Overview

Self-assembly in nature: using the principles of nature to create complex nanobiomaterials

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Self-assembly is a ubiquitous process in biology where it plays numerous important roles and underlies the formation of a wide variety of complex biological structures. Over the past two decades, materials scientists have aspired to exploit nature's assembly principles to create artificial materials, with hierarchical structures and tailored properties, for the fabrication of functional devices. Toward this goal, both biological and synthetic building blocks have been subject of extensive research in self-assembly. In fact, molecular self-assembly is becoming increasingly important for the fabrication of biomaterials because it offers a great platform for constructing materials with high level of precision and complexity, integrating order and dynamics, to achieve functions such as stimuli-responsiveness, adaptation, recognition, transport, and catalysis. The importance of peptide self-assembling building blocks has been recognized in the last years, as demonstrated by the literature available on the topic. The simple structure of peptides, as well as their facile synthesis, makes peptides an excellent family of structural units for the bottom-up fabrication of complex nanobiomaterials. Additionally, peptides offer a great diversity of biochemical (specificity, intrinsic bioactivity, biodegradability) and physical (small size, conformation) properties to form self-assembled structures with different molecular configurations. The motivation of this review is to provide an overview on the design principles for peptide self-assembly and to illustrate how these principles have been applied to manipulate their self-assembly across the scales. Applications of self-assembling peptides as nanobiomaterials, including carriers for drug delivery, hydrogels for cell culture and tissue repair are also described.

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INTRODUCTION

Molecular self-assembly is the spontaneous organization of molecules, due to their mutual interactions (from the noncovalent type) into ordered aggregates (spatial and/or temporal ordering) without external control.[1–4] Supramolecular chemistry provides the basis of self-assembly where the instructions of how to assemble larger entities are coded in the structural motifs of individual molecules.[5] According to Whitesides,[6] the components of a molecular self-assembling system consist of a group of molecules or segments of a macromolecule that interact with one another. These molecules, or molecular segments, may

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be the same or different. Their interaction starts from a less ordered state (a solution, disordered aggregate or random coil) leading to a final state (a crystal or folded macromolecule) that is more ordered. Although the definition of self-assembly still remains ambiguous, being used inconsistently in the literature, the above described definition will be used throughout this article. Within the definition of self-assembly, Whitesides and Grzybowski have been considering two main classes of self-assembly: static and dynamic.[1,2,7] In static self-assembly, components form ordered static structures (structure does not change in time) without energy exchange with the environment. In contrast, dynamic self-assembly refers to ordered nonequilibrium structures, which are maintained far from equilibrium through an input of energy from an external source and subsequent dissipation into the environment.

SUPRAMOLECULAR INTERACTIONS

The interactions relevant in molecular self-assembly are noncovalent forces (electrostatic, hydrophobic, hydrogen bonding, van der Waals interactions, aromatic stacking, metal coordination) (Figure 1). These interactions are individually weak (2–250 kJ mol^{-1}) when compared with covalent bonds (100–400 kJ mol^{-1}) but collectively, if in sufficient number, they can generate highly stable assemblies and their subtle balance govern the shape and function of the final assembly.

Electrostatic Interactions

Coulombic interactions are long range nonselective interactions that can result in attractive or repulsive effects. Ionic self-assembly has been employed as straightforward and reliable method for the organization of different building blocks (e.g., polyelectrolytes, charged surfactants, peptides, and lipids) as described in the following sections.

Hydrophobic Effects

The hydrophobic effect is a unique organizing force based on repulsion of solute by the solvent. Nonpolar molecules tend to avoid an aqueous surrounding.

Aromatic Stacking (π-π Stacking)

Aromatic stacking (also called π-π stacking) refers to attractive interaction between aromatic rings when oriented face-to-face as in a stack of coins, being responsible for intramolecular stability.

Hydrogen Bonding

Hydrogen bonding is a form of association between an electronegative atom and a hydrogen atom attached to a second, relatively electronegative atom. Both electronegative atoms are usually N, O, or F. The atom to which the hydrogen is connected is referred as hydrogen-bond acceptor and the hydrogen is called hydrogen-bond donor (Figure 1). Hydrogen bonding (H-bonding) interactions are highly directional and can be of either short- or long-range nature. Hydrogen bonds may be intermolecular or intramolecular. The strength of single hydrogen bonds depends mainly on the nature of donor or acceptor, although it is influenced by a larger extent by the solvent. Combining several hydrogen bonds in a functional assembly strengths the interaction and their spatial arrangement enhances its specificity.

BIOLOGICAL SELF-ASSEMBLIES

Biology is replete with examples of highly functional complex nanoscale structures formed by self-assembly.[4] Self-assembly is of central importance to life. It generates much of the functionality of the living cell. A variety of biological structures, ranging from proteins and nucleic acids to viruses and cell membranes (Figure 2), possess a highly precise organization on the nanometer scale which derives from specific interactions at molecular level. Being this organization critical for their function, biological self-assembly uses biomolecular building blocks of precisely defined shape, size, hydrophobicity, and spatial distribution.

Protein folding (Figure 2(a)) clearly illustrates the noncovalent interactions involved in the self-assembly in aqueous solution. The peptide sequence (primary structure) is synthesized from the 20 amino acids by translation of a sequence present in a messenger ribonucleic acid (mRNA). When viewed in a simplistic perspective, proteins appear to be an assembly of linear strands of covalently linked amino acids. Precise folded proteins involve, however, the assembly of prefolded intermediates before yielding perfectly functioning proteins. The secondary structure of proteins may be constituted by α-helices (stabilized by intramolecular hydrogen bonds between the carbonyl oxygen atoms C=O with amide NH groups, four residues away in the helix) and β-sheets consisting of several β-strands, kept together by a network of intermolecular hydrogen bonds.

The formation of protein tertiary structure is mediated by how α-helices and β-sheets bend and pack into each other originating a protein subunit. The conformation of the tertiary structure is

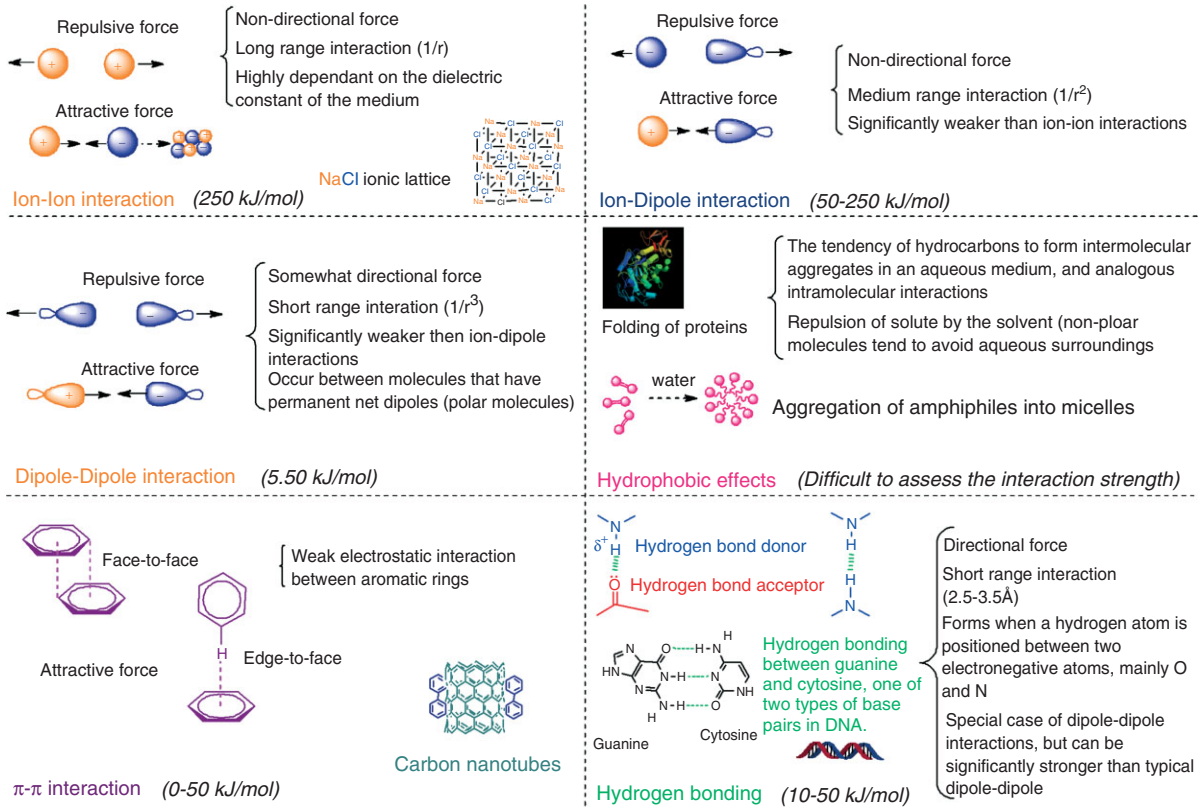


FIGURE 1 | An illustration showing the noncovalent interactions involved in supramolecular chemistry and their strength.[2,8]

provided by the number and type of interactions (e.g., hydrogen bonds, hydrophobic, and ionic interactions) and it is at this stage of protein folding that the functional regions of the protein (e.g., catalytic and binding points) become assembled. Lastly, the quaternary structure of proteins is formed when the subunits previously formed are interacting together. Nevertheless, it is the primary structure, containing the specific order of amino acids that primarily dictates how the folding will occur. Consequently, the same sequence will give every time the same structure, except when disturbance is introduced into the system.[9] Besides their structural function, proteins also serve as transporting agents, scaffolds of biological catalysis (enzymes), protection agents and hormone receptors.[9,10]

A particularly important example of self-assembly is provided by the double-stranded (ds) *DNA* molecule. The building blocks of DNA are nucleotides, consisting of a 5-carbon sugar (deoxyribose), a nitrogen containing base attached to the sugar, and a phosphate group. There are four different types of nucleotides [adenine (A), cytosine (C), guanine (G), and thymine (T)] found in DNA, differing only in the nitrogenous base. The DNA backbone is a polymer with an alternating sugar-phosphate sequence where A, G, C, and T bases extend away from the chain and stack on the top each other. Complementary pairs of nucleobases (A–T and C–G) from two antiparallel strands wrap around one another in the double helix. This configuration allows the formation of stable hydrogen bonds that sustain the strands together, reinforced by π-π stacking interactions between cyclic purine (A and G) and pyrimidine (C and T) bases (Figure 2(b)). The stability resulted from the combination between these two pairs of nucleobases is unique and responsible for the effectiveness of DNA replication, transcription, and ribosomal translation. DNA helix has a diameter of approximately 2 nm, a helical repeat of 3.4 nm (10.5 base pairs) and persistent length of about 50 nm.[11]

Viruses are protein and nucleic acid based supramolecules, with different shapes and variety of sizes, that manifest their functionality through self-assembly. A typical virus possesses a protein outer shell (capsid) composed of multiple copies of coat proteins arranged in a symmetrical fashion, housing the viral genome. In general, viruses adopt an icosahedral or helical structure, giving a spherical or rod-shape particle.[12] To assemble into an icosahedral geometry, the capsomers, the structural units of icosahedral viruses, are

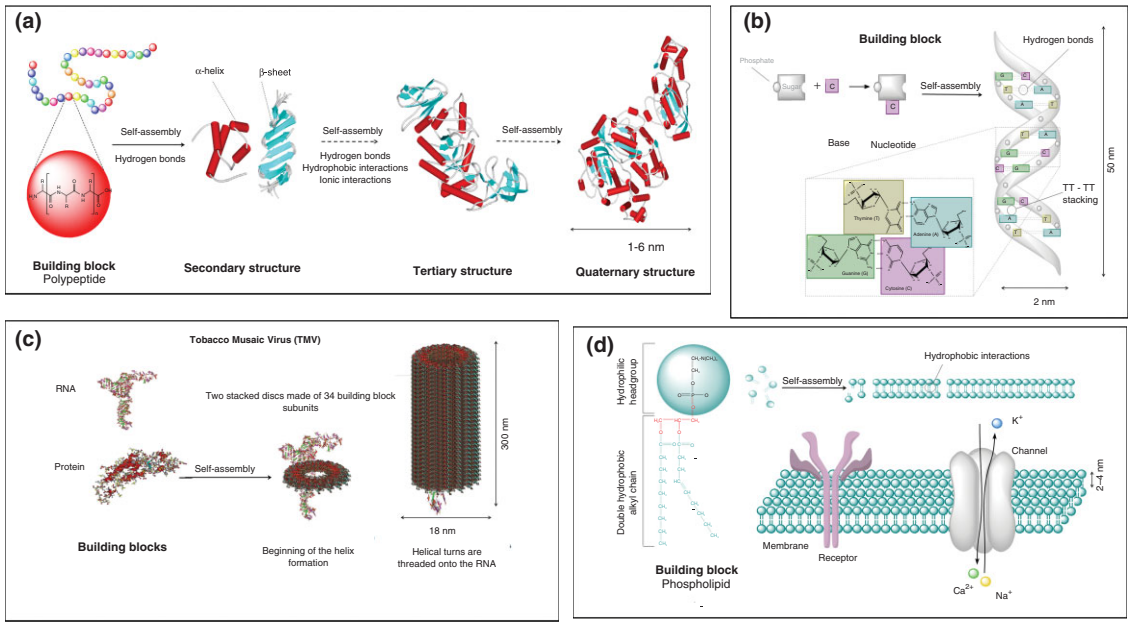


FIGURE 2 | Examples of biological self-assembled structures showing the building blocks and the relevant interactions involved in the self-assembly process. (a) Protein folding; (b) ds-DNA; (c) tobacco mosaic virus (TMV); and (d) cell membrane.

distributed symmetrically despite their self-assembly from asymmetrical components. The tobacco mosaic virus (TMV), which self-assembles into rod-like structures,[9,13] represents an excellent example of effective control over size of self-assembled structures. First, the TMV protein forms a two-layer disk, with 17 proteins in each ring. A special initiation sequence in the RNA then binds in the hole at the center. This cause the disk to dislocate, forming a lockwasher shaped ring with 16 1/3 subunits pert turn. The remaining subunits then stack in this structure elongating until the RNA is covered. Thousands of coat proteins (2130 identical protein units each with 158 amino acid residues) self-assemble into 300 nm long cylinder (protein shell—capsid) around a single RNA molecule (that comprises 6400 nucleotides) which acts as a template (Figure 2(c)). The length of the assembly is determined by the length of the enclosed RNA template. In the absence of the RNA template, the TMV coat proteins assemble into cylinders with the same diameter (18 nm) as the templated capsids but with variable length. The RNA template limits the self-assembly of the capsid proteins through specific molecular interactions. The head to tail configuration of TMV represents a superb example of a biological self-assembled system. One of the interesting features of the TMV is that if the virus is broken into its sub-unities, when re-mixed in physiological conditions, the virus has the ability to self-assemble exactly into the previous structure constituting a full replica.

The *cytoplasmic membrane* (Figure 2(d)) is a phospholipid bilayer that serves to create a confined space (the cell) isolated from the external environment. The phospholipid bilayer is arranged so that the polar ends of the molecules (the phosphate and glycerol portion of the phospholipid that is hydrated in water) form the outermost and innermost surface of the membrane, while the nonpolar ends (the fatty acid portions of the phospholipids that are insoluble in water) cluster in the nonpolar region of the membrane. This membrane is stable enough to serve as a barrier to the transport of water soluble ions (Na^+, K^+, and Ca^{2+}) and molecules and has the fluidity to allow the entrance of particles inside the cell by endocytosis (the process by which cells take in solutes or particles by enclosing them into vesicles or vacuoles pinched off from the cytoplasmic membrane).

The previously described examples clearly show how sophisticated self-assembly can be and how nature's assembly principles are used to create supramolecular materials.

DESIGN OF SUPRAMOLECULAR MATERIALS

From a materials science point of view, self-assembly can be a simple and low cost bottom-up fabrication process to obtain functional supramolecular materials from pre-existing components (building blocks) involving a reduced number of steps.[3] Different

TABLE 1 | Synthetic and Biological Building Blocks Used in Supramolecular Self-Assembly for Obtaining Diverse Complex Structures and Their Potential Biomedical Applications[4,15–20]

Building-Blocks			Supramolecular Assemblies	Applications
Synthetic	Polymers	Linear (e.g. block-co-polymers) AB ABA ABC	Micelles Vesicles Tubes	Nanoreactors; artificial organelles; nanocarriers drug delivery[21, 22]
		Branched (e.g. dendrimers) Dendrons	Nanoparticles Nanofibers	Nanocarriers for drug and gene delivery[23–25]
	Surfactants	Anionic Cationic Neutral	Micelles Vesicles	Drug and gene delivery systems; antimicrobial and antifungal activity[26, 27]
	Others	Graphene Porphyrin Rotaxane	Nanotubes Toroids Carbon nanotubes	Nanomedicine; drug delivery; hydrogels[8, 28, 29]
Biological	Viruses	CPMV λ phage hHPBV	Aligned phage film Fibrils Particles	Biomaterials; cell culture substrates[30–33]
	Nucleic acids	RNA DNA	DNA origami	Therapeutics (vehicles for drug delivery); diagnostics (biosensing)[11, 34, 35]
	Lipids	Fatty acid Phospholipid Cholesterol	Lipid bilayer Vesicles Films	Nanoreactors; artificial organelles; controled drug delivery[19, 36–37]
	Saccharides Amylose (helical)	Cyclodextrin (cyclic)	Double helix Nanotube Spherical micelle	Drug delivery; biosensors[38,39]
	Peptides	VSYK EACQ	Random coil β-sheet α-helix Helix protein	Hydrogel biomaterials; drug delivery; tissue engineering; 3D cell culture[40–48]

CPMV, cowpea mosaic virus; λ phage, lambda bacteriophage; hHPBV, human hepatitis B virus.

building blocks (synthetic and biological, Table 1) have been extensively studied for this purpose. These building blocks can be atoms, small molecules or macromolecules, and be designed (chemical nature of the blocks, composition, length, and molecular architecture) to contain all the necessary information that encodes their self-assembly for specific applications. In most cases, their self-assembly is reversible and allows the combination of different building blocks. The instructions for assembly lie on the molecular structure of the building components, namely on the precise geometrical positioning of functional groups, which provides multiple intra- and intermolecular interactions. Tuning the strength and directionality of interactions among building blocks is the key feature that defines the field of supramolecular materials.[14] The interactions that provide high degree of directionality to the assembly process of the different building blocks are potential elements in the design. By controlling the multiple interactions during assembly will lead to a wide range of ordered structures. The supramolecular structures that emerge through self-assembly are triggered by controlling environmental variables, which create a driving force that pushes the system to a new thermodynamic minimum. Each of these characteristics of the self-assembling system is described in detail in the following sections.

Self-Assembling Building Blocks

A wide range of different molecules (Table 1) can be used to form supramolecular nanostructures in a variety of different assembling conditions. The molecules must have the potential to form multiple noncovalent interactions in a given solvent.

Synthetic block copolymers have been widely used for fabricating a variety of organized assemblies

with distinct morphologies (e.g., micelles, vesicles, and tubes). Self-assembly of these supramolecular monomers relies on properties such as molecular dimension (including the relative volume fractions of the blocks), composition, solubility, stability, and stimulus responsive behavior.[49]

Dendritic molecules (e.g., dendrons) are synthetic molecules which have a well-defined (monodisperse) 3D branched architecture. Dentritic building blocks can assemble into more complex nanoscale assemblies (nanoparticles, nanofibers) by noncovalent interactions.[25]

Surfactants are compounds that consist of a hydrophobic (usually a long hydrocarbon chain) and a hydrophilic (ionic or polar group).[26] Examples of surfactants are the well-known sodium dodecylsulfate (SDS) and sodium oleate. Numerous studies have demonstrated that surfactants self-assemble in aqueous solution into micellar, vesicle, and multilayer structures.

Viruses have been used as robust building blocks in nanosciences due to their canonical shape (rod or sphere) and size uniformity. The surface properties of viruses can be manipulated genetically or chemically without disrupting their integrity and morphology. They have been employed to generate long-range ordered assemblies for biomaterials development.[30,33]

In biology, *nucleic acids* (DNA and RNA) are fundamental molecules of life as carriers of molecular information. DNA's base sequence stores and imparts instructions, while RNA's sequence plays the role of a messenger and a regulator of gene expression. As biological polymers, nucleic acids possess recognition capabilities and interesting chemical properties which depend on their base sequence. Nucleic acids can be synthesized with a nearly infinite number of sequences.[11] As referred before, the nucleobases present in DNA and RNA molecules can form hydrogen bonds and π-π stacking interactions and these interactions can be engineered for the bottom-up fabrication of a variety of nanostructures.[50] DNA double helix is inherently a nanoscale object. Although being more chemically labile, RNA molecules have structural components that are very similar to DNA. In addition, most RNAs are single-stranded molecules with nanoscale motifs that mediate precise intra- and intermolecular interactions. Two strands of DNA with completely complementary sequences can bind to each other and form a fully bases-paired duplex structure.

Lipids are the most important building blocks of cell membranes. Generally, they are composed of a hydrophilic head group and a hydrophobic tail region.[51] Due to the large variability in the head group and tail chemistries, lipids are often categorized into polar and nonpolar. The hydrophobic nature and rigid structure of lipids creates a tendency of these molecules to aggregate into larger structures in water in which the position and orientation of the molecules is organized. In addition, the structural diversity of lipids (fatty acids, triglycerides, phospholipids, and cholesterol) with different polarities, charged groups and lengths allows the formation of dynamic (e.g., monolayers, bilayers) and compartmentalized (e.g., micelles, vesicles) structures in water, acting as barriers, within which isolated processes can occur.

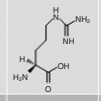
High-molecular weight *saccharides* (polysaccharides) are produced by a broad variety of plants and microorganisms with remarkable chemical (neutral, charged, and linkage type) and structural diversity (linear or branched, random coil or helical conformation). Polysaccharides contain multiple OH groups capable of hydrogen bonding. In addition, carbohydrates play key roles in many molecular recognition processes (e.g., bacterial and viral infection, cancer metastasis, and inflammatory reactions),[52] are highly adaptable and can introduce densely charged templates into nanostructured assemblies.[53]

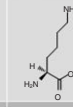
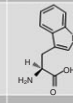
Amino acids are naturally occurring molecules being the constituents of *peptides* and proteins. There are 20 natural (L-form) amino acids that are used by cells to synthesize peptides and proteins. Their names, three-letter and one-letter codes and structures are given in Table 2.

With the exception of glycine (G), all amino acids are chiral and have the same basic structure, a central α carbon atom to which a hydrogen atom, an amino group, a carboxyl group and side chain R group are attached. The nature of their side chain (charged, polar, nonpolar, aliphatic, aromatic, Table 2) contributes to their biochemical mode of action and dictates the conformation assumed by peptides and proteins. The aliphatic residues (A, I, L, M, V) provide a hydrophobic environment while amino acids with aromatic (F, W, Y) side chains can be engaged in π-π stacking. Neutral polar residues (N, Q, S, T) can be involved in the formation of hydrogen bonding through OH (S, T) or CONH (N, Q) groups. Basic (H, K, R) residues can be positively charged and acidic amino acids (D, E) can carry a negative charge. The presence of charged groups in these residues can be used to create electrostatic interactions that are important to drive the self-assembly process.

There are other amino acids that contain functional groups with the ability to bind other compounds. For example, cysteine (C) can bind to gold surfaces and histidine (H) has high affinity for binding Ni^{2+} ions. Additionally, cysteine contains thiol groups (SH) available for the formation of

TABLE 2 | The Twenty Gene-Coded/Natural L-Amino Acids: Structure and Properties (Nature and pKa Values for the Ionizable Side Chains and Their Tendency for α-Helix-Promoting/Breaking and β-Structure Promoting/Breaking when Part of a Peptide. Neutral: 0.8–1.00, No Tendency Either Way)[17,54]

Amino acid	Three/ One-letter code	Structure	Nature/ pKa side chain	α-Helix promoting/ breaking	β-Sheet promoting/ breaking
L-Alanine	Ala (A)		Hydrophobic Aliphatic	1.45 (promoting)	0.97 (neutral)
L-Arginine	Arg (R)		Hydrophilic Basic (+) pKa ~ 12.4	0.79 (neutral)	0.90 (neutral)
L-Asparagine	Asn (N)		Polar Neutral	0.73 (breaking)	0.65 (breaking)
L-Aspartic Acid	Asp (D)		Hydrophilic Acidic (-) pKa ~ 3.8	0.98 (neutral)	0.80 (neutral)
L-Cysteine	Cys (C)		Polar Can form disulfide bond upon oxidation pKa ~ 8.2	0.77 (neutral)	1.30 (promoting)
L-Glutamic Acid	Glu (E)		Hydrophilic Acidic (-) pKa ~ 4.2	1.53 (promoting)	0.26 (breaking)
L-Glutamine	Gln (Q)		Polar Neutral	1.17 (promoting)	1.23 (promoting)
Glycine	Gly (G)			0.53 (breaking)	0.81 (neutral)
L-Histine	His (H)		Hydrophilic Basic (+) pKa ~ 6.1	1.24 (promoting)	0.71 (breaking)
L-Isoleucine	Ile (I)		Hydrophobic Aliphatic	1.00 (neutral)	1.60 (promoting)

Amino acid	Three/ One-letter code	Structure	Nature/ pKa side chain	α-Helix promoting/ breaking	β-Sheet promoting/ breaking
L-Leucine	Leu (L)		Hydrophobic Aliphatic	1.34 (promoting)	1.22 (promoting)
L-Lysine	Lys (K)		Hydrophilic Basic (+) pKa ~ 10.5	1.07 (neutral)	0.75 (breaking)
L-Methionine	Met (M)		Hydrophobic Aliphatic	1.20 (promoting)	1.67 (promoting)
L-Phenylalanine	Phe (F)		Hydrophobic Aromatic	1.12 (promoting)	1.28 (promoting)
L-Proline	Pro (P)			0.59 (breaking)	0.62 (breaking)
L-Serine	Ser (S)		Polar Neutral	0.79 (neutral)	0.72 (breaking)
L-Threonine	Thr (T)		Polar Neutral	0.82 (neutral)	1.20 (promoting)
L-Tryptophan	Trp (W)		Hydrophobic Aromatic	1.14 (promoting)	1.19 (promoting)
L-Tyrosine	Tyr (Y)		Hydrophobic Aromatic pKa ~ 10.1	0.61 (breaking)	1.29 (promoting)
L-Valine	Val (V)		Hydrophobic Aliphatic	1.14 (promoting)	1.65 (promoting)

intra and intermolecular disulfide bonds (crosslinking) upon oxidation, while serine (S), threonine (T) and tyrosine (Y) provide chemical groups for chemical or enzymatic modification (e.g., OH groups in serine can be phosphorylated to yield phosphoserine). The absence of a side chain in glycine (G) allows a higher degree of flexibility compared with other residues. On the contrary, proline (P) having its side chain attached to the amino terminus, can add structural rigidity due to its locked conformation.[17] Amino acids are therefore simple building blocks that provide relevant noncovalent interactions to build complex supramolecular assemblies. Peptides are formed by linking a series of amino acids by peptide (amide) bonds. Just using natural amino acids, the number of possible combinations that can be obtained for a single peptide or protein is extremely large (20^n, where n is the number of amino acids in the sequence). Having the flexibility of using 20 chemically different amino acids, peptides are versatile assembly components due to the intrinsic functional diversity of amino acids and have been widely studied as self-assembling systems. The unique self-assembling properties of peptides and their ability to form emergent structures are increasingly being exploited for the development of new bioactive materials. A number of very important physiological and biochemical functions of life are influenced by peptides. For example, peptides are involved in receptor-mediated signal transduction, influencing cell–cell communication upon interaction with receptors. Another important feature of peptides is related with their aggregation state and its implications in human diseases. Long, unfolded polypeptides have an innate tendency to form aggregates, such as amyloid fibrils which are known to be involved in Alzheimer's disease. The self-assembly process involved in collagen fibrillogenesis is also determinant for proper animal development and may be associated with certain pathological situations.

The isolation of peptides from natural sources is often problematic and the demand of synthetic peptides in biological applications is steadily increasing. Peptides can be obtained through chemical synthesis or genetic engineering.

Synthetic routes include conventional synthesis in solution or solid-phase chemistry which allows for the synthesis of short peptides (2–30 amino acids). Several reviews covering solid-phase peptide synthesis have been published.[55–57] The easy preparation of short peptides by solid phase has provided ample opportunities for peptide design and to study the effect of molecular design on their properties.

As self-assembling building blocks, peptides are readily accessible through chemical synthesis; the information required for their self-assembly is encoded within their sequence; their self-assembly is usually spontaneous (simple method to develop nanostructured materials), instantaneous (milliseconds), and reproducible (defined stable structures). Furthermore, by varying systematically the chemical structure (e.g., sequence size and nature of the amino acid side groups) during synthesis it is possible to adjust the self-assembling properties of the building blocks as well as to produce a variety of diverse nanostructures (e.g., micelles, fibers, vesicles).[58] The specific field of peptide self-assembly will be the focus of this review.

Classes of Self-Assembling Peptides

Different peptide sequences have been designed to yield peptides with self-assembling properties. The most widely described in the literature are discussed below.

α-Helix/Coiled-Coil Forming Peptides

α-Helical coiled-coil motifs are highly diverse, in terms of structure and function, and are therefore excellent starting units to form fibrous structures.[15] Most coiled coils are based on heptad sequence repeat (abcdefg). The first and fourth positions (a and d) are usually hydrophobic residues and the remaining sites largely polar. This configuration results in a rope-like assembly that measures 1 nm per heptad. Excellent reviews on coiled coil based systems have been described by Woolfson and colleagues[59,60] and such systems are not discussed in detail here. Due to their supramolecular arrangement, coiled-coil peptides can be used to present biological epitopes with high density.[59] Villard et al.[61] showed that α-helical coiled-coil peptides carrying the RGD motif at the N-terminal ($GRGDSPSG_2QLAQ_2L(Q_2LAQ_2L)_4$) could be used as soluble antagonists or surface-immobilized agonists to inhibit or promote integrin-mediated cell adhesion.

Replicating the helical structure of collagen is very appealing and Chaikof[62] and Hartgerink[63] have developed a strategy that employs electrostatic interaction to guide the self-assembly of heterotrimeric triple helices using aminoacid triplets $(Pro\text{-}Arg\text{-}Gly)_n$, $(Glu\text{-}Hyp\text{-}Gly)_n$, $(Pro\text{-}Hyp\text{-}Gly)_n$ on their peptide chain design. In a recent paper, Hartgerink[64] described the synthesis of collagen mimetic peptides $(Pro\text{-}Lys\text{-}Gly)_4(Pro\text{-}Hyp\text{-}Gly)_4(Asp\text{-}Hyp\text{-}Gly)_4$ that was shown to replicate the self-assembly of collagen (from peptide chain to triple helix formation to nanofiber and finally to a hydrogel). This finding demonstrates the utility of electrostatic interactions for stabilizing triple helices.

β-Sheet Forming Peptides

A wide range of natural proteins possess intrinsic propensity to self-assemble into fibrillar nanostructures that are rich in β-sheet secondary structure.[65] The β-strands in the nanofibrils are organized perpendicularly to the fibril axis and connected through a dense hydrogen-bonding network between amides and carbonyls in the protein backbone.[66] Several different β-sheet forming peptides have been designed sharing a common motif of alternating hydrophobic and hydrophilic residues.

Self-Complementary Peptides

The group of Zhang at MIT has developed a class of self-assembling peptides termed as self-complementary ionic peptides inspired by the segment $(RERERKRK)_2$ found in the Z-DNA binding protein zuotin.[67] The peptides, termed as RAD16-I ($(RADA)_4$), KLD-12 ($(LKLD)_3$), EAK16-II ($(AE)_2(AK)_2(AE)_2(AK)_2$) are composed of alternating hydrophilic and hydrophobic amino acids residues (Figure 3(a-i)), present β-sheet configuration and ability to form hydrogels through changes in ionic strength or pH (addition of salts or buffers).[68]

Glutamine-Rich Peptides

The Ac-Q_2RFWQFEQ_2-Am (P_{11}-2) β-strand forming sequence was first described by Aggelli and co-workers.[69] The design is based on alternating hydrophilic (R, Q, E) and hydrophobic (F, W) residues in the core which favored a β-strand conformation. An odd number of amino acid residues were selected to encourage a complete register of the adjacent amphiphilic peptide strands in an antiparallel β-sheet tape. Side-chain interactions are another important factor in driving strand assembly. Dimethylene groups on the glutamine residues provide hydrophobic side-chain interactions, while the hydrophobic aromatic residues additionally interact via π-π stacking interactions. Aqueous solubility of peptide tapes is promoted through the presence of hydrophilic residues. Finally, the positively charged arginine and the negatively charged glutamic acid residues are positioned such that the complementary charges of arginine and glutamic acid on neighboring strands are adjacent to each

other if the peptide is in an antiparallel β-sheet configuration. P_{11}-2 was found to form self-supporting gels at a concentration of approximately 15 mg/mL in pH-neutral water. Following the initial design, other β-sheet fibrillizing peptides have been designed. For example, the 11-residues sequence rich in glutamine (Q11 peptide), containing arginine and glutamic acid at positions 3 and 9 ($AcQ_2RQ_5EQ_2$-$CONH_2$) was shown to form antiparallel β-sheets that at higher concentrations (0.01 mM) form semi-flexible tapes (Figure 3(a,b-ii)).[70]

β-Hairpin Peptides

β-hairpin peptide sequences have been explored by Schneider and Pochan[71,72] due to their ability to self-assemble into gels by its intramolecular folding propensity. The design of β-hairpin peptides comprises a tetra-peptide turn sequence (–V^DPPT–) and two neighboring β strands of alternating hydrophobic valine (V) residues and hydrophilic lysine (K) residues [$(VK)_4V^DPPT$-$(KV)_4$-NH_2] (Figure 3(a-iii)).[72,73] This initial peptide sequence has been referred as MAX1. At acidic pH, the lysines are protonated preventing peptide self-assembly. Neutralizing the charge, by addition of counterions or increase of pH, results in the formation of self-supporting rigid hydrogels by self-assembly. Replacing the lysine residue at position 15 with a negatively charged glutamic acid (E) residue [$(VK)_4V^DPPT$-$K(VE)(VK)_2V$-NH_2], the gelation kinetic of the resultant peptide (MAX8) increased significantly in response to identical cell culture conditions.[74] It has been demonstrated that the gel properties (e.g., gelation kinetics, stiffness, network mesh size) of the β-hairpin peptides have the ability to be modulated for cell encapsulation[74] or controlled release[75] purposes by changing the peptide sequence, concentration, ionic strength and/or temperature.[76]

Amphiphilic Peptides

Amphiphilic peptides contain both hydrophobic and hydrophilic segments on their structure. This class of self-assembling peptides includes surfactant-like peptides and peptide amphiphiles (PAs) and is beginning to attract great interest in bionanotechnoogy, due their versatility and potential to form bioactive nanostructures by self-assembly. Their self-assembly properties have been investigated and exploited by several groups.[77]

Surfactant-Like Peptides. Zhang's group designed surfactant-like peptides in which the amphipathic nature of the peptide molecules derives solely from the sequence of amino acids. These peptides have a hydrophobic tail and a hydrophilic head and are able to self-assemble into organized nanostructures including micelles, vesicles, and tubes in water (Figure 3(a,b-iv)).[43,78] The nonpolar tail consists of six amino acids with variable hydrophobicity, such as G, A, V, I, L, and F, followed by charged amino acids (D, K) as the hydrophilic head to ensure solubility in water. Examples of surfactant-like peptides are: Ac-A_6D-OH, Ac-A_6K-NH_2, Ac-V_6K_2-NH_2, DA_6-NH_2, Ac-A_6K-OH, Ac-I_6K_2-NH_2, AcV_6D_2-OH, KA_6-NH_2, and Ac-V_6R_2-NH_2. By varying the number of glycines from 4 to 10 as the component of the hydrophobic tails and aspartic acid as the hydrophilic heads (G_nD), they showed that these peptides formed nanotubes and nanovesicles in water at neutral pH and the obtained nanostructures became more polydisperse as the length of the glycine tails increased.[79] A different design proposed by Zhang includes cone-shaped amphiphilic peptide (e.g., Ac-GAVILRR-NH_2) with a hydrophilic head of two charged amino acids and a tail with decreasing hydrophobic and side chain (R) size.

Peptide Amphiphiles. PAs are peptide-alkyl-chain surfactants in which a peptide segment is covalently bond to a very hydrophobic segment, usually a simple alkyl tail found in lipid molecules (Figure 3(a-v)). Their surfactant nature orients the more hydrophilic peptide segment into the periphery, and the hydrophobic segment to the core of the assembly. The laboratory of Stupp has given important contributions in understanding the self-assembly of PAs.[42] The peptide segment can be divided in two or three domains.[41] The sequence close to the hydrophobic segment was designed to promote β-sheet formation between PA molecules and is the driving force for the formation of cylindrical nanostructures instead of spherical micelles. The presence of charged elements into the peptide block, not only enhances their solubility in aqueous solutions, but also suppress their self-assembly as a result of charge repulsion. The net charge also endows the PA responsive to pH variations and salt addition and drives their self-assembly into cylindrical nanofibers (Figure 3(b-v)) upon neutralization or charge screening. The nanofibers intertwine into 3D networks resulting into a self-supporting gel matrix. Additionally, a bioactive peptide sequence (e.g., cell-adhesive sequence—RGD), can be incorporated on the other side of the PA molecule, allowing its display at the water interface upon self-assembly, being accessible to be recognized by or to recognize

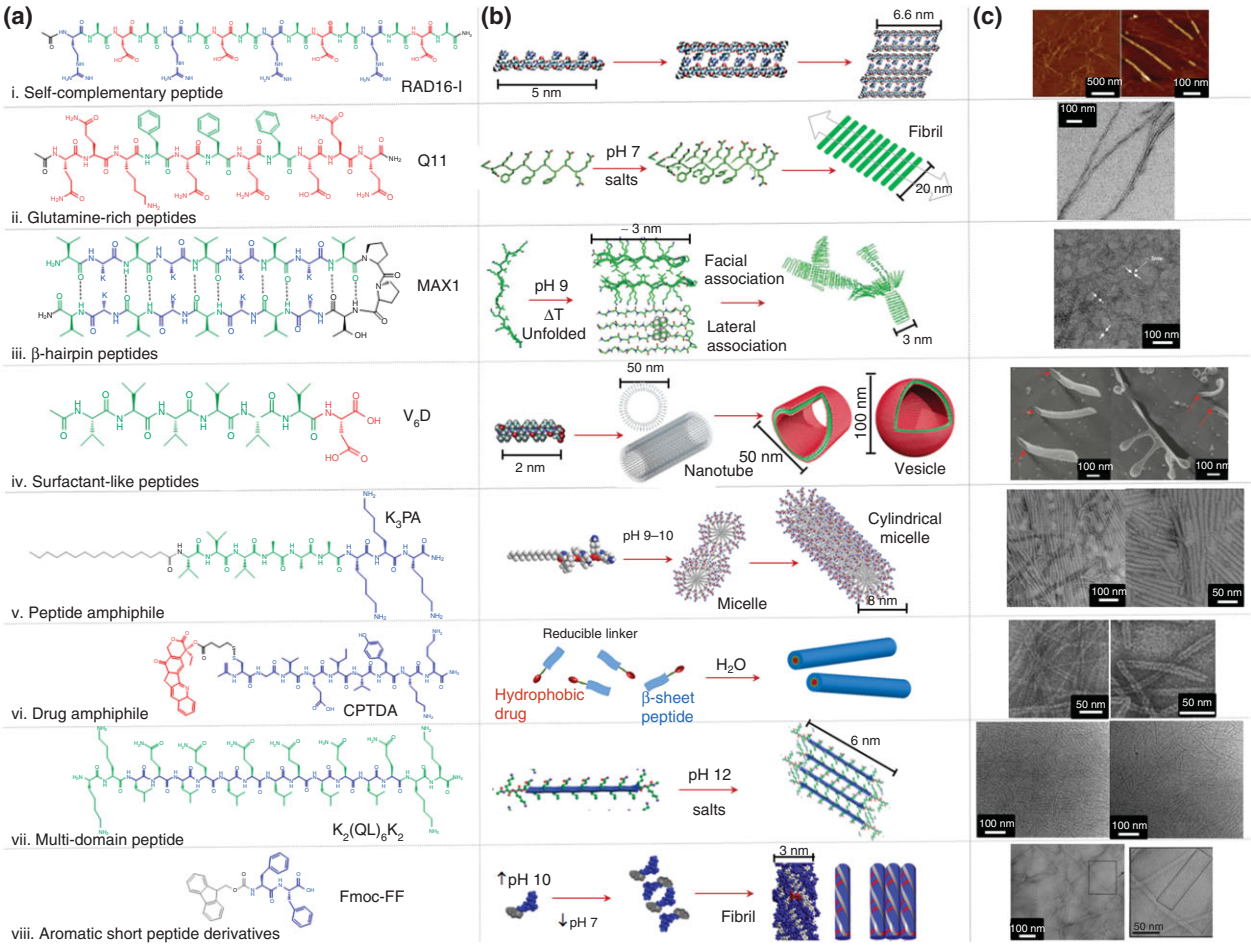


FIGURE 3 | Examples of self-assembling peptides with β-sheet secondary structure: Molecular structure (a) of peptide building blocks, molecular graphics (b) showing their self-assembly mechanism and TEM/AFM images (c) of corresponding self-assembled nanostructures. (b-i, c-i: Reprinted with permission from Ref 101. Copyright 2005 National Academy of Sciences, USA; c-ii: Reprinted with permission from Ref 102. Copyright 2010 National Academy of Sciences, USA; b-iii, c-iii: Reprinted with permission from Ref 103. Copyright 2009 American Chemical Society; b-iv, c-iv: Reprinted with permission from Ref 78. Copyright 2002 National Academy of Sciences; b-iv: Reprinted with permission from Ref 104. Copyright 2002 Elsevier and Reprinted with permission from Ref 79. Copyright 2002 American Chemical Society; b-vi, c-vi: Reprinted with permission from Ref 91. Copyright 2013 American Chemical Society; b-vii, c-vii: Reprinted with permission from Ref 94. Copyright 2007 American Chemical Society; (b-viii, c-viii: Reprinted with permission from Ref 100. Copyright 2009 Elsevier)

other biomolecules. The nature of the cylindrical assembly of the PA system allows the presentation of high density of biological signals perpendicular to the long axis of the nanofiber, an external presentation that is favorable for cell signaling.[80] The Stupp lab has designed a variety of PA molecules with high control over their nanostructure morphology and functionality.[41,42,81] The amino acids in the peptide segment and the type of alkyl tail can be varied to change the pathway of self-assembly and the physical properties of the final self-assembled structure.

The group of van Hest has also created amphiphilic peptides using the $GANPNA_2G$ sequence (derived from the CS protein of the malaria parasite) acylated at the N or C termini with alkyl chains of variable length (C_n-$GANPNA_2G$-OH, $n = 6$, 10, 12, 14, 16, 18)[82] and with diacetylene functionality[83,84] to tune their assembly behavior. When the peptide was coupled to short alkyl chains (containing 12 or less carbons) no aggregates were formed, whereas with longer tails (containing more than 16 carbons) the PAs formed highly stable assemblies. Tirrell and co-workers have investigated PA molecules that can self-assemble into micellar structures.[85–89] A different PA design, containing a pentapeptide head group based on a sequence from procollagen I attached to a hexadecyl lipid chain (C_{16}-KT_2KS) was shown to assemble into extended nanotapes in aqueous

solution.[90] This is the only PA currently in use in the market.[77]

Drug Amphiphiles. A new type of PAs, known as drug amphiphiles (DAs), has been reported recently.[91,92] A typical DA combines a hydrophobic drug (e.g., camptothecin, CPT) with a small β-sheet forming peptide sequence (VQIVYK) derived from the Tau protein, conjugated through a linker (Figure 3(a-vi)). These amphiphilic molecules were shown to self-assemble into discrete filamentous nanostructures (nanofibers or nanotubes, Figure 3(b,c-vi)). These nanostructures can act as self-delivering drugs, without the need for additional carriers, allowing the precise control of drug content by attaching one or more drug molecules.

Multi-Domain Peptides

Hartgerink's group has proposed a distinct design of peptides termed as multi-domain peptides (MPDs). The MDP consists of an ABA block motif[93,94] in which the B block is composed of alternating hydrophilic and hydrophobic amino acids (glutamine or serine and leucine, respectively, Figure 3(a-vi)) surrounded by the charged flanking A blocks (e.g., glutamate or lysine). $K_2(QL)_6K_2$, $K_2(SL)_6K_2$, $E(QL)_6E$, $E(SL)_6E$ are examples of MDPs investigated by the Hartgerink group. The A block provide water solubility and counteract fiber assembly via electrostatic repulsion. This design allows all glutamine or serine side chains to lie on one face of the peptide and leucine side chains on the other. In aqueous environment, there will be a significant driving force for two of these hydrophobic faces to pack against one another forming a hydrophobic sandwich which consequently stabilizes the fully extended conformation. An intermolecular β-sheet hydrogen-bonding network can then be created between two or more pairs of these sandwiches (Figure 3(b-vii)). Different variations of these MPDS have been previously synthesized and demonstrated to self-assemble into well-defined nanofibers (Figure 3(c-vii)) with various bioactive epitopes[95–97] and form self-supporting gels when the charge is screened (e.g., in the presence of multivalent anions such as phosphate).[94]

Aromatic Short Peptide Derivatives

Another class of self-assembling peptides is short aromatic peptides based on the amyloid-forming peptides containing the diphenylalanine (FF) polypeptide recognition motif.[16] These short peptides form highly stable hollow nanotubes in solution. Different variations of these low molecular weight peptides have been investigated, including di- or tripeptides conjugated with bulky aromatic groups, such as fluorenylmethoxycarbonyl (Fmoc), or naphthalene, at the N-terminus. Self-assembly occurs by a combination of hydrogen bonding and π-π stacking of the aromatic rings. Fmoc-peptides have been investigated by Gazit[65,98] and Ulijn[99] (Figure 3(a-viii)). For example, Ulijn's group has investigated the self-assembly of Fmoc-dipeptides with varying hydrophobicity (Fmoc-FF-OH, Fmoc-FG-OH, Fmoc-GF-OH, Fmoc-LG-OH, Fmoc-AA-OH, Fmoc-AG-OH, Fmoc-GG-OH).[40] An interesting feature of these systems is the formation of stable hydrogels which can be used to encapsulate cells. Therefore, these peptide systems have been functionalized with biological motifs (Fmoc-FRGD-OH, Fmoc-RGD-OH).[98,100] This class of peptides consists on much shorter and simpler peptide sequences as compared to those described in the other categories, which might lower their costs and facilitate their commercialization.

Methods and Techniques for the Characterization of Self-Assembled Peptide Nanostructures

The function of self-assembling peptides clearly depends on their structural properties. There are a number of techniques that have been used to characterize the structure of self-assembled peptides and study their assembly. This structural information is important to understand the mechanism of peptide self-assembly and determine potential applications. For example, circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies can provide information about the self-assembly behavior (folding) of peptides in solution, while atomic force microscopy (AFM) and Transmission electron microscopy (TEM) have been used to provide high-resolution information on the size and nanoscale morphology of the assembled structures (Figure 4).[46,105]

Solution and solid state NMR have been used to determine secondary structure of peptides and their self-assembly mechanism, as it allows to characterize aggregation at the atomic level. Solution state NMR can measure bulk solution properties, such as aggregation of complex molecules in solution over time (early events of aggregation) and probing the effect of the environment on the self-assembly (e.g., pH, solvent, temperature).[107–109] In addition, it has the advantage of preserving the native peptide conformations. 2D NMR spectroscopy was used to elucidate the assembly mechanism during the co-assembly of oppositely charged PAs.[110] By providing evidences on close intermolecular contacts between amino acids of different PA molecules, it was hypothesized that the two oppositely charged

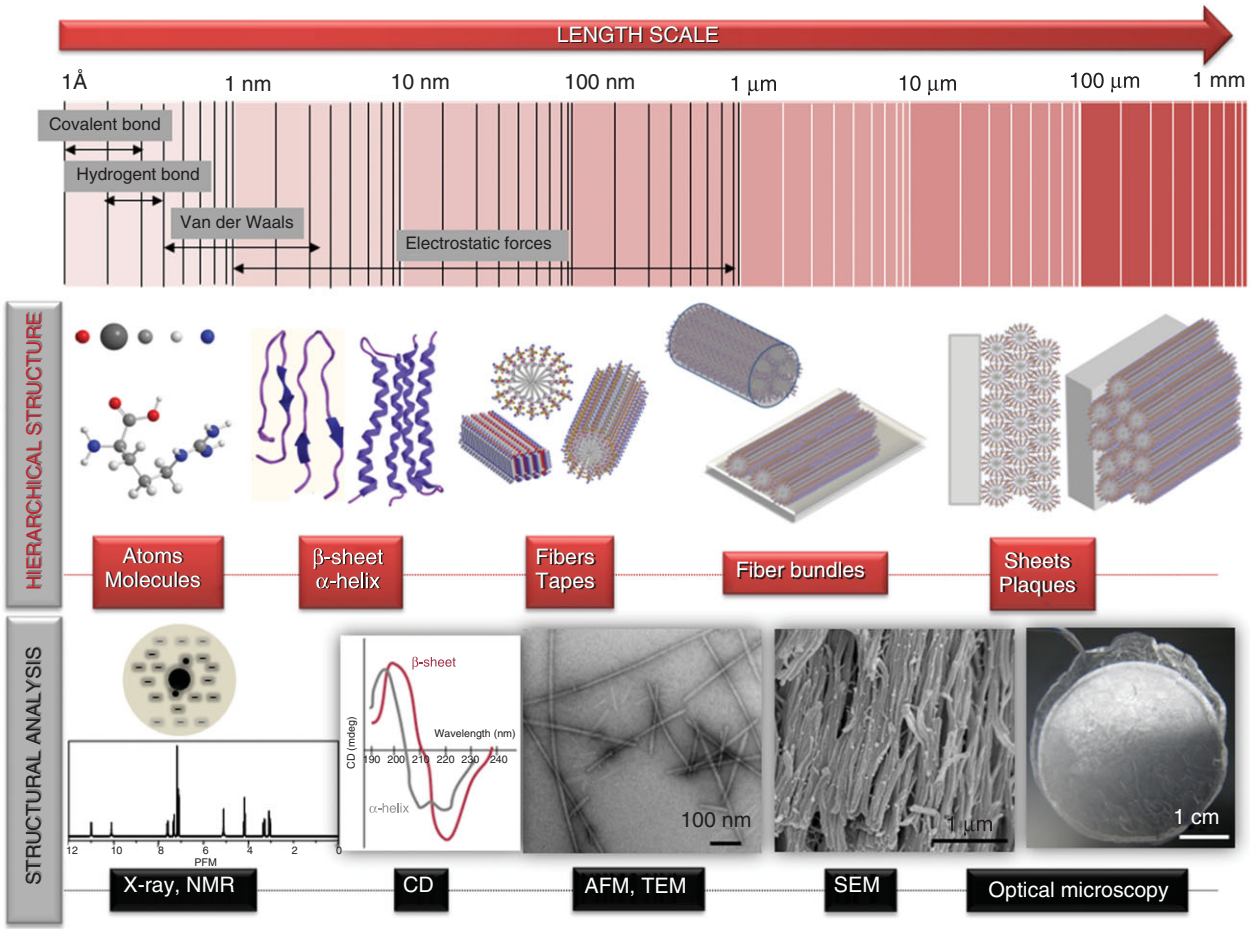


FIGURE 4 | Length scales of the forces involved in self-assembly[106] (first panel) and the hierarchical complex structures generated by peptide self-assembly (second panel). Spectroscopy and microscopy techniques used for structural characterization of peptide molecules and assemblies from the nanometer to centimeter length scales (third panel). NMR, nuclear magnetic resonance; X-ray, X-ray diffraction; CD, circular dichroism; AFM, atomic force microscopy; TEM, transmission electron microscopy; SEM, scanning electron microscopy.

molecules are thoroughly mixed within any given nanofiber as opposed to molecules segregating into mixtures of homomeric fibers.

The conformation of peptides in solution can be determined by CD spectroscopy and this technique has been widely used to study peptide self-assembly.[111–114] β-sheet structures show a strong maximum at 195 nm in positive CD, zero CD at 207 and 250 nm and a maximum at 217 nm with medium intensity in the negative CD. A characteristic CD signature of α-helices shows maximum at 191 nm (positive CD) and 208, 222 nm (negative CD) with zero CD at 202 and 250 nm. An unordered (random) conformation exhibits a maximum (weak) at 218 nm in the positive CD, zero CD at 211, 234, and 250 nm, with a strong CD signal at 197 nm and very weak at 240 nm in the negative CD. The secondary structure content in peptides can be calculated using CD data and Chou-Fasman rules.[115]

X-ray diffraction techniques have been widely used to measure the degree of order in self-assembled structures and include analysis in the small-angle (small-angle X-ray scattering, SAXS) and wide-angle (wide-angle X-ray scattering, WAXS). SAXS allows determination of order in the range 1–100 nm, whereas WAXS is used on atomic length scale (<1 nm). For example, *in situ* SAXS and WAXS were used, respectively to characterize the wall thickness (1–100 nm) and 2D molecular crystal structure (angstrom scale) of crystalline membranes obtained by co-assembly of oppositely charged amphiphiles.[116]

Optical polarization microscopy can also be used to quickly observe structure orientation and obtain information about liquid crystalline-like structures.[117] Electron microscopy is useful to supplement SAXS

analysis as it can provide information on the morphology of the self-assembled nanostructures (Box 1).[118]

BOX 1

ADVANCED MICROSCOPY TECHNIQUES FOR THE CHARACTERIZATION OF SELF-ASSEMBLED PEPTIDE NANOSTRUCTURES

Advances in electron microscopy [scanning electron microscope (SEM) and transmission electron microscopy (TEM)] have permitted the visualization of nanostructures in their natural state with minimal artifacts or interference from sample preparation. In cryo-SEM and -TEM, the examination is done below ambient temperature (typically between −100°C and −175°C), allowing the structure of the sample to be preserved and recorded in the fully hydrated and chemically unmodified state. For example, cryo-TEM provides a method to directly visualize nanostructures in the solution state through a thin film of vitrified solvent.[119,120] AFM, together with TEM, has been used to characterize the morphology[113] and dimensions (e.g., width and height of nanofibers)[121,122] of self-assembled peptide nanostructures. Nanoindentation experiments on single peptide nanofibers have been performed using AFM to evaluate the nanomechanical properties of the assemblies.[122,123] In addition, AFM allows specimen imaging in wet conditions, avoiding artifacts caused by specimen drying, and enabling live observation.[118]

Strategies to Control the Self-Assembly Process

The self-assembly of molecules is normally carried out in solution (Figure 5(a)) or at an interface (Figure 5(c)) to allow the required motion of the components. The interaction of the components with their environment can strongly influence the course of the process. A driving force causes the movement of the system through the different possible configurations into a ordered final state. For self-assembly to occur, molecules must be mobile. In solution, thermal motion provides the major part of the motion required to bring the molecules into contact. Therefore, one of the challenges consists on assuring the mobility of the components; as they become larger than molecules, Brownian motion rapidly becomes irrelevant, and gravity and friction become important. The concentration of self-assembling units is expected to influence the diffusion and the mobility of the components. Increasing the concentration will drive assembly of larger structures.

For self-assembly to generate ordered structures, the association either must be reversible or must allow the components to adjust their positions within an aggregate once it has been formed. The strength of the bonds between the components, therefore, must be comparable to the forces tending to disrupt them.

Environment and Driving Force

There are many pathways to trigger and/or drive the self-assembly by changing gradually the environmental conditions (e.g., building block concentration, pH, temperature, ionic strength, solvent, Figure 5(a)) or using an external input (e.g., light, enzyme activity). The presence of ions in solution, as well the pH and solvent polarity, are significant factors that affect the noncovalent interactions and hence the assembly routes. The pH environment can modulate the degree of protonation/deprotonation of amino acids with ionizable groups (carboxyl and amino side chains, Table 2) in peptides and proteins and thus control their electrostatic interactions (attraction/repulsion). Similarly, ionic strength also affects the strength of ionic interactions. Repulsive forces, due to ionic interactions, can be neutralized due to changes in pH, or addition of multivalent ions or charged polymers. In an early paper, Stupp and co-workers showed three different modes of self-assembly of PA molecules, using pH control, addition of divalent ions and concentration.[124] At neutral pH, the PAs have a net negative charge, which keeps PA molecules from self-assembly as a result of electrostatic repulsion. When exposed to HCl vapor, and as the acid diffused into the solution, the negative charge is eliminated with the consequent aggregation of the alkyl tails and gel formation. In a similar effect of charge screening, the addition of a divalent ion, such as Ca^{2+}, causes gelation of the solution. Increased PA concentration due to solvent evaporation was also shown to drive the PAs self-assembly. In another example, heparin, a sulfated glycosaminoglycan, was used to induce the self-assembly of a positively charged PA.[125] pH variation has been used in other self-assembling peptide systems for inducing gelation, including Ulijn's Fmoc-dipeptides.[40] More recently, Adams's group has exploited salt-induced gelation of functionalized-dipeptides to form rigid hydrogels.[126,127] The effects of solvent type and final concentration on the hydrogel strength were studied by Gazit and colleagues using aromatic dipeptides.[128]

The peptides were first dissolved in an organic solvent (hexafluoroisopropanol or acetone) and then diluted with water, resulting in the formation of a strong and rigid hydrogel. As expected, the peptide final concentration influenced the hydrogel strength, the storage modulus increased with an increasing peptide concentration, in the range 2–10 mg mL^{-1}. Aromatic dipeptides (e.g., Fmoc-LD) also showed thermoreversible behavior.[129] When such peptides are dissolved in water at concentrations below 1% at 100°C, and cooled below 60°C, they formed turbid solutions.

Bulk self-assembly of small molecules frequently yields structures that exhibit a high degree of order on the nanometer scale, whereas at the micrometer scale they exhibit more disordered morphologies. A method to dynamically control the self-assembly process consist of designing self-assembling molecules that are sensitive to external stimulus, such as light or catalytic activities. This external stimulus can convert a nonassembling precursor into self-assembling element which is produced locally. A major challenge in self-assembly is to control the kinetics of the process (nucleation and growth) and avoid defects in the formed assemblies. There is, therefore, a need to design self-assembling systems (building blocks and assembling pathways) that allow the spatiotemporal control of the process. Light-triggered self-assembly was demonstrated by the Stupp group by conjugation of a 2-nitrobenzyl group with the amide nitrogen of the amino acid closest to the hydrophobic segment (alkyl tail) of PA[130] This chemical configuration protects the amide nitrogen from hydrogen bonding. The 2-nitrobenzyl group can be cleaved photochemically (upon light irradiation at 350 nm) restoring a standard PA.

The specific activities of enzymes have been also explored in the context of dynamic self-assembly by designing self-assembling building blocks that are sensitive to enzymatic activities under controlled conditions of pH, temperature, and ionic strength. Enzyme-directed self-assembly can be achieved either by catalyzing the synthesis of self-assembling molecules, or by removing a blocking from the molecules.[17] For that, several enzymes have been used to generate supramolecular assemblies via bond cleavage (proteases, phosphatases, and esterases, which catalyze hydrolysis reactions) or bond formation (e.g., kinases, which catalyze the transfer of a functional group from one molecule to another). A PA was designed containing a sequence which is a substrate of a protein kinase A (PKA).[131] Upon treatment with PKA, the PA molecules became phosphorylated causing the disassembly of the original cylindrical structures. Subsequent treatment with alkaline phosphatase enzyme, which cleaves the phosphate groups, resulted in PA reassembly. The laboratory of Ulijn has provided very interesting examples on enzymatically controlled self-assembly.[113,132–134] They have used a protease enzyme (subtilisin) to produce building blocks in reversible and spatially confined manners using aromatic PA (Fmoc-dipeptide methyl esters) as self-assembling precursors. Subtilisin catalyzes the hydrolysis of these esters to yield a Fmoc-peptide that self-assembles into hollow nanotubular structures.[113] Because the rate of formation of the self-assembling building blocks is determined by the kinetics of the enzymatic reaction, different folded structures may be formed, hence offering additionally the possibility of controlling the mechanical properties.

Directed Self-Assembly

In directed self-assembly (DSA), the positions of self-assembling building blocks are guided by an external input to introduce hierarchical organization in supramolecular materials. Examples of external inputs include temperature gradients, radiation, mechanical, magnetic, and electric forces (Figure 5(b)) and chemically or topographically patterned substrates (Figure 5(c)).

Temperature is known to play a role on the kinetics of self-assembly by regulating the formation and size of ordered aggregates.[135] An interesting study reporting the use of temperature control on PA assembly at the micro and macroscale by the formation macroscopic bundles of aligned nanofibers has been demonstrated by Stupp and co-workers.[117]

Field-Directed Self-Assembly

Externally applied fields can also be used to direct the self-assembly process. Field-directed self-assembly has the added advantage that fields can be switched on/off and tuned dynamically. Therefore, these external forces can be used to template assemblies with improved long-range order and controlled orientation. Time varying fields can be used to agitate an equilibrium structure in order to allow kinetically trapped defects to be removed from the system or to establish and sustain a dynamically self-assembled system. For example, the van Hest group has shown the alignment of diacetylene-containing PA nanofibers in the presence of a strong magnetic field (20 T).[136] They found that it was necessary to first disassemble the fibrous structures by heating the sample to 90°C and after cooling down in a magnetic field resulted in significant alignment

of the PA assemblies and the fibers were oriented parallel to the applied magnetic field. Furthermore, the alignment on a molecular level was passed on to the high-molecular-weight chains that formed upon light-induced polymerization of the diacetylene groups and was reflected in optical properties of the material. This study shows that through a convenient hierarchical build up of self-assembling materials, structural information on a molecular scale is transferred to macroscopic properties, allowing the development of new materials that are well defined on all dimension scales. Later, the same group reported the patterning of a solid film using polarization holography (variation of the light polarization direction) and diacetylene-containing PA fibers aligned in a strong magnetic field, with no template, mask, or mechanical contact with the film.[137] Using polarized light, spatially confined polymerization was achieved because polymerization only took place at positions where the polarization of the incident light was parallel to the fiber orientation, yielding a polymer pattern.

X-ray irradiation was also shown to induce the crystallization of like-charge supramolecular peptide filaments by repulsive forces at low peptide concentrations (at concentrations greater than or equal to 1 wt% the crystallization is spontaneous).[138] The X-ray triggered organization of peptide filaments from a disordered state to a hexagonally ordered state was attributed to an increase in charge density caused by X-ray irradiation leading to enhanced electrostatic repulsions among filaments. Addition of ions that screened the charges on the filaments suppressed the formation of crystalline structures. More recently, Stupp and co-workers reported the effect of an electrical field during the dynamic self-assembly of a negatively charged polyelectrolyte and a positively charged PA in water leading to the formation of an ordered membrane.[139] The superposition of an external electric field during molecular co-assembly and diffusion of the charged species allowed controlling both growth rate and directionality of the fibrils that made up the membrane. Depending on the strength and orientation of the field, they observed a significant increase or decrease of up to nearly 100% in the membrane thickness, or the controlled rotation of nanofiber growth direction by 90°, which led to a significant increase in mechanical stiffness. These results suggest the possibility of using electric fields to control structure in self-assembly processes that involve the diffusion of oppositely charged molecules.

Zhang first reported the use of microcontact printing, together with self-assembling oligopeptides containing cell adhesion sequences, to fabricate a variety of surface patterns.[140] They showed the formation of complex cell patterns on the engineered surfaces. Stupp and co-workers have also used surface patterning techniques, including dip-pen nanolithography[141] and soft lithography[142–144] to control the self-assembly of PAs on different substrates to align and generate precise patterns of self-assembled peptide nanofibers over large areas (Box 2). The final morphology of assembled peptide nanostructures and organization are likely to be mediated by the surface charge and evaporation conditions. Steric confinement is also expected to play a role, promoting the transition to a lyotropic liquid crystalline phase as solvent evaporates. More recently, Mendes et al.[145] have successfully used conventional lithography techniques to guide the self-assembly of a positively charged MPD with the anionic biopolymer hyaluronic acid, to generate different regular patterns with microsized dimensions on self-supporting membranes. They demonstrated that integration of bottom-up and top-down fabrication processes allowed the development of membranes with a hierarchical structure composed of self-assembled nanofibers and well-defined microtopographies (Figure 5(c-iii)). Another approach toward TSA is the use of geometric

BOX 2

TEMPLATED SELF-ASSEMBLY

In templated self-assembly (TSA), a prefabricated template (2D substrate chemically or mechanically patterned) orients and directs the assembling components to (1) create structures with better long-range order than their nontemplated counterparts, (2) control the morphology of the resulting assemblies, and/or (3) form novel structures/phases that would not be favorable in the absence of the template.[2] The patterned substrate interacts selectively with the assembling components and their organization near the substrate is governed largely by the patterns on the template and this organization is propagated into the bulk of the assembling structure via the interaction between the components. TSA can be used to hierarchically organize self-assembled structures for higher order assemblies. In the TSA approach, specific physical and chemical interactions of the molecules and components with the surfaces are the driving forces for the formation of stable organized films at solid substrates (Figure 5(c)). Vertical and horizontal segregation of components, their chemical binding and anchoring density determine supramolecular organization in a self-assembled film.

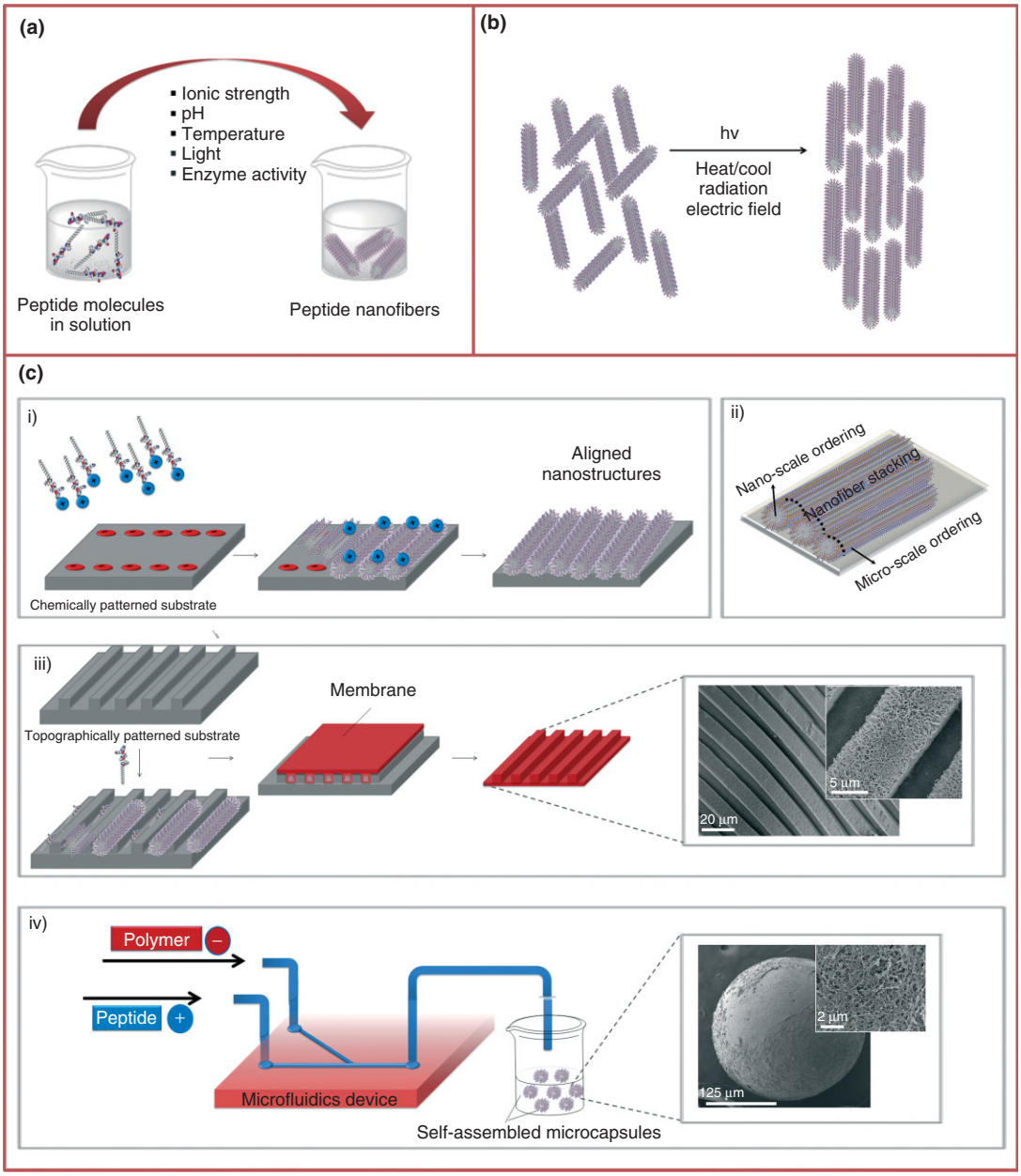


FIGURE 5 | Examples of spontaneous self-assembly in solution (a) and directed by external forces (b) or by a chemical/physical template (c). DSA can be used to guide the self-assembly process and lead to specific orientation, alignment, ordering, or new microdomain structures. (b) DSA using energy (electric and magnetic fields, shear, temperature gradients). (c-i) TSA on chemically patterned substrates. (c-ii) Self-assembly across the length scales, from nano to microscale. (c-iii) TSA of a multi-domain peptide in the presence of hyaluronic acid on topographically patterned soft substrate to fabricate freestanding thin membranes.[145] (c-iv) TSA using microfluidics (confined space) to precisely generate spherical capsular structures with monodispersed size.[146]

confinement to orient bulk structures or to induce the formation of novel morphologies not found in bulk systems. For example, our group has used microfluidics to generate polymer microdroplets to serve as templates for the formation of polymer-peptide microcapsules which are formed by self-assembly upon contact of the polymer droplets, containing the anionic polysaccharide xanthan, with positively charged a MPD (Figure 5(c-iv)).[146]

Combining external fields with templates will allow a better control over the spatial distribution and orientation of self-assembling building blocks toward the fabrication of complex hierarchical materials.

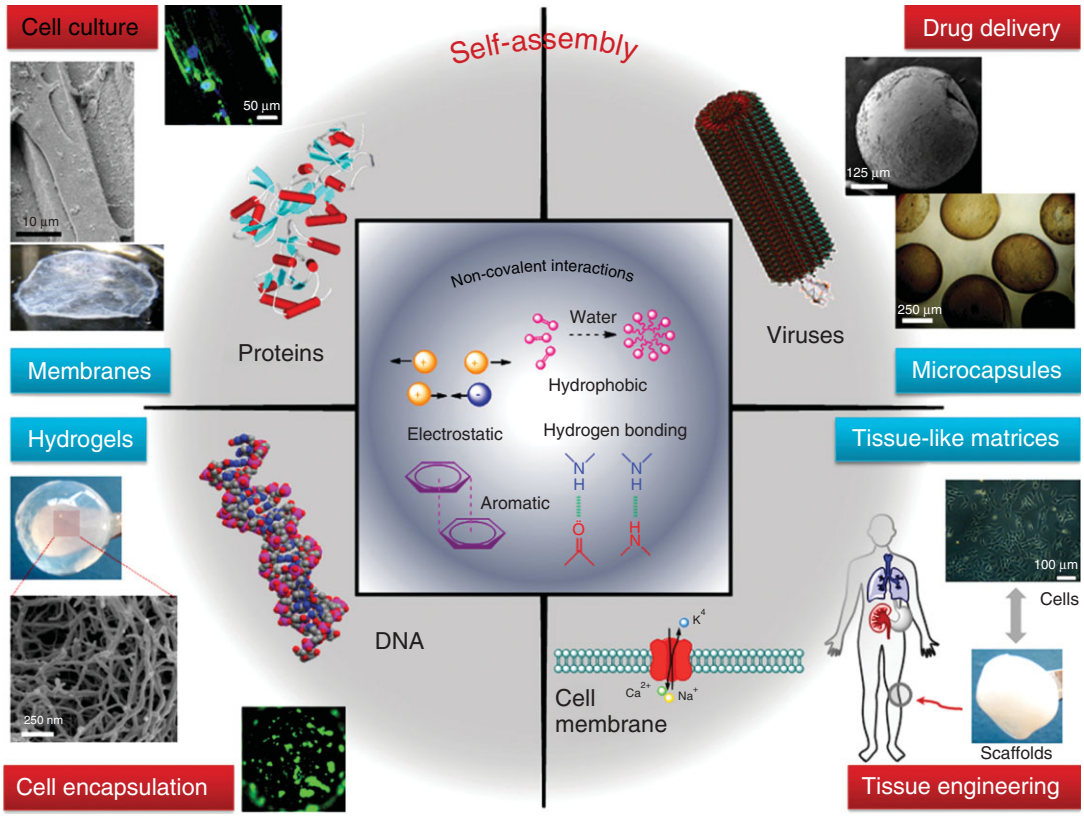


FIGURE 6 | Advances in peptide self-assembly have contributed to the generation of nanostructured biomaterials enabling their expansion into different applications in regenerative medicine.

APPLICATIONS OF SELF-ASSEMBLING PEPTIDE BIOMATERIALS IN REGENERATIVE MEDICINE

The extracellular matrix (ECM) of tissues is a dynamic and hierarchically organized nanocomposite that regulates essential cell functions such as adhesion, migration, proliferation, and differentiation. Engineering complex tissues requires biomaterial scaffolds that recapitulate the structure and composition of ECM to provide cells with physical and biochemical signals that are important for tissue development and organization (functional tissue). 3D scaffolds, in which cells are randomly distributed, offer limited control over spatial cell organization and tissue architecture. To recreate the structure and function of natural ECMs, self-assembling peptides enable the creation of scaffolds based on a network of nanofibers. At sufficiently high concentration, the fibrillization of peptides is accompanied by gelation. Nanofibers obtained by self-assembly can become entangled to form network hydrogels. The nanofibers can have diameters as small as 10 nm (significantly smaller that those obtained by electrospinning, a top-down fabrication technique) and be designed with bioactivity to provide cells with instructive cues toward tissue formation. Cell adhesion or macromolecular-binding sequences can be incorporated into the peptide structures and are natural choices to recreate bioactive artificial ECMs. For effective display of these signals on the nanoscale, it is also necessary a careful synthetic design for presenting the peptide signals in a proper supramolecular setting that allows better interaction with target cell receptors. Peptide nanofibers can display high epitope densities that facilitate receptor clustering for signaling and simultaneously maximize successful binding. Peptide self-assembly technology can further provide additional routes for the spatial and temporal program of selective binding of relevant biomolecules to nanofiber networks for stimulating and guiding tissue regeneration. Such class of materials would be useful throughout medicine. This part of the review describes self-assembled peptide systems designed for applications in regenerative medicine, including artificial matrices for cell culture, controlled drug delivery, and tissue engineering (Figure 6). The most relevant works that the authors considered of particular interest and significance in the field are highlighted. Several excellent reviews are cited

that are sources of more detailed descriptions and discussions.

Peptide Hydrogels for 3D Cell Culture

Traditional cell culture is performed on 2D plastic surfaces which are far from the complex 3D environment where cells naturally reside. 3D environments provide another dimension for external mechanical and chemical inputs, which dramatically affects integrin ligation, cell contraction, and associated intracellular signaling. Furthermore, 3D models might help to isolate and investigate specific cell–cell and cell–ECM interactions. Well-defined synthetic 3D systems require control not only of cell adhesion sites and matrix viscoelasticity, but of nano and microporosity (which regulates cell motility and the transport of soluble molecules), growth factor binding and matrix degradation. Peptide hydrogels offer significant potential to recreate 3D cell culture biomimetic environments because the formation of these self-assembled matrices occurs under physiological and cytocompatible conditions enabling the direct encapsulation of cells in a 3D environment. Zhou and co-workers, developed a peptide-based bioactive hydrogel as 3D scaffolds for anchorage-dependent cells.[100] This hydrogel is formed through self-assembling of two aromatic short peptide derivatives: Fmoc-FF (fluorenylmethoxycarbonyl-diphenylalanine) and Fmoc-RGD (arginine-glycine-aspartate) and exhibited a highly hydrated, stiff, and nanofibrous network comprising bioactive ligands at the fiber surface, mimicking some features of the ECM. Encapsulated dermal fibroblasts were observed to adhere, spread, and proliferate within this hydrogel (Figure 7(a)), suggesting the use of these self-assembled matrices as suitable model scaffolds for 3D cell culture.

To provide a proper environment for encapsulating chondrocyte cells, Sinthuvanich et al.[147] designed peptide sequences based on MAX8 self-assembling peptide to form 3D hydrogels for cell culture and to examine the effect of charge on their behavior.

Several works describing the use of self-assembled peptide hydrogels for cell encapsulation and 3D cell culture (Figure 7, Table 3) have been reported in the literature.[17,70] Some examples will be also described in the topic peptide hydrogels as scaffold for tissue repair.

Peptide Hydrogels/Nanostructures for Delivery of Bioactive Factors

Peptide-based matrices have also been used for the delivery of therapeutic agents such as proteins and small molecules (e.g., drugs). Physical encapsulation/entrapment of proteins (e.g., lysozyme, trypsin inhibitor, bovine serum albumin (BSA), and immunoglobulin (IgG)) was reported by Koutsopoulos using self-complementary ionic peptide gels (Figure 7(d)).[148,149] It was demonstrated that the protein release through the peptide hydrogels was dependent on the protein size and the density of the peptide nanofibers. Similar strategy was applied by Branco and co-workers[150] for entrapping proteins (BSA, IgG, lysozyme, α-lactalbumin, myoglobin, and lactoferrin) within β-hairpin gels. The release rate of the protein was shown to be dependent on the protein charge density. These systems have also been used for the controlled release of small drugs. Herein, the drug is encapsulated by physical entrapment within the gel, as reported by Altunbas et al.[75] in the encapsulation of the hydrophobic polyphenol curcumin within β-hairpin gel. The release rate was modulated as function of peptide concentration. Webber[131] also demonstrated the ability to encapsulate chemotherapeutics, such as doxorubicin (anticancer drug) in the core of PA nanofibers. The drug was released through the disassembly of peptide nanofibers triggered by enzymatic phosphorylation of a serine residue. To increase the drug loading efficiency, Cui and co-workers have developed supramolecular nanostructures formed by DAs.[91,92] Using anticancer drugs (e.g., camptothecin and paclitaxel) on their DA formulation, they showed high drug loading contents (23–41%). Since most of the anticancer drugs need to be internalized by cells to exert its cytotoxic effect, a reducible linker (disulfylbutyrate, a molecule that breaks down in the presence of glutathione a reducing agent present in the cytosol) was incorporated between the hydrophobic drug and the peptide segment, to allow intracellular drug release. Assuming that the hydrophobic drug and the linker are buried in the core of the filamentous nanostructures, the supramolecular morphology of DAs provides protection from the external environment and a mechanism for drug controlled release. *In vitro* toxicity experiments with different cancer cell lines revealed identical toxicity of the synthesized DAs compared to free drug.

Self-assembling peptides have also been designed to act as reservoir for the local and temporal release of growth factors (GFs) through the incorporation of GF binding sequences. Stupp and co-workers[111] found peptide sequences by phage display with binding affinity for BMP-2 (e.g., YPVHPST) and TGF-β1 (e.g., LPLGNSH) GFs with potential use in bone and cartilage regeneration, respectively. The use of heparin binding peptide sequences has been also

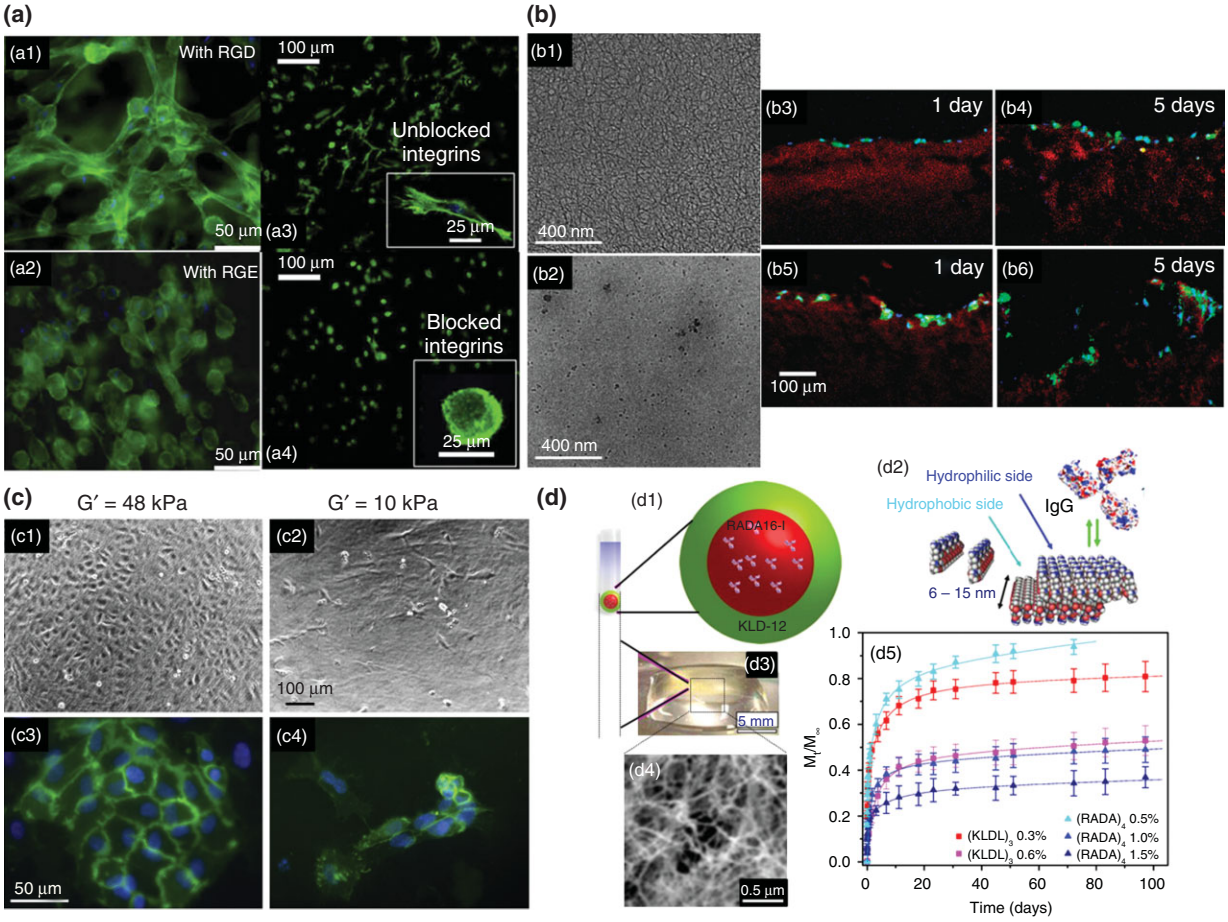


FIGURE 7 | Examples of peptide hydrogels used for *in vitro* 3D cell culture and delivery of protein molecules. (a) Cell (human dermal fibroblasts) adhesion and morphology in Fmoc-FF/Fmoc-RGD (a1) and Fmoc-FF/Fmoc-RGE (a2) hydrogels; Cells are well spread in the Fmoc-FF/Fmoc-RGD hydrogels while in Fmoc-FF/Fmoc-RGE maintain a round morphology. Integrin ($\alpha5\beta1$) blocking experiments proved direct interaction of the cells with RGD after 20 h (a3, a4). (Reprinted with permission from Ref 100. Copyright 2009 Elsevier) (b) Enzymatically degradable peptide hydrogels allows cleavage of self-assembled peptide structures by specific enzymes, as visualized by cryo-TEM images which show intact nanofibers of a multi-domain peptide containing enzyme cleavage sites before (b1) and after incubation with MMP-2 (b2) resulting in the disintegration of the nanofibrous network; The presence of a MMP-2 cleavage site allows cell migration into the peptide hydrogels (b5, b6) whereas in nondegradable hydrogels green-fluorescent cells remain as monolayer on top (b3, b4). (Reprinted with permission from Ref 96. Copyright 2010 American Chemical Society) (c) Effect of mechanical properties of self-assembled peptide (Q11) hydrogels on the growth and proliferation of primary human umbilical vein endothelial cells (HUVECs) seeded on top of the gels; HUVECs were nearly confluent on the stiffer gels (c1, storage modulus $G' = 48$ kPa) and expressed significantly higher levels of the cell–cell adhesion protein PECAM (CD31, expression of CD31 at cell–cell contacts is expected in normally functional endothelial cells) (c3) whereas on less stiffer gels ($G' = 10$ kPa) cells are sparse and spindle-shaped (C2) (green-CD31, blue-DAPI); (Reprinted with permission from Ref 151. Copyright 2008 Elsevier) (d) Two-layered (d1) self-assembling peptide (d2) nanofiber (d4) hydrogel (d3) for the long-term sustained delivery of antibodies such as immunoglobulin G (d5). (Reprinted with permission from Ref 149. Copyright 2012 Elsevier)

applied for the controlled release of angiogenic GFs for therapeutic applications in islet transplantation and cardiovascular disease.[48]

Peptide Hydrogels as Scaffolds for Tissue Repair

Bone

Despite four decades of research on bone regeneration therapies, the gold standard in bone replacement therapy is still the use of autografts (patient's own bone) or allografts (donor bone). However, both approaches present drawbacks such as morbidity and severe pain complications at the donor-site of harvesting, risk of septic complications, viral transmission, or disease transfer and limited bone volume at donor site.[152] In that respect, there is an enormous need for synthetic replacements for bone repair and regeneration. The use of self-assembling peptides for bone repair applications has been reviewed elsewhere.[68,81] Several studies demonstrated the potential of these materials for bone

regeneration. For example, 3D self-assembled peptide nanofibers were shown to enhance significantly the *in vitro* proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs) when compared with conventional cell culture plate.[153] Moreover, it was found that self-assembling peptide sequences containing the phosphoserine (S(P)) residue and the RGD cell adhesion sequence ($C_{16}O$-$C_4G_3S(P)RGD$) have the ability to induce hydroxyapatite mineralization in 2D.[154] Later, this combination of signaling epitopes (5% RGDS and S(P) 95%) simultaneously with pre-osteoblastic cells were loaded as a gel into a porous titanium foam in order to increase biocompatibility of typical metallic implants.[155,156] The ability of these amphiphilic self-assembled matrices ($GS(P)E_2L_3A_3$-OC_{16} and $SDGRK_2L_3A_3$-OC_{16}) to induce faster bone regeneration at tissue interfaces was demonstrated. The same nanofiber matrix was tested in a rat femoral critical-size defect and showed significant bone formation (Figure 8(a)).[157] In another study, peptide functionalized with RGDS ($CH_3(CH_2)_{14}$CONH–GTAGLIGQ–RGDS) and DGEA ($CH_3(CH_2)_{14}$ CONH–GTAGLIGQ–DGEA) sequences were investigated as an ECM-mimicking biomaterial to provide an instructive microenvironment for osteogenic differentiation of human MSCs,[158] showing osteogenic differentiation with and without the addition of osteogenic media. Additionally, self-assembled nanostructures consisting of BMP receptor-binding peptides,[159,160] as mentioned previously, have been explored due to its osteoinductive potential to be applied in bone repair therapies. RAD16 peptide-based gels have also been demonstrated to be valuable in enhancing bone regeneration.[68,161,162]

Cartilage

Cartilage is an avascular tissue with low capacity of self-repair mainly due to the reduced availability of chondrocytes and absence of progenitor cells in cartilage tissue, and restricted mobility of chondrocytes in the dense ECM. Cartilage lesions, caused by trauma, or disease, results in the loss of partial or complete tissue functionality. Thus, one of the approaches in regenerative medicine for repairing cartilage defects consist on the use of an injectable system capable of gelling in situ, to deliver autologous chondrocytes and stimulating cells to produce cartilage. Significant advances have been made in developing self-assembling gels for creating well-controlled 3D environments for encapsulating chondrocytes to foster the formation of cartilage. For instance, a self-assembling KLD-12 peptide hydrogel was used as a 3D scaffold for encapsulation of chondrocytes.[163] This self-assembling matrix was found to support cell survival and to retain the chondrocytic phenotype with increased production of glycosaminoglycans and type II collagen (components of cartilage ECM). Moreover, an increase in dynamic stiffness of the matrix material during the culture period was observed. In another study, bone marrow stromal cells were encapsulated within a self-assembling peptide hydrogel containing two peptide sequences (KLD-12 and RAD16-I) known to enhance chondrogenic differentiation.[164] Chondrogenesis in these self-assembling peptide matrices was shown to be superior when compared with agarose hydrogels, as shown by ECM production, DNA content, and aggrecan molecular structure.

Also in the context of cartilage repair using self-assembling peptide hydrogels, a PA (Table 3) was designed to form nanofibers for cartilage regeneration and displaying a high density of TGFβ-1 binding sequence. These materials were shown to support the survival and promote the chondrogenic differentiation of MSCs.[165] Moreover, regeneration of articular cartilage was also observed in a full thickness chondral defect (microfracture in a rabbit model) treated with this PA gel, with or even without the addition of exogenous GF (Figure 8(b)).

Central Nervous System

The central nervous system (CNS) is a dynamic organ which is prone to injury and degeneration. Stimulation of the CNS to regenerate after trauma (e.g., after spinal cord injury, SCI) both structurally and functionally, still remains a significant challenge due to the inherent complexity of the adult CNS, as well as the inability of the central neurons to regenerate correct axonal and dendritic connections. Strategies for regenerating the adult CNS include cellular replacement, neurotrophic factor delivery, axon guidance, and removal of growth inhibition, manipulation of intracellular signaling, cellular bridging using artificial substrates, and modulation of the immune response.[166] Artificial substrates may be useful for repair of lesions where cellular bridging is necessary. The ideal artificial substrate will have a molecular composition that is easily manipulated and immune tolerant, and will contain a porous structure for nerve regeneration and cell repopulation that will be easily absorbed by the CNS. Different types of self-assembled peptide hydrogels have been investigated for CNS repair. A PA containing the IKVAV epitope (Table 3) and able to form 3D networks of nanofibers by self-assembly, was used to encapsulate neural progenitor cells. The IKVAV sequence is derived from the protein laminin responsible for neurite growth.[80] This artificial nanofiber scaffold was shown to induce very rapid differentiation of cells into

neurons, while discouraging the development of astrocytes comparatively to laminin. These findings had led the same authors to use a similar PA molecule containing the IKVAV epitope ($C_{16}O$-$(SL)_2A_3$EIKVAV) *in vivo* using a mouse model of SCI.[167] The peptide solution was injected at the site of injury where gelation took place. It was found that treatment with the PA reduced astrogliosis, reduced cell death, and increased number of oligodendroglia at the injury site. In addition, the IKVAV based peptide gel promoted the regeneration of injured motor and sensory axons, while in injured spinal cords treated with peptide gel (without IKVAV sequence) axons were unable to traverse the lesion. Later, using two SCI models (compression and contusion) and two different species (mice and rats), the injection of IKVAV PA on the animal functional recovery was investigated.[168] This study confirmed the regeneration potential of the IKVAV PA in improving behavioral outcome by stimulating axon regeneration through the lesion (Figure 8(c)).

Peptide hydrogels, formed by self-assembly of ionic self-complementary peptides (RAD16-I $(RADA)_4$ and RAD16-II $(RARADADA)_2$) at physiological conditions, were also shown to perform successfully in neurorepair strategies.[169] It was demonstrated the ability of these gels to support neuronal cell attachment, differentiation, and extensive neurite outgrowth, being permissive substrates for functional synapse formation between the attached neurons. The authors postulated that the bioactivity of these peptides is due to the similarity of the tripeptide arginine-alanine-aspartate (RAD) sequence in the peptide with the RGD motif which is known as a binding site for some cell integrins. The ability of these peptide hydrogels for brain repair was also demonstrated using a severed optic tract in a hamster model. Injection of peptide solution at the lesion site created a permissive environment for regenerated axons to reconnect to target tissues with sufficient density to promote functional recovery, as demonstrated by a return of lost vision.[170] In another study, $(RADA)_4$ peptide was used as a scaffold for transplantation of neural progenitor cells and Schwann cells into the transected dorsal column of spinal cord of rats to evaluate its potential for SCI repair.[171] The self-assembled matrix was shown to promote survival, migration, and differentiation of both cells. In addition, migration of host cells, growth of blood vessels, and axons into the scaffolds was also observed. The same hydrogels were also investigated on the reconstruction of acutely injured brain. Increased brain tissue restoration was observed in groups treated with this hydrogel.[172]

Revascularization Therapies

Angiogenesis, the process of new blood vessels formation from existing ones, assumes great relevance in wound healing. Heparin based molecules have shown to play an important role in angiogenesis due to their ability to bind, stabilize, and protect proangiogenic proteins such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2). On the basis of this knowledge, heparin was used to trigger the self-assembly of PA molecules[125,173] containing heparin binding sequences (Table 3) demonstrating the ability of these systems to stimulate new blood vessel formation *in vivo*. In diabetes type I, loss of vascular networks during islet isolation from donor tissue is common. However, implanting heparin-containing peptide gels delivering VEGF, FGF-2 in diabetic mouse significantly increased vascular density in the transplant site and improved islet engraftment, as evidenced through the higher cure percentages and shorter times to achieve normoglycemia.[174] These heparin binding peptide nanofibers with heparin displayed on their surfaces has shown to increase islet survival and insulin secretion and if combined with angiogenic GFs lead to enhanced levels of islet endothelial cells sprouting[175] that may be valuable in islet transplantation to treat diabetes type I.

There is a need of cell-based therapies for ischemic tissue repair in cardiovascular diseases, as result of limited regeneration of cardiomyocytes.[176] To overcome this limitation, a biocompatible matrix is normally required to support cell functions during the transplantation, once the direct cell transplantation (embryonic stem or endothelial progenitor cells) results in low cellular viability and minimal retention.[177] A scaffold constituted of self-assembled peptide nanofibers containing fibronectin-derived RGDS cell adhesion epitope was used to encapsulate bone marrow-derived stem and progenitor cells *in vivo*.[177] Enhanced viability, proliferation, and adhesion of encapsulated cells suggested the potential of these materials to be applied in cell therapies for ischemic diseases. Self-assembled RAD16 based gels have also shown to support the survival of encapsulated endothelial and myocardial cells[178] and the potential to create a 3D microenvironment when injected in myocardium by recruiting both endogenous endothelial and smooth muscle cells with stimulation of vascularization.[176] Very recently, self-assembling peptide Ac-$(RA)_2(DA)_2(RA)_2(DA)_2$) nanofiber scaffolds containing VEGF were shown to create a microenvironment for arteriogenesis and cardiac repair when injected into the heart tissue after myocardial infarction (Figure 8(d)).[179]

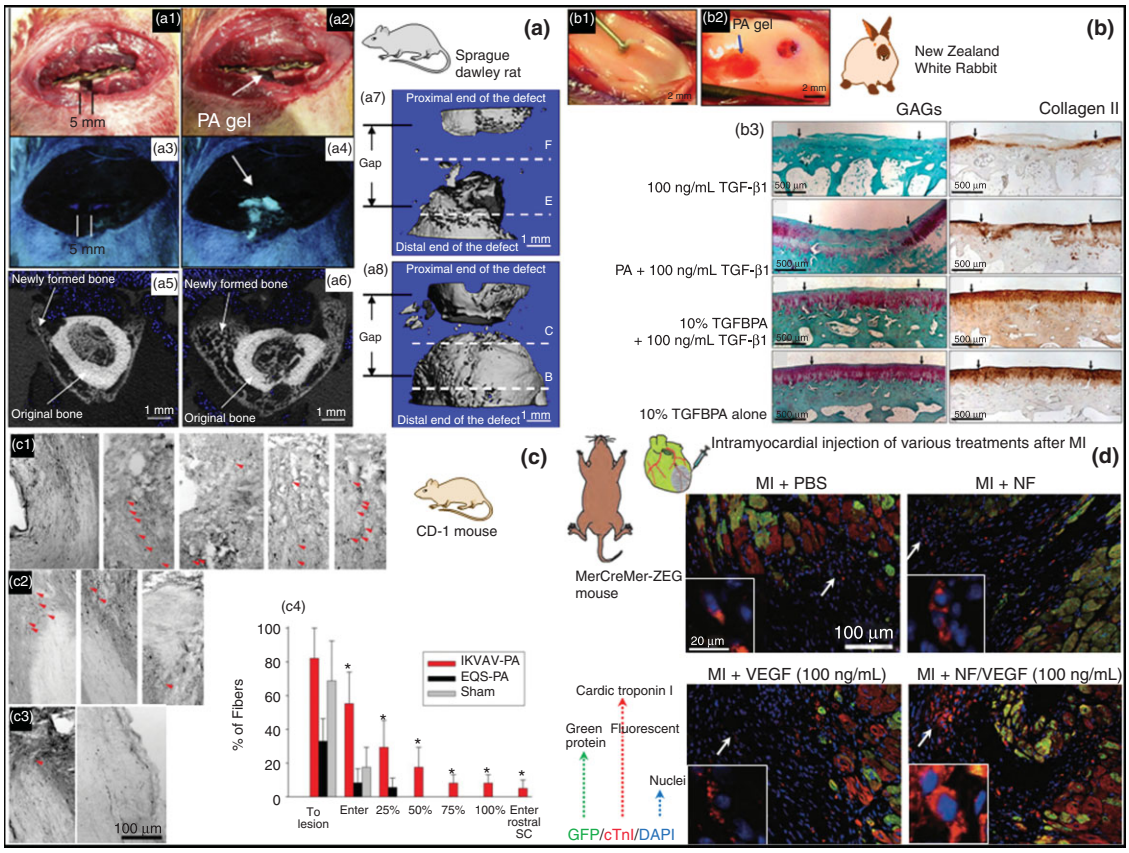


FIGURE 8 | *In vivo* studies showing the potential of self-assembling peptides as regenerative biomaterials and their advanced testing stages toward clinical applications. (a) Bone regeneration mediated by a self-assembling peptide nanofiber gel matrix that has the capacity to mineralize; A critical-size (5 mm wide) defect (a1, a3 empty defect) in a rat femoral was treated with these gels (a2, a4); Microcomputed tomography analysis of the rat femurs after 4 weeks of gel implantation revealed the formation of new bone (a6, a8) when compared with empty defects (a5, a7). (Reprinted with permission from Ref 157. Copyright 2010 Elsevier) (b) Cartilage regeneration in a full thickness articular cartilage defect (microfracture rabbit model, b1) treated with a PA gel (b2) displaying a high density of binding epitopes to transforming growth factor β-1 (TGFβ-1) a growth factor known to maintain articular cartilage in the differentiated phenotype; Histological evaluation of cartilage samples 12 weeks after treatment with PA gels (b3), containing the TGFβ-1 epitope (TGFBPA) with or even without the addition of exogenous growth factor, showed formation of hyaline-like tissue within the defect space, as observed by the glycosaminoglycans (GAGs) and collagen II stainings. (Reprinted with permission from Ref 165. Copyright 2010 National Academy of Sciences, USA) (c) IKVAV PA promotes regeneration of sensory axons after spinal cord injury (SCI); Brightfield images of biotinylated dextran amine-labeled tracts from IKVAV PA-injected (c1), EQS PA (nonbioactive PA)-injected (c2) and sham (c3) animals at 11 weeks postinjury; Sensory axon tracing (fibers through the lesion indicated by red arrows) was only observed in the IKVAV PA-injected group and just up to and slightly into the lesion in EQS PA-injected and sham groups; Approximately 55% of labeled dorsal column axons in the IKVAV PA group entered the lesion compared with only about 18% of the fibers in sham controls and less than 10% in EQS PA injected animals (c4). (Reprinted with permission from Ref 168. Copyright 2010 John Wiley and Sons, Inc.) (d) Nanofiber (NF) peptide (RAD16-II) scaffolds with vascular endothelial growth factor (VEGF) create a microenvironment for cardiac repair; Immunostained images at myocardium border zone for each treated group at 28 h after myocardial infarction (MI) shows a higher number of newly generated cardiomyocyte-like cells ($GFP^-/cTnI^+$, small cells indicated by arrows and magnified in the inset images) derived from endogenous stem/progenitors cells in the animals treated with NF/VEGF that is favorable for induction of endogenous cardiomyocyte regeneration. (Reprinted with permission from Ref 179. Copyright 2012 AAAS)

Heparin binding peptide nanofiber gels were observed to have binding ability for paracrine factors from hypoxic conditioned stem cell media.[180] When injected in coronary artery ligation, the preservation of hemodynamic function in a mouse ischemia (reperfusion model of acute myocardial infarction) was observed and revascularization in chronic rat ischemic hind limb models was stimulated.

Peptide nanostructures containing bioactive signals offer exciting novel therapies with potential impact in regenerative medicine. Table 3 displays some examples of self-assembling peptide structures containing different biological epitopes for specific applications.

As described previously, self-assembling peptides offer numerous advantages for biomedical

TABLE 3 | Self-Assembling Peptide Biomaterials Presenting Different Functionalities and Biological Epitopes for a Variety of Regenerative Applications

Peptide Sequence/Design Self-Assembling Block + Functionality + Epitope	Supramolecular Material Self-Assembling Mode	Application	Refs.
$C_{16}O$-A_4G_3E-IKAV Negative PA+LM cell adhesioin sequence	Gel formed by addition of culture medium.	Selective differentiation of neural progenitor cells.	80
$C_{16}O$-A_3L_3-E_2-S(P) Negative PA + Phosphorylated seriner residue for hydroxyapatite mineralization	Gel formed by the addition of Ca^{2+} ions.	Bone regeneration.	155–157, 181
$C_{16}O$-V_3A_3-E_3-RGDS Negative PA + FN cell adhesion sequence	Gel formed by the addition of Ca^{2+} ions.	Cell delivery in cell-based therapies.	177
$C_{16}O$-A_3L_3-LRK_2LGKA Positive PA + Heparin binding sequence	Gel formed by the addition of heparin.	Delivery of angiogenic factors for enhancing angiogenesis is islet transplantation, wound healing, treatment of cardiovascular diseases.	125, 175, 180, 182
HSNGLPL-GGGS-E_3-A_3V_3(K)-$CO(CH_2)_{10}CH_3$ TGFβ1 binding sequence + Reverse negative PA	Gel formed by the addition of Ca^{2+} ions.	Cartilage regeneration.	165
$C_{16}O$-$V_2A_2K_3$-GKLTWQELYQLKYKGI-NH_2 Positive PA + VEGF-mimetic peptide	Non-assembled material.	Ischemic tissue repair.	183
RGDS-KKLLA(K)-(COC_3H_{16})-diacetylene-(C_2H_{25}) FN-derived cell adhesion sequence + Positive diacetylene PA	Patterned gel formed by diffusion of NH_4OH vapor and further polymerized by UV radiation.	Substrate to study behavior of human mesenchymal stem cells.	142
$C_{12}O$-V_2AG-EGDK(sulfobenzoic acid)S-NH_2 Heparin mimetic PA	Gel formed by mixing with PA of opposite charge (C_{12}-O-V_2AGK-NH_2)	Promote angiogenesisavoiding the use of heparin and exogenous growth factors.	184
$C_{16}O$-GTAGLIGQE-RGDS Negative PA + MMP-2 cleavage + FN cell adhesion sequences	Gel formed by the addition of CA^{2+} ions.	Dental tissue regeneration.	95
Ac-$K(SL)_3$-RG-$(SL)_3K$-GRGDS-$CONH_2$ Positive MDP + MMP-2 Cleavage + CN cell adhesion sequences	Gel formed by the addition of phosphate or heparin.	Dental pulp tissue engineering.	97
Ac-GGRDS-GGG-QQKFQFQFEQQ-$CONH_2$ FN cell adhesion sequence + β-sheet fibrillizing peptide	Microgel formed by the addition of PBS in a water-in-oilemulsion.	Cell encapsulation.	185
Ac-GG-IKVAV-GGG-Q_{11}-$CONH_2$ LM cell adhesion sequence + β-sheet fibrillizing peptide Ac-GG-YIGSR-GGG-Q_{11}-$CONH_2$ LM cell adhesion sequence + β-sheet fibrillizing peptide Ac-GG-REDV-GGG-Q_{11}-$CONH_2$ FN cell adhesion sequence + β-sheet fibrillizing peptide	Hydrogel formed by the addition of PBS.	Defined matrices for endothelial cell growth.	186
Ac-GRGDS-PGG-$(RADA)_4$-$CONH_2$ FN cell adhesion sequence + Self-complementary peptide	Gel formed by addition of hepatocyte culture medium.	Peptide immobilized on synthetic membranes for hepatocyte culture.	187
Ac-YIGSR-GG-$(RADA)_4$-$CONH_2$ Ac-RYVVLPR-GG-$(RADA)_4$-$CONH_2$ LM 1 cell adhesion sequence + Self-complementary peptide Ac-TAGSCLRKFSTM-GG-$(RADA)_4$-$CONH_2$ Coll IV cell adhesion sequence + Self-complementary peptide	Gel formed by addition of PBS.	Substrate for endothelial cell culture.	188
Ac-$(RADA)_4$-GG-ALKRQGRTLYGF-$CONH_2$ Ac-$(RADA)_4$-GG-DGRGDSVAYG-$CONH_2$ Self-complementary peptide + Osteogenic growth peptide and OPN cell adhesion motifs	Gel formed by the addition of maintenance culture medium.	Peptide nanofiber scaffolds for osteobast proliferation and differentiation.	162
Ac-$(RADA)_3$-PVGLIG-$(RADA)_3$-$CONH_2$ Self-complementary peptide + MMP-2 cleavage sequence	Gel formed by addition of PBS.	Bifunctional scaffolds mimicking the remodeling of natural ECM.	189
Ac-$(RADA)_4$-GG-SKPPGTSS-$CONH_2$ Ac-$(RADA)_4$-GG-PFSSTKT-$CONH_2$ Self-complementary peptide + Bone marrow homing peptide	Gel formed by addition of basal medium.	3D cell culture system.	190
Fmoc-FF, Fmoc-RGD Fmoc-protected dipeptide + FN cell adhesion sequence	Gel formed by pH and temperature changes.	Cell encapsulation/3D culture.	100

Coll, collagen; Fmoc, fluorenylmethoxycarbonyl; FN, fibronectin; LM, laminin; MDP, multi-domain peptide; MMP-2, matrix metalloproteinase-2; OPN, osteopontin; PA, peptide amphiphile; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor (angiogenic signaling protein); TGFβ1, transforming growth factor β 1 (chondrogenesis promoter protein).

applications as versatile and efficient biomaterials. In addition to biological epitopes, different fluorescent compounds can be easily attached to the peptides during synthesis to enable their visualization and detection by fluorescence microscopy in *in vitro* and *in vivo* studies.[157,175] Similarly, contrast agents have been incorporated to provide a noninvasive visualization method to *track in vivo* the integration of the material by magnetic resonance imaging.[191]

An important feature of biomaterials for applications in regenerative medicine is biodegradability. Peptide biomaterials can degrade overtime by hydrolytic and enzymatic processes into amino acids which are nontoxic and easily cleared in the body. To control the degradation of self-assembled peptide matrices, peptides can be designed to be susceptible to enzymatic cleavage. For example, proteolytic degradation of MPDs containing the MMP-2 consensus cleavage motif (Figure 7(b), Table 3) by collagenase IV was demonstrated using mass spectrometry and cryo-TEM.[96]

Despite the promising results obtained in cell culture studies,[192] there are not many reports on the *in vivo* stability/degradation, biocompatibility and immunogenicity[193] of this class of supramolecular peptide-based biomaterials. Detailed studies to evaluate the risks associated with their use *in vivo* are necessary to consider their clinical application in the near future.

CONCLUSION AND OUTLOOK

Understanding how to control the fabrication and organization of matter across multiple length scales is of growing interest to construct complex materials with practical utility in regenerative medicine. Hierarchical structures are of great advantage for tissue engineering applications as they provide a more natural environment for cells to grow and develop into tissues. The exciting findings on peptide self-assembly suggest that this bottom-up technology can offer tools to the 3D assembly of supramolecular objects at scales and with geometrical complexity and regularity not accessible to top-down technologies. Despite the level of precision, it has been difficult to construct self-assembling systems across length scales. The integration of self-assembly into existing microtechnologies could offer new possibilities to fabricate reproducible hierarchical biomaterials with precise biomolecular and physical properties and the opportunity to better tune them with spatiotemporal control.[194]

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