improve electrostatic cell-surface interactions [117], [118] and Tryptophan enhances cellular uptake of CPPs due to their capability of interacting with lipid/cholesterol molecules inside the membrane [119]. The helical conformation of CADY also plays a significant role in the process of cell penetration and interactions with the cell membrane.

Multicomponents have been evaluated for gene delivery, since they can perform a series of tasks in the multi-step process of drug or gene delivery as well as cell targeting. A multicomponent gene carrier composed of numerous combinations of the CPP TAT peptide (amino acid 48–60 of the HIV TAT protein) (Gly-Arg-Lys-Lys- Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln), a lysine-based cationic dendrimer (DEN), and a nuclear localization signal coming from the SV40 Large T protein (Pro-Lys-Lys-Lys-Arg-Lys-Val) were evaluated for their potential to deliver DNA to human HeLa cells [120]. The function of DEN is to form condensed particles with DNA, which is necessary for effective uptake [121]. TAT was used to promote uptake from cellular membranes [122], and finally NLS was used to improve nuclear translocation [123]. The most efficient particle for DNA delivery was shown to be a multicomponent of DEN, TAT, and NLS. A fusogenic agent such as chloroquine was required to improve gene expression. The results showed that the function of chloroquine is not only to aid in endosomal escape, but also to protect DNA from degradation [124].

Moreover, synthetic peptides were constructed to mimic the fusogenic properties of virus-based peptides [125]. Perhaps the most revolutionary synthetic peptide is the GALA, which has pH-sensitive and amphiphilic properties. The GALA sequence consists of 30 amino acid residues (WEA ALA EAL AEA LAE HLA EAL AEA LEA LAA) and has a repeating

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series of Glu, Ala, Leu, and Ala. Therefore, at pH 7.4, GALA forms a coil structure due to repulsive interactions of negatively charged groups of Glu, while at pH 5, it forms an amphipathic α helix due to a protonated Glu residue. GALA exhibits pH-responsive properties with membrane fusion activity at pH 5. Therefore, GALA can induce an assistive process to release drugs from endosomes, like other virus-derived fusogenic peptides [126]. In terms of DNA delivery, GALA has limitations associated with its anionic properties, which prevent formation of condensed particles with DNA [127]. A combination of GALA with cationic complexes has been proven successful as a multifunctional carrier for gene delivery. In this regard KALA was synthesized in which Glu was substituted with Lysine, making it possible to condense and protect DNA. However when Lysine is present in high amounts, the responsive activity of the peptide compared to GALA, which is highly active in low pH, is altered. When exposed to the negative charge of the membrane, increased protonation of KALA allows the peptide to permeate membranes. In contrast, in a low-pH environment, increased net charge disrupts α-helical structures and impedes permeation. Endosomal agents should be active only at low pH in order to avoid disturbance of other membranes, and this behavior contributes to the lack of specificity and toxicity of the peptide [128]. In order to overcome this limitation, KALA was modified, and Lysine was substituted with Arginine in order to form complexes with DNA [109]. The so-called peptide referred to as RALA, was synthesized, and RALA-DNA complexes were developed with pCMV-Red Firefly Luc. For distribution studies, pCMV-Red Firefly Luc or RALA/p pCMV-Red or Firefly Luc complexes were injected into a mouse and its bio-distribution was imaged (Fig. 12).

Biodistribution analysis has shown that naked DNA is localized at the site of injection, whereas RALA/pCMV-Red Firefly Luc nanoparticles have been dispersed (Fig. 12Ai). However, stronger gene expression was detected in the lungs and liver after systemic injection of RALA/pCMV-Red Firefly Luc nanoparticles compared to empty vectors. The transfection efficacy realized by the RALA/pDNA nanoparticles in vitro was comparable to commercial transfecting agents with significantly lower toxicity, thus demonstrating the effectiveness of RALA as a DNA delivery platform with potential to reach the clinic.

According to studies using self-assembled peptide structures as a platform for DNA delivery, the main function of the peptide is to form nanoparticles with DNA, facilitate the uptake of DNA intracellularly, enable cargo escape from endosomal vesicles, and prompt gene expression both in vitro and in vivo [110] [74]. These findings have allowed researchers to develop vectors with improved efficiency, specificity, and safety. However, the use of peptides as delivery vectors is still in its infancy and has the potential to grow exponentially.

4. Conclusion and future outlook

The examples presented in this review highlight the potential role of self-assembled peptides and their smart activity for advanced drug delivery. A major advantage of using self-assembled peptides is that in many cases the carrier is the therapeutic itself. Multiple functionalities could be included in a single self-assembled carrier, including targeting, cell permeation, endosomal escape motifs, release-responsive characteristics, and efficient ionic binding with therapeutic payloads. The benefits of peptide-based carriers are many, since

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peptides make up the most important signaling language in the ECM [6], and once the peptides self-associate into defined aggregates, they gain the other important functions of a self-assembled nanostructure (e.g., nanotubes and nanofibers, etc.). In recent years, peptides have progressed from basic studies focused on introducing various PAs and their self-assembly mechanisms to advanced studies that test their function in in vitro systems and translationally relevant in vivo models in order to develop novel therapies [6]. Based on these studies, many peptides have been studied or tested with in vivo model systems, and a diverse range of potential biological medical targets have been introduced. As building blocks, SAPs have significant advantages in nanomedical applications due to their biocompatibility, responsiveness to environmental change, and ability to be functionalized with targeting ligands. These advantages make SAPs essential elements for advanced in-vivo drug delivery applications. However, several issues must be addressed before clinical trials begin, including precise size control during synthesis, stability of hydrophobic peptides in water-based solutions, and the long-term effects of nanosized structures on cells. For improving control over the synthesized SAPs' size, changes in the fabrication parameters or the use of templates could represent novel routes in obtaining highly reproducible nanostructures. In order to enhance the stability of these self-assembled nanostructures in liquid-based solutions, studies in this field have shown that changes in pH most likely increase the stability of FF aromatic diphenylalanine peptides in liquid solutions. However, future works should be directed towards a more complete study of the biocompatibility and immunogenicity of this biomaterial in order to clarify the consequences of exposure [7]. Two main routes for peptide synthesis are recombinant technology and solid-phase chemical methods. Even the smallest-scale GMP-grade manufacturing lot by fermentation of a recombinant organism can cost over US$ 1 million. The solid-phase method is still the manufacturing method of choice for peptide synthesis [42-44]. Current challenges exist for manufacturing peptides at large scale, which is essential for their clinical translation; additional challenges include peptide impurities and failure to detect and remove them chromatographically. Lack of regularity in the rules for defining peptide purity for therapeutic compounds and for synthesis of highly purified peptide might lead to loss of economic viability. The future outlook of self-assembled peptides in the area of drug delivery is that a growing number of peptide-based drugs will soon enter clinical trials. There will likely be a massive upsurge in peptide formulations with PEG, CPP for intracellular delivery, and short-sequence peptides as carriers [141]. Similarly, several self-assembled peptides with anti-bacterial properties are expected to enter clinical trials [18]. Furthermore, self-assembled membrane-penetrating peptides have the potential to increase the number of intracellular targets. Supramolecular hydrogelators formed from small peptides are already commercially available drugs that act both as carriers and the drug itself [37]. One of these hydrogelators has already been approved by FDA for clinical use [142]. In the future, peptides could act not only as the active ingredients of new drugs but as multifunctional “add-ons” to other pharmaceutical agents to direct them to their targets, to ferry them across cellular membranes, and/or to modify their biological action. In conclusion, we anticipate that the future for peptide-based self-assembled nanostructured therapeutic compounds is bright and promising.

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Biography

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Spaller. He was a postdoctoral fellow in Chicago with Brian Kay at the University of Illinois. He was previously a visiting assistant professor of toxicology and pharmacology at Dartmouth Geisel Medical School. He joined King Abdulaziz University in 2010, was promoted to associate professor of nanotechnology and is concurrently a Lecturer on Medicine at Harvard Medical School. His research focuses on bioactive molecule discovery, and includes the design and development of biomaterials, chemical and peptide analog libraries, protein and antibody engineering towards solving challenges in targeted drug delivery, development of biosensors, and tissue engineering and regenerative medicine applications.

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Highlight

- Through self-assembly, peptides can generate well-defined nanostructures such as nanotubes, nanofibers, nanoparticles, nanotapes, gels and nanorods.

- Mild synthesis conditions, relatively simple functionalization, low-cost and fast synthesis process are among several advantages of peptides for drug delivery applications.

-The self-assembled structure could be designed with peptide motifs, making them smart and stimuli-responsive

- Self-assembled peptide matrices will be essential elements in developing next generation biomaterials and the future of advanced drug delivery.

- Comprehensive overview on the most recent studies on self-assembled peptides and their role toward targeted and responsive carriers.

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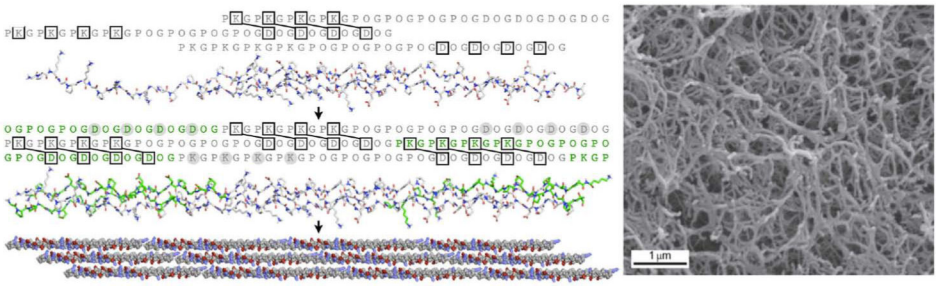

Fig. 1.

Design of a collagen peptide that self-assembles into fibrous hydrogels by the aim of electrostatic interaction initiated between Lys and Asp at the ‘sticky end’ of a collagen triple helix [12] Printed with permission from ACS.

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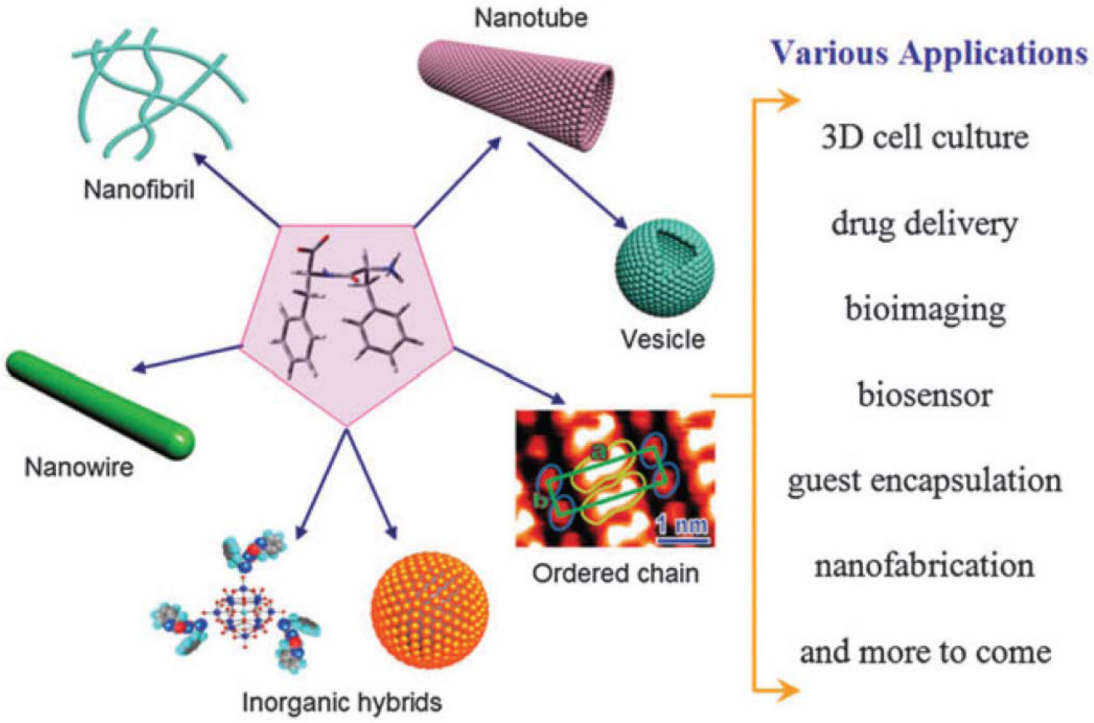

Fig. 2.

Schematic representation of various nanostructures formed by self-assembly of FF-based building blocks and their potential applications [5] Printed with permission from RCS.

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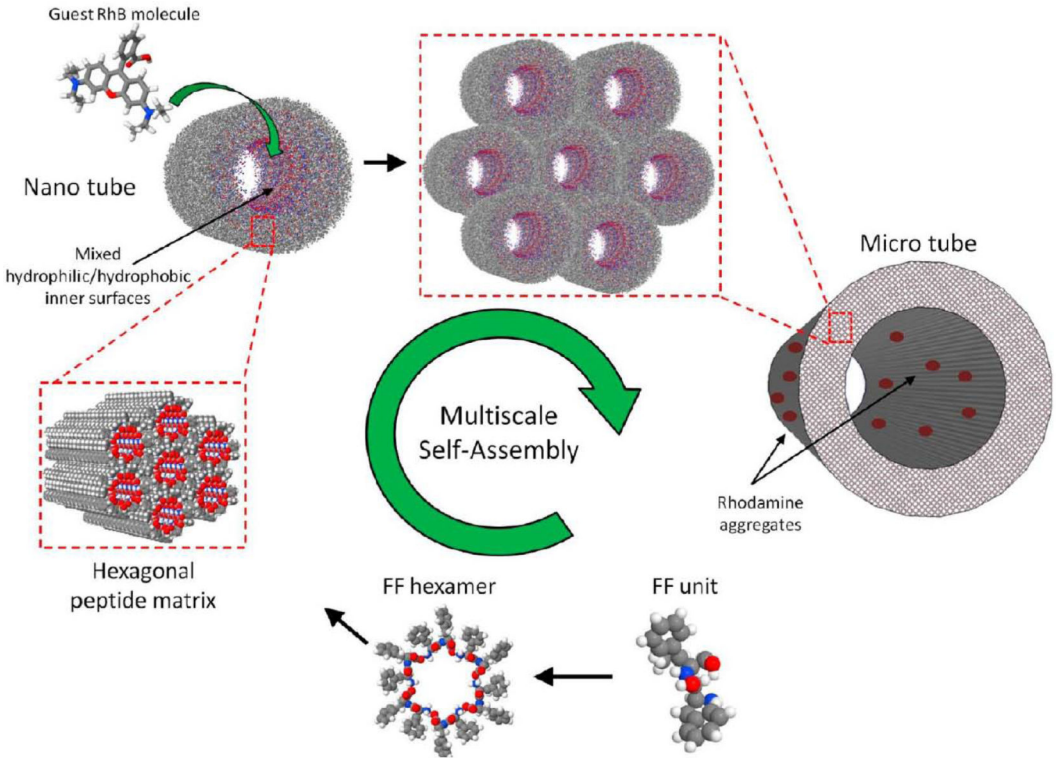

Fig. 3.

Schematic representation of the multiscale self-assembly of the FF-microtubes and their conjugation to RhB. FF hexamers self-associate to form narrow channel arrays, which give rise to nanotubes. Subsequently, these nanotubes cluster into larger microtubes. The inner surfaces of the nanotubes exhibit both hydrophobic and hydrophilic groups, with the latter being able to trap polar species [24] Printed with permission from ACS.

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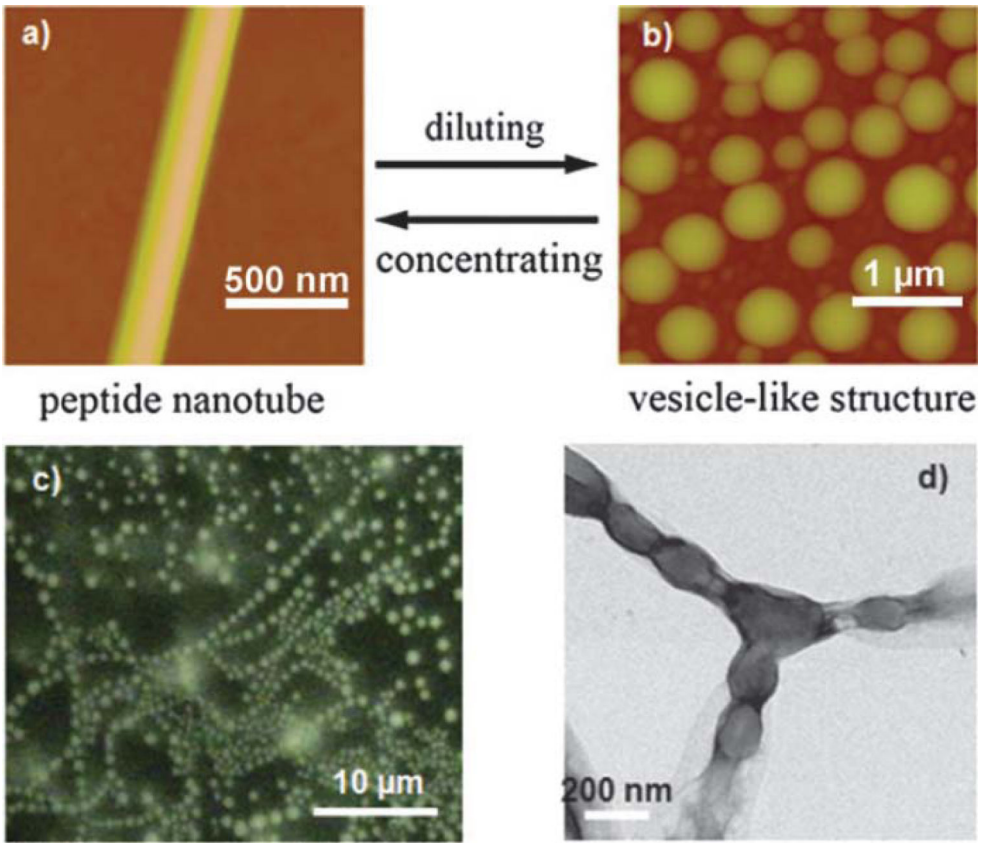

Fig. 4.

AFM height image of the reversible transition from: a) peptide nanotubes to b) vesicle-like structures by diluting FF solution; c) Fluorescently-labeled ss-DNA joined necklace-like structures; d) TEM image of the joined necklace-like structures [5] [25][108] Printed with permission from RCS and Wiley-VCH.

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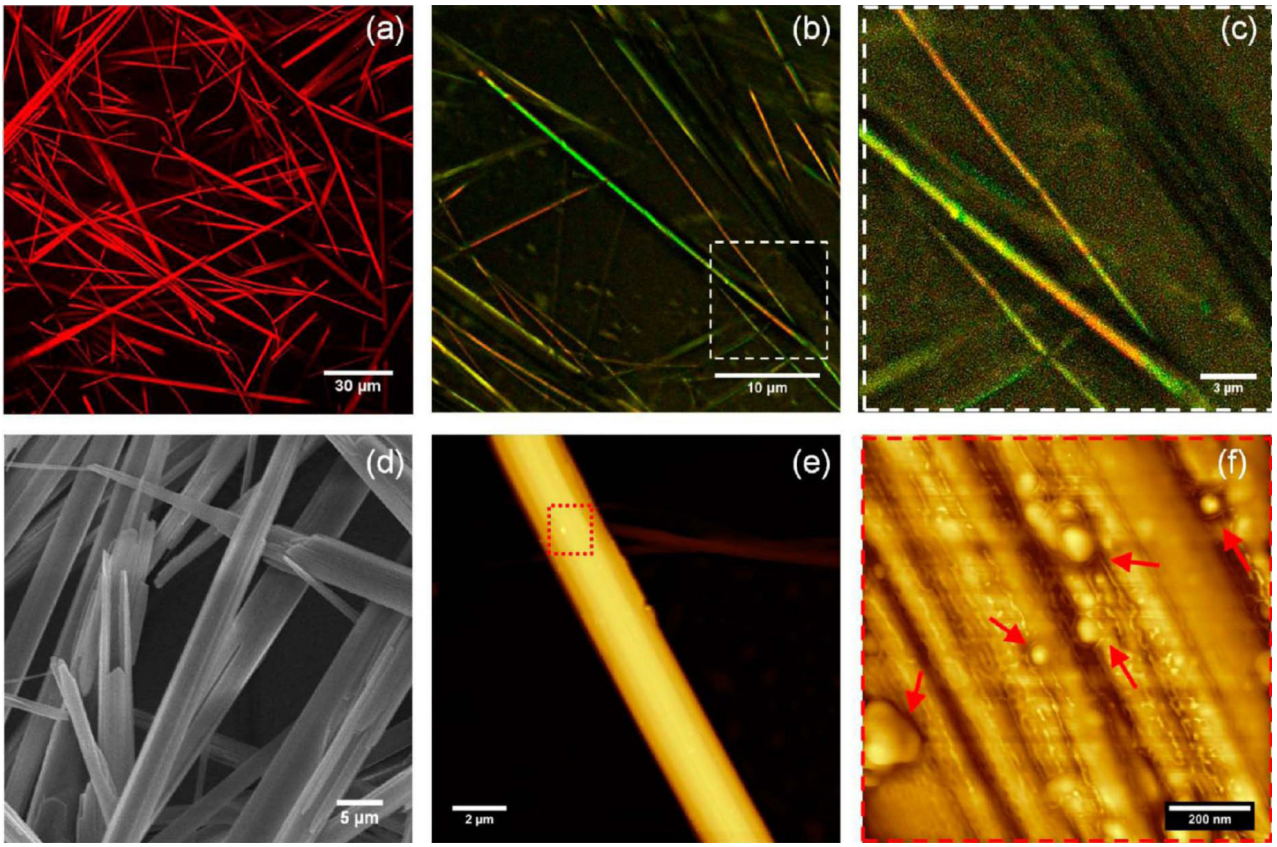

Fig. 5.

Micrographs of the FF-MT/RhB samples: a) fluorescence image representing peptide needles with high aspect ratios; b) colocalization micrograph exhibiting the location of fluorescence RhB (in red) and ZcPc (false-colored in green); c) detail of the region marked by a white dashed square in panel b; d) SEM images of open ended nanotubes; e) and f) AFM images of RhB aggregates on nanotube surfaces [24] Printed with permission from ACS.

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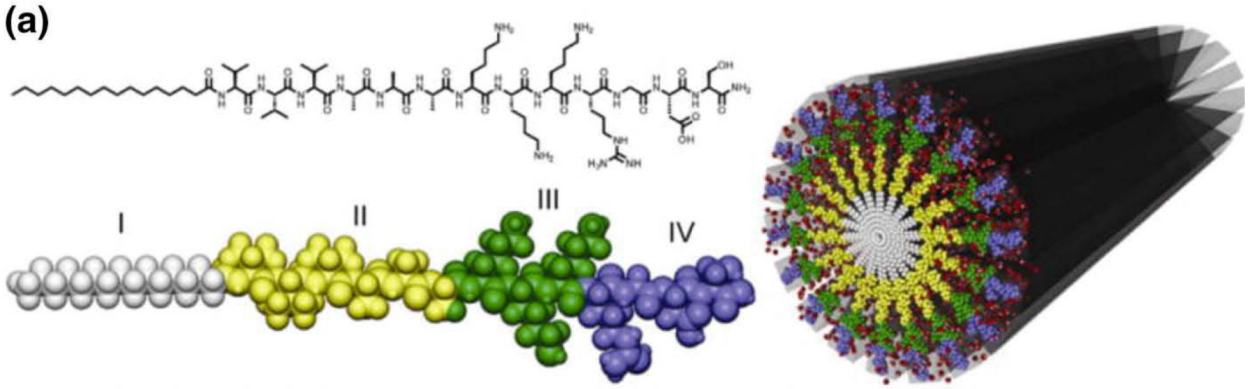

Fig 6.

Chemical structure and space filling model of PA molecule, and Illustration of a self-assembled PA nanofiber (red spheres representing water molecules). The four regions represent the unbranched alkyl (usually palmitic acid) tail (region I), a β-sheet amino acid sequence to promote formation of fibril structures through H-bonding (region II), charged amino acids to induce solubility and fiber crosslinking (region III) and a peptide signaling epitope for biological response (region IV) [8] [131] Printed with permission from ELSEVIER.

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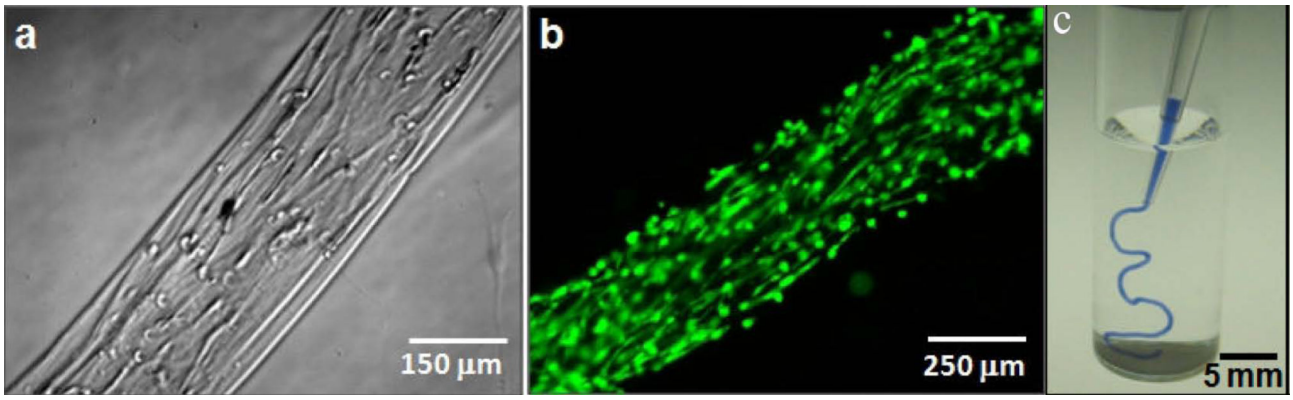

Fig. 7.

a) Alignment of encapsulated human mesenchymal stem cells (hMSCs) along the string axis; b) Calcein-labeled aligned cells cultured in string PA; c) solution colored with trypan blue injected into phosphate buffered saline after heat treatment [31] Printed with permission from NIH.

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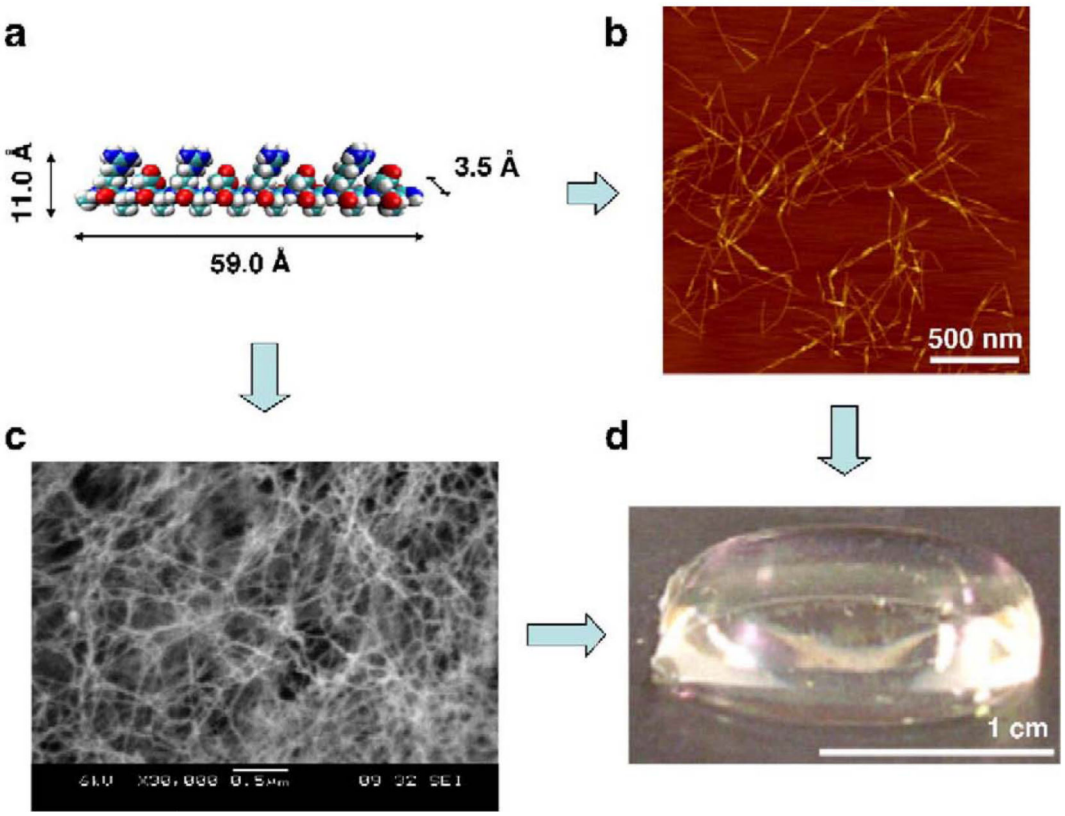

Fig 8.

a) Schematic illustration of the RADA16 molecule composed of negative (Asp) and positive (Arg) residues driving the assembly of the peptide by means of electrostatic interactions into b) and c) fibers and d) macroscopic hydrogels in buffer [50] Printed with permission from ELSEVIER.

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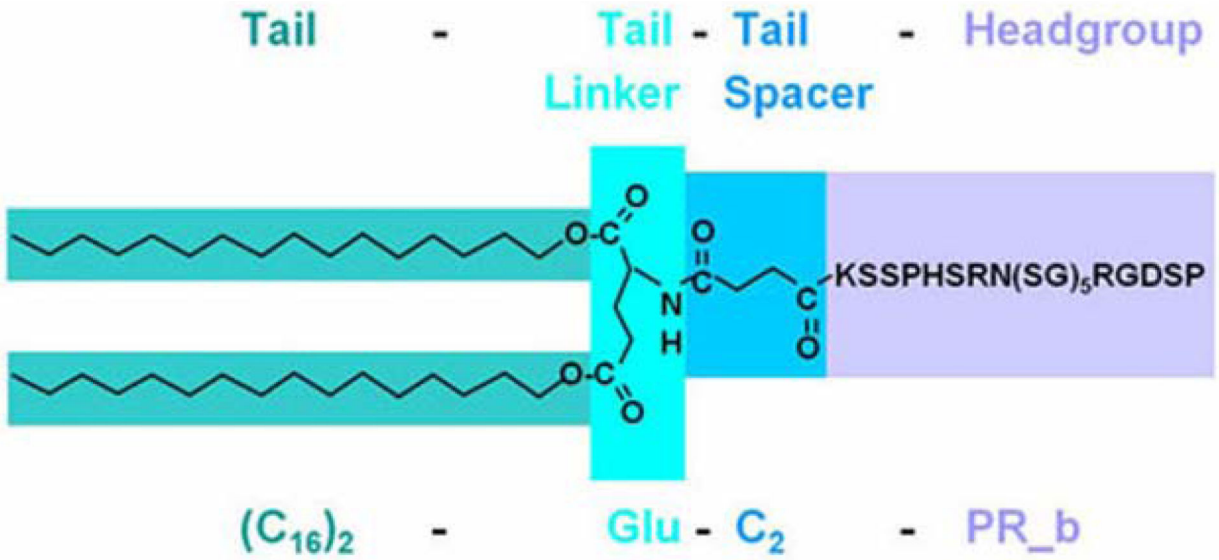

Fig. 9.

Structure of PR_b peptide-amphiphile containing C16 dialkyl ester tail, a glutamic acid (Glu) tail connector, a – (CH2)2– tail spacer, and the peptide headgroup [65] Printed with permission from ELSEVIER and PUBMED.

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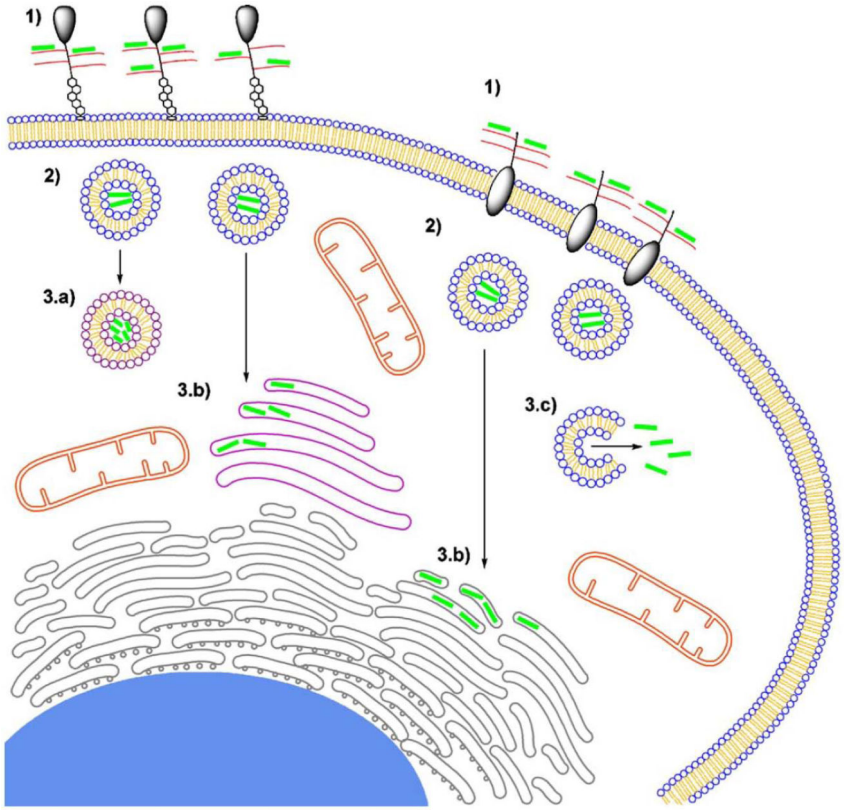

Fig. 10.

Different steps in CPP-mediated intracellular delivery: (1) Interaction of the CPP (represented as a green bar) with the cell-surface proteoglycans (in red). (2) Endocytic pathway. (3a) Degradative route to lysosomes in clathrin-mediated endocytosis. (3b) CPP ultimately reach the Golgi apparatus (in purple) or endoplasmic reticulum (ER, in grey) in caveolin-mediated endocytosis. (3c) Endosomal release [10] Printed with permission from ELSEVIER.

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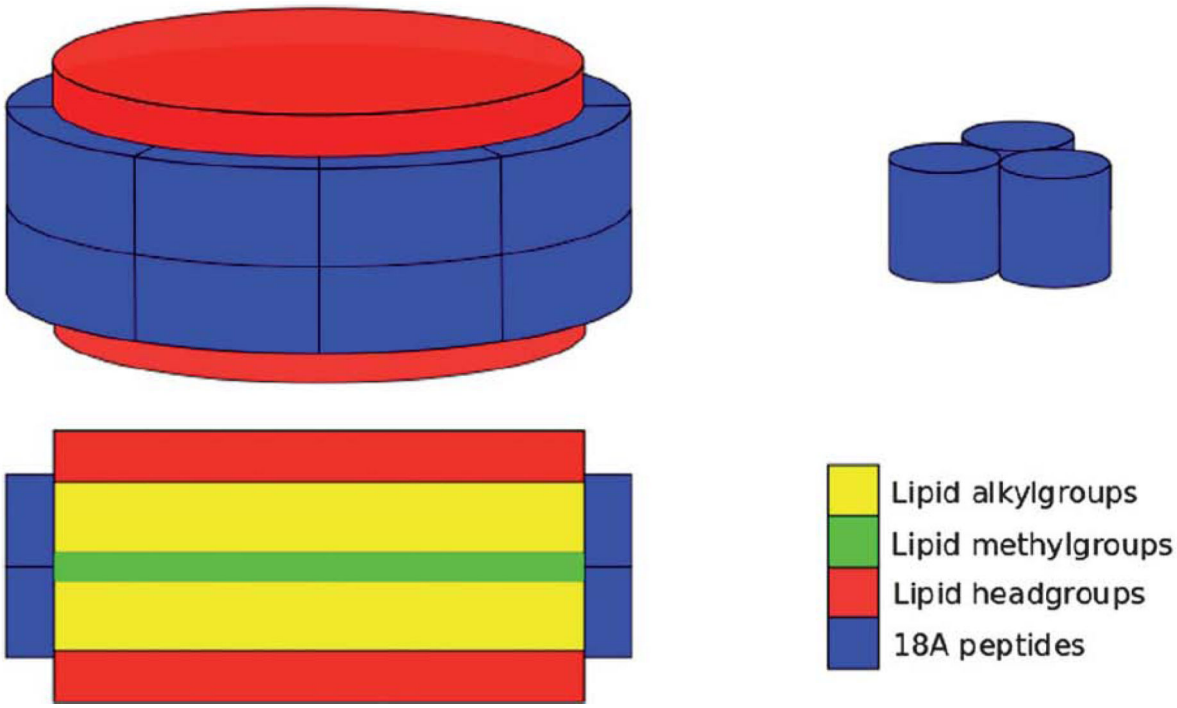

Fig. 11.

Upper left: schematic illustration of 18A:DMPC-disc model. Lower left: the cross-section of the same model showing the internal layers. Upper right: a representation of the 18A peptide trimer in solution [86] Printed with permission from RSC.

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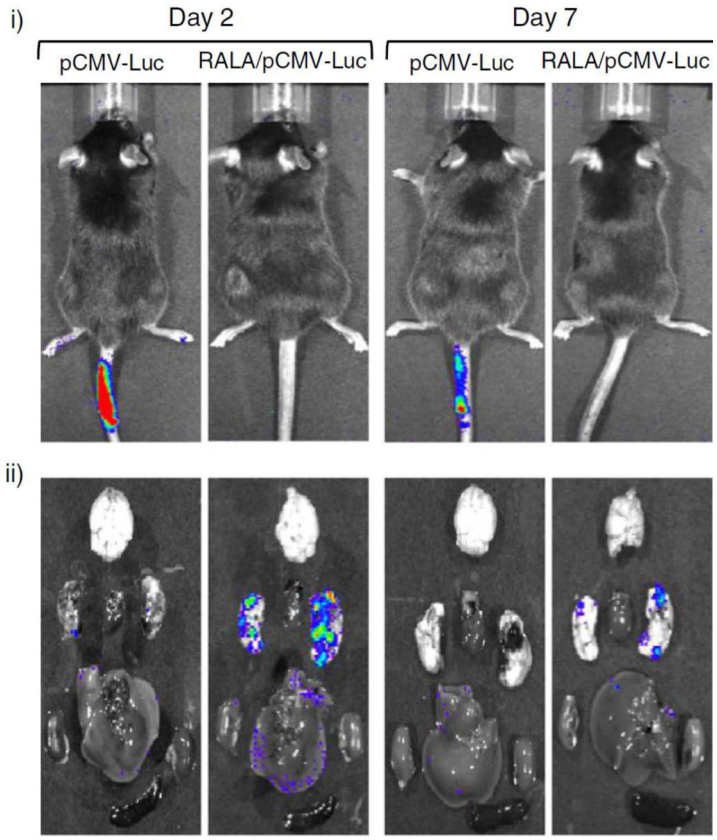

Fig. 12.

In vivo and ex vivo biodistribution of gene expression following administration of RALA/pCMV-Luc complexes. Representative images of pDNA (i) Dorsal; (ii) Harvested organs captured via IVIS imaging of C57BL/6 mice 2 and 7 days post administration of pCMV-Luc or RALA/pCMV-Luc N:P 10 nanoparticles containing 50 μg. (N = 3 ± SEM) Printed with permission from ELSEVIER [109].

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Table 1

summarizes different types of self-assembled peptides with biomedical application.

Peptide type	Nano structure	Biomedical applications	References
L-Phe-L-Phe (FF)	Nanotubes, β-sheets, Nanovesicles, Nanofibrils, Ordered chains, Peptide-nanoparticle hybrids, Dendrite nanostructures,	3D cell culture, Gene delivery, Anticancer drug delivery, Bioimaging, Biosensors, Guest encapsulation	[5] [129] [24]
Peptide amphiphilic (PA)			
C16-Cysteine-Glycin- Serine-RGD	Nanofibers	Mineralization of hydroxyapatite	[21]
C16- V3A3F3	Nanofibers	Cell encapsulation	[31]
C(16)V2A2E2-hydrazone	Hydrogel	Controlled release of prodan	[130]
C12-PPPPRRRR-NH2	Nanofibers	Doxorubicin and paclitaxel delivery with intracellular uptake	[33]
PA-IKVAV C16-(A4G3) (Isoleucine-lysine-valine-alanine-valine)	Nanofibers	Artificial 3D scaffold, Encapsulation and differentiation of neural progenitor cells	[131]
C16-V3A3K3-lysin-folate Alginate core -PA shell	Micellar core- nanofibers Shell	Doxorubicin delivery with folate targeting	[66]
SC4 (KLFKRHLKWKII)-C16	Helical structures	Anti-bacterial activity	[77]
RAD16-II Arg - Ala - Asp - Ala - Arg - Ala - Asp - Ala - Arg - Ala - Asp - Ala - Arg - Ala - Asp - Ala -NH_2	Nanofibers	Hydrophobic drug (pyrene) delivery	[35] [132] [64]
RADA16 I (AcRADARADARADARADA-CONH2)	Hydrogels	Controlled release of small molecules, functional proteins and anticancer drugs	[50] [64]
EAK16-II	Hydrogels	Delivery of anticancer agents (Ellipticine)	[97] [133]
(D-Ala-Glu-D-Ala-Gln)2	β-sheets Cylinders Nanotubes		[134]
(Trp-D-Leu)4-Gln-D-Leu	Cyclic nanotubes	Delivery of anticancer drug (5-Fu)	[135]
mPEG-b-P(Glu-co-Phe) poly(ethylene glycol)-b-poly(L-glutamic acid-co-Lphenylalanine)	Micellar-type nanoparticles	Anticancer drug doxorubicin delivery	[136]
Poly (L-lysine) poly (L-leucine)	Dendritic vesicles	pH-triggered responsive smart nano-vesicles for controlled intracellular drug delivery	[75]
(ELP) I	Elastin-like polypeptide	Inhibitor of tumor growth	[137]
$KLDL_3$ (AcN-KLDLKLDLKLDL-CNH2)	Hydrogels	Controlled release of doxorubicin and Smac-derived pro-apoptotic peptide, Protease sensitive hydrogel	[51] [52] [47]
RGD- PA NTFR - PA	Nanofibers	Cell response studies, Melanoma and endothelial cell adhesion, Enhanced liposome uptake, Mineralization of hydroxyapatite, Pyrene encapsulation	[9] [80] [21] [138]
Collagen mimetic self-assembled peptides IV-H1 peptide-amphiphiles (Gly-Pro-Hyp)4-[IV-H1]	Triple-helix structure	Targeting fibroblast cell lines, Enhanced uptake of liposomes	[139] [68]
Collagen IV - $a_2\beta_1$ integrin and CD44/CSPG receptors	Triple-helix structure	Melanoma cell activation	[140]