Recent advances in self-assembled peptides: implications for targeted drug delivery and vaccine engineering

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Abstract

Self-assembled peptides have shown outstanding characteristics for vaccine delivery and drug targeting. Peptide molecules can be rationally designed to self-assemble into specific nanoarchitectures in response to changes in their assembly environment including: pH, temperature, ionic strength, and interactions between host (drug) and guest molecules. The resulting supramolecular nanostructures include nanovesicles, nanofibers, nanotubes, nanoribbons, and hydrogels and have a diverse range of mechanical and physicochemical properties. These molecules can be designed for cell-specific targeting by including adhesion ligands, receptor recognition ligands, or peptide-based antigens in their design, often in a multivalent display. Depending on their design, self-assembled peptide nanostructures have advantages in biocompatibility, stability against enzymatic degradation, encapsulation of hydrophobic drugs, sustained drug release, shear-thinning viscoelastic properties, and/or adjuvanting properties. These molecules can also act as intracellular transporters and respond to changes in the physiological environment. Furthermore, this class of materials has shown sequence- and structure-dependent impacts on the immune system that can be tailored to non-immunogenic for drug targeting, and immunogenic for vaccine delivery. This review explores self-assembled peptide nanostructures (beta sheets, alpha helices, peptide amphiphiles, amino acid pairing, elastin like polypeptides, cyclic peptides, short peptides, Fmoc peptides, and peptide hydrogels) and their application in vaccine delivery and drug targeting.

Graphical abstract

Self-assembled peptides fo targeted delivery
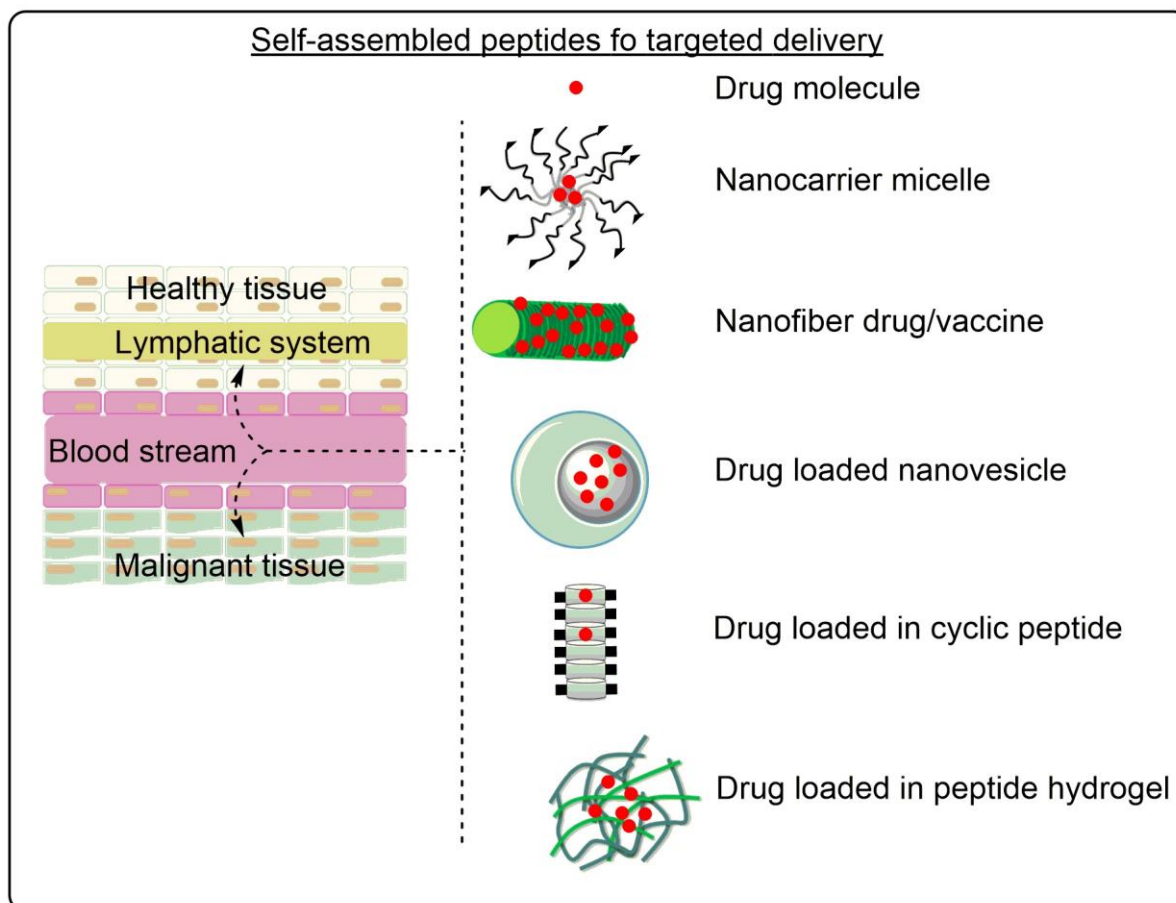
Keywords: Biomaterial, peptide design, drug delivery, peptide hydrogel, self-adjuvant, self-assembled peptide, supramolecular nanostructure, vaccine delivery.

Chemical compounds studied in this article:

Glucono-δ-lactone (PubChem CID: 736); dehydrophenylalanine (PubChem CID: 5702627); Fmoc-phenylalanine (PubChem CID: 978331); Glucomannan (PubChem CID: 24892726); Doxorubicin (PubChem CID: 31703); cis- dichlorodiamine platinum (PubChem CID: 92026269); Ellipticin (PubChem CID: 3213); Curcumin (PubChem CID: 969516); Dalargin (PubChem CID: 6917894); 5-Flurouracil (PubChem CID: 3385)

CONTENTS

1. Introduction

Drug delivery systems can target the drug to specific tissues, minimise side effects, overcome solubility problems and toxicity and be tailored to have the appropriate immunogenicity and metabolic stability. Despite the use of rational design principles, developing a drug delivery platform often relies on synthesising and screening a library of drug derivatives to find a suitable candidate. The ability to predict the physicochemical and biological properties of peptide nanomaterials from the sequence alone is a continuing field of research. Ideal nanomaterials would allow the delivery of several active pharmaceutical ingredients with different release profiles. Ongoing research aims to reformulate existing drugs using smart materials to control the drug structure and function at the molecular level to mimic the three-dimensional structure of biological proteins [1, 2]. In the absence of predictable structure-activity outcomes, a wealth of research has been conducted to develop platforms for a diverse range of drug and vaccine candidates on a case-by-case basis.

Self-assembled peptide nanoparticles form part of a bottom-up strategy to create reproducible nanosized delivery systems [3]. Molecular self-assembly is a practical approach in which molecules are spontaneously organized into ordered structures using processes driven by free-energy that include, Van der Waals, electrostatic, hydrogen bonding, and π-π stacking interactions [4]. The balance of attractive and repulsive forces within (and between) molecules affects their arrangement and is dependent on molecular composition, assembly kinetics, and variation in assembly environment (pH, solvent, co-assembling molecules, temperature, and ionic strength) [4, 5]. Peptide-based drug and vaccine carrier molecules can deliberately be designed to spontaneously self-assemble under specific environmental conditions [6]. For example, peptide scaffolds that spontaneously assembled into a beta sheet structure were isolated from the yeast protein Zuotin and contained alternating hydrophobic and chargedhydrophilic amino acids, lysine and glutamic acid, with 50% charged residues (EAK16-II, AEAEAKAKAEAEAKAK) [7]. Synthetic polypeptides derived from large or natural amino acids are very useful building blocks for fabricating self-assembling structures for medical and pharmaceutical applications due to their high physicochemical stability, diversity in sequence and shape, suitability for large scale synthesis, biodegradability, and bio-compatibility [4]. The self-assembly of various amphiphilic molecules including micelles, polymeric vesicles, microemulsions, liposomes, and nanoparticles has been widely studied for drug delivery applications. Nevertheless, increased control over the structure and biofunctionality of self-assembled peptide delivery systems would improve their application to the delivery of drug and vaccine candidates [8].

Peptide delivery systems have advantages over liposomes or nanoparticles because they can be composed of amphiphilic prodrugs with high drug loading, low drug leakage, biodegradability, and high permeability to bio-membranes of the target cells [3]. In addition, the formation of stable colloidal suspensions allows for the stabilization of hydrophobic compounds as micro- and nano-crystals more effectively than micelles [8]. These systems efficiently adopted the desired stimuli responsiveness that resulted from their molecular

design, biological environment, and interactions. For example, the pH triggered release of an encapsulated drug or targeted delivery with a recognition pattern led to a more efficient delivery system with less side effects [1]. Moreover, peptide delivery systems have been shown to form a protective coat on the surface of hydrophobic compounds, resulting in increased control of drug release, protecting the drug from exposure to degradation agents and reducing exposure of normal tissues to toxic drugs [1, 8, 9]. Depending on the choice of starting materials, self-assembled particles have been shown to display large numbers of biologically active peptides on their surface enabling them to be recognized by cell surface receptors, thus serving as both drug and drug delivery agents simultaneously without the need to further encapsulate drugs within their nanostructure. The diversity of peptides that can be employed in drug and vaccine delivery highlights the importance of ongoing research in this field.

The effect of a peptide on the immune system is dependent on sequence and physicochemical properties. Strategies for either avoiding or specifically inducing immune response are the subject of continuing research. New delivery systems must be tailored to elicit the desired immune response: non-immunogenic peptides for drug delivery applications and immunogenic self-adjuvanting peptides for vaccine delivery. Therefore, investigation into the correlation between the self-assembled peptide sequence and its immunogenicity will facilitate the design of self-assembled peptides for both applications [10-16]. This review outlines recent advances in self-assembled peptides for drug delivery, focusing on peptide designs that produced nanostructures for the targeted delivery of therapeutics. In addition, applications of peptide-based self-assembly for the development of peptide subunit vaccines have also been outlined.

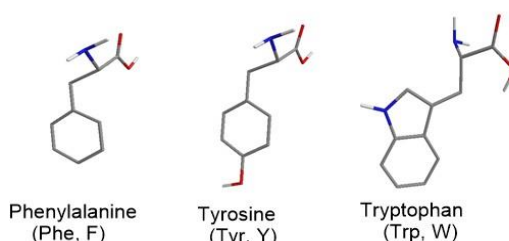## 2. Self-assembling peptide designs and hydrogel formation

Amino acids provide the primary structure and site for chemical modification when designing peptide nanomaterials [17]. Amino acid side chains have different charge, hydrophobicity, size, and polarity. The number, type, and sequence of amino acids can be manipulated to design unique self-assembled peptide nanostructures with specific secondary structures and physicochemical properties [4]. Figure 1 summarises amino acid properties and their role in self-assembly. Variation in peptide length, the percentage and number of repeats of hydrophobic residues (i.e., alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan) and the difference in charge distribution significantly influences the mechanical properties of nanostructure scaffolds and the speed of self-assembly [7, 18]. Known peptide ligands can be added to biologically inert substrates to target the nanomaterial to the appropriate tissue or receptor *in vivo* [17, 19]. This strategy includes adding pattern recognition receptor ligands to stimulate specific immune responses through toll-like receptor pathways. Cellular adhesion ligands (e.g. RGD) can also be added to promote cellular interaction through transmembrane receptors (integrin proteins) that mediate cell-cell and extracellular matrix (ECM) interactions. The ability to include targeting

or tissue-specific peptide sequences highlights the importance of rational sequence selection when designing self-assembling peptides.
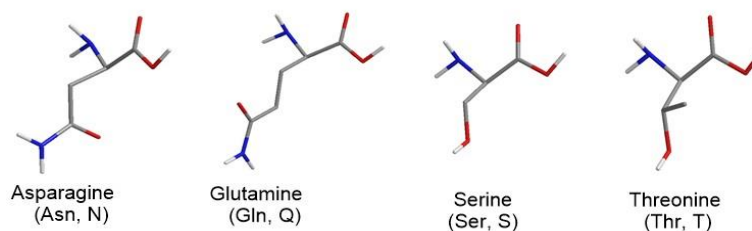
A   **Aliphatic hydrophobic residues:** Provide a hydrophobic environment.

Alanine
(Ala, A)

Leucine
(Leu or L)

Isoleucine
(Ile, I)

Valine
(Val, V)

Methionine
(Met, M)
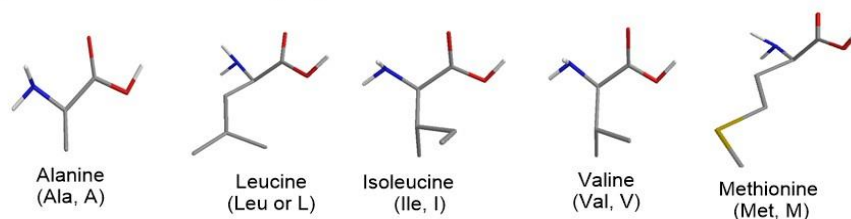
B   **Aromatic hydrophobic residues:** Involved in π−π stacking. Important in peptide and protein folding.

Phenylalanine
(Phe, F)

Tyrosine
(Tyr, Y)

Tryptophan
(Trp, W)

C   **Hydrophilic residues**: Involved in hydrogen bonding interactions.

Asparagine
(Asn, N)

Glutamine
(Gln, Q)

Serine
(Ser, S)

Threonine
(Thr, T)

D   **Charged residues:** Involved in charge-charge interactions.

Basic:

Acidic:

Histidine
(His, H)
pKa=6.5

Arginine
(Arg, R)
pKa=12.48

Lysine
(Lys, K)
pKa=10.7

Glutamic acid
(Glu, E)
pKa=4.4

Aspartic acid
(Asp, D)
pKa=4.4
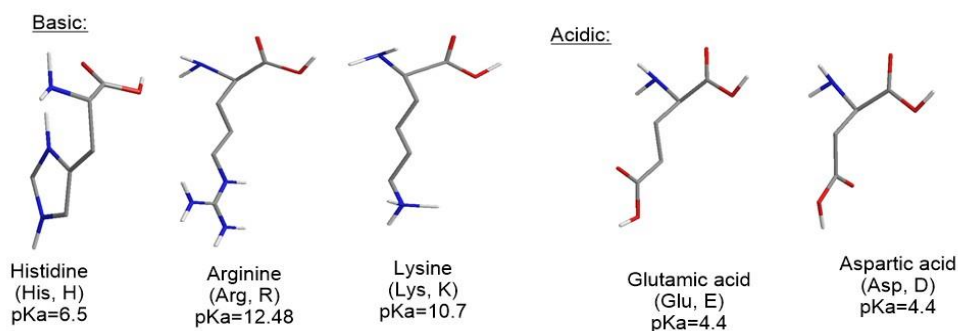
E   **Others:**

A target for chemical modification, inter-peptide crosslinking and binding to gold surfaces. Used to conjugate peptides to proteins and drugs.

Reduces steric hindrance and introduces a high degree of flexibility.
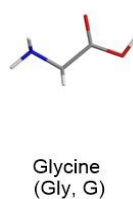
Introduces structural rigidity.

Cysteine
(Cys, C)

Glycine
(Gly, G)

Proline
(Pro, P)
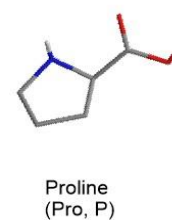
Figure 1. Amino acid structures, including common abbreviations, pKa of acidic and basic amino acids, and properties that are used in the design of self-assembled peptides for

biomedical applications [17]. Reproduced and adapted by permission from The Royal Society of Chemistry.

Cell adhesion and integrin binding are an important component of drug targeting. Molecular binding at one end of an integrin mediates interaction with intracellular signalling pathways, including calcium channels, kinases, phosphatases and other binding proteins, and also recruits intracellular non-receptor tyrosine kinases. These changes affect cell cycle regulation, cell shape and motility, and can result in up-regulation of integrin receptors on the cells surface [20]. ECM proteins are an important feature for development and morphogenesis because they act as ligands for integrins, including: fibronectin, vitronectin, collagen, and laminin [21, 22]. Peptide-based biomaterials can be engineered to optimize the spatial organization, effective density, and accessibility of the ligand to modify its bioactivity and binding to an integrin [20]. Table 1 outlines the functional peptide sequences responsible for cell adhesion in integrin proteins, neuron growth, and cell adhesion in endothelial cells. Applications of these functional peptide sequences in designing targeted drug delivery have been described in more detail in Section 4. Prodrug self-assembled delivery systems containing matrix metalloproteinases (MMP)-specific targeting sequences (GTAGLIGQ, PVGLIG) have been shown to utilize in potent tumor targeting drug release systems [23]. Here, MMPs are a class of protease enzymes that are abnormally elevated in MMP-overexpressing tumor cells and have been shown to contribute to cancer cell metastasis [24]. These important tumor-associated enzymes contribute to drug release by directing cleavage at the site of the tumor [24-27]. Cui and co-workers applied two MMP-2 peptide substrates, GPQG-IAGQ and IPVSLRSG in the design of peptide cross linkers over peptide amphiphile filaments. The MMP-2 cross linked filaments introduced a stable carrier for targeted protein and drug delivery [28].

Table 1. Biologically active peptide ligands for cell adhesion

Peptide Sequence	Derived Protein	Biological Activity	Ref.
RGD	Laminin, collagen I, fibronectin	Integrin mediated cell adhesion	[29, 30]
IKVAV	Laminin	Neuron growth and development, integrin mediated cell adhesion	[29, 31]
YIGSR	Laminin	Integrin mediated cell adhesion, endothelial cell-adhesive ligand	[29, 32, 33]
LGTIPG	Laminin	Integrin mediated cell adhesion	[29, 34]
PDGSR	Laminin	Integrin mediated cell adhesion	[29, 35]
LRE	Laminin	Integrin mediated cell adhesion	[29, 36]
LRGDN	Laminin	Integrin mediated cell adhesion	[29, 37]
IKLLI	Laminin	Integrin mediated cell adhesion	[29, 37]
DGEA	Collagen I	Integrin mediated cell adhesion	[20, 38]
KQAGDV	Fibronectin	Integrin mediated cell adhesion	[20, 39]
REDV	Fibronectin	Integrin mediated cell adhesion	[20]
PHSRN	Fibronectin	Integrin mediated cell adhesion	[20]

Peptides can be designed to self-assemble into different supramolecular nanostructures with specific properties. Peptides that were deliberately engineered to have beta sheet or

alpha helix secondary structure and amphiphilic peptide monomers have shown promise in self-generating supramolecular assemblies, with applications in vaccine design and targeted drug delivery. Peptide molecules that self-assembled in aqueous media to form cylindrical nanofibers were shown to also assemble into a higher network arrangement of peptide nanofiber-based hydrogels, where the bioactive peptide was displayed on the surface.

Hydrogels are polymeric networks with three-dimensional configurations which have the capacity to absorb large quantities of water or biological fluids due to the presence of hydrophilic groups (amine, hydroxyl, ether, sulphate, or carboxyl) in their structural networks [40]. Peptide-based hydrogels have prominent advantages over traditional polymeric hydrogels which include biodegradability, low bioaccumulation and toxicity, spontaneous formation without the use of harmful reagents (such as chemical cross-linkers), and the facile incorporation of cell-specific bioactive moieties. Peptide-based hydrogels are also cost effective, easily synthesized and responsive to external stimuli, including temperature, ionic strength, pH, light, enzyme and magnetic fields [41-47]. The responsiveness of these peptide hydrogels to biological stimuli attributed to their ability to sense changes to the local environment and release therapeutics in a controlled manner [47].

 The hydrogelation of peptide hydrogels is easily modified through the attachment of chemical and biological moieties [48]. Peptide hydrogels are similar in scale to natural ECM and provide an *in vivo* cell environment with properties that favour cellular adhesion [49]. These peptide hydrogels were engineered to encapsulate hydrophobic guest molecules, target specific cell types, and improve stability, thus overcoming the peptides susceptibility to enzymatic degradation *in vivo* [50, 51]. Hydrogel drug delivery vehicles can be designed to entrap drug molecules through physical or covalent bonds.

The noncovalent interaction between drug and peptide structure with a net hydrophobicity can be used to stabilise the entrapped therapeutic molecule in micelles or porous nanoparticles. These systems must be rationally engineered and optimised to load and release the drug appropriately [52, 53]. Chemical conjugation of the drug into the peptide molecule via a biochemically cleavable linker has been shown to provide more control over the triggered release of the drug. The bioactivity of the drug depends on how effectively it is cleaved from the carrier at the target tissue or site of action [53, 54].

Therapeutic agents released from peptide hydrogels are controlled by the mesh size of the network to diffuse encapsulated drugs, and the self-supporting properties of the hydrogel to sustain itself and any active component. In the case of drug conjugation to polypeptides, network degradation has been shown to control the release of a drug [55]. The optimal design parameters of synthetic peptide hydrogels and their environment have been shown to dictate their molecular structure (e.g, beta sheet secondary structure), biological function, mechanical properties, and stability [55].

2.1. Beta sheets

A series of hydrogen bonds between residues in different polypeptide chains or between residues in different sections of a folded polypeptide produced parallel or anti-parallel

sheet-like structures. The alternating hydrophobic-hydrophilic residues assembled into sheets with a hydrophobic and hydrophilic face, where two sheets came together to exclude the surrounding aqueous media from the hydrophobic face. Thus, depending on the number of packed sheets, a variety of different hierarchical structural arrays can be designed, including tapes, ribbons, fibrils, and fibers (Figure 2) [1]. The principle arrangement of amino acids to form beta sheet nanoribbons is alternating placement of charged (or polar) and hydrophobic amino acids to promote tailored hydrogen bonding between hydrogen donors and acceptors within the peptide. Furthermore, peptide structures that contained alkyl chains, 9-fluorenylmethyloxycarbonyl (Fmoc) peptides, and cyclic peptides (e.g. cyclo[($_D$-Ala-Glu-$_D$-Ala-Gln)$_2$]), have the ability to form beta sheets through hydrophobic collapse and stacking on top of each other, thereby adopting a low energy, ring shaped conformation resulting in nanotube formation [4]. Mechanical properties of self-assembled beta sheets can be controlled by adjusting the molar ratio of enantiomeric peptides (e.g. V$_D$PPT) as a design tool. The use of chirality in peptide design has been shown to greatly improve the stability of peptide scaffolds against enzymatic degradation [56, 57].

Moreover, *Bombyx mori* silk fibroin—a natural resource and an alanine glycine (AG)-rich polypeptide—was shown to form extended beta strands. The alanine glycine -rich peptides inspired synthetic polypeptides that contained a repetitive sequence of [(AG)xEG]n (where x was 3-6 amino acids long) that was attributed to the formation of these extended beta sheets [58]. Peptides that form beta sheets have applications in drug delivery as nanowires, nanofibers or hydrogel scaffolds, where the ability to encapsulate hydrophobic guest molecules, such as pyrene or nile red, between the two beta sheets enhanced their cell-penetration and uptake [59-64]. In comparison with spherical particles, beta sheet filamentous particles (including cylindrical micelles, nanotubes, nanoribbons, and nanobelts) were able to improve drug bioavailability by increasing circulation times and the maximum tolerable dose, achieving a higher tumor cell apoptosis, delayed clearance of the drug by the liver and spleen, and minimal interactions with serum proteins due to their charge-neutral surface [61].

A
X: Hydrophilic residue
Y: Hydrophobic residue
X Y X Y X Y X Y X Y
Beta sheet building block

B
Hydrophilic face
Hydrophobic face
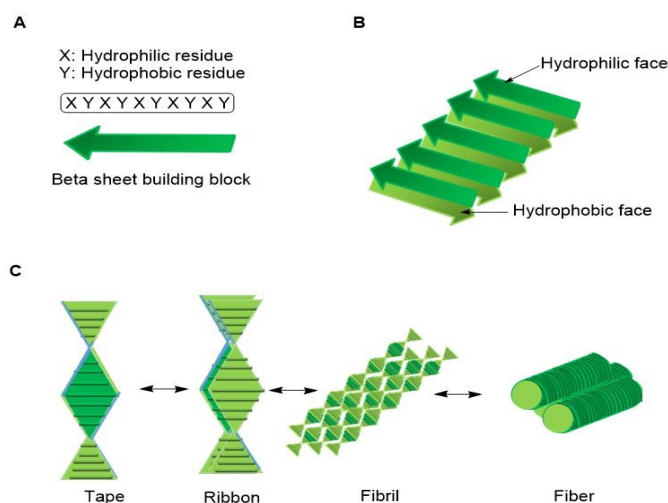
C
Tape   Ribbon   Fibril   Fiber

Figure 2. Schematic representation of peptides that form beta sheets and the self-assembled structures that can be formed. A: a peptide sequence with alternating hydrophilic (X) and hydrophobic (Y) residues. B: assembly of the beta sheet peptides into a molecule that contains both a hydrophilic and hydrophobic face. C: self-assembly of the beta sheet forming peptide into a tape, ribbon, fibril, and fiber based on their packing density [65]. Reproduced and adapted with permission from Elsevier.

2.2. Alpha helices and c oiled coils

Alpha helix assemblies are used as components of coiled coils which are a basic folding pattern found in native proteins [17]. A characteristic coiled coil sequence is made of seven-residue (*abcdefg)* repeats, referred to as a heptad (Figure 3) and the number of repeats can differ within a protein [66]. Positions *a* and *d* (Figure 3) are occupied by hydrophobic amino acid residues (such as L, I and V) while positions *e* and *g* are often charged amino acids (such as L and E). The hydrophobic interaction between residues *a* and *d,* and the electrostatic interaction between residues *e* and *g* contribute to the stability of the coiled coil structure [17, 66, 67], and is often represented by a helical-wheel diagram (Figure 3).
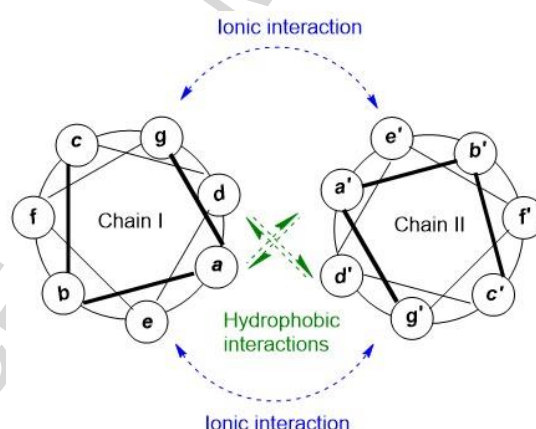

Figure 3. Helical-wheel of two coiled coil chains (chain I and II) showing inter-helical hydrophobic core and ionic interactions [66]. Reproduced with permission from Elsevier.

Expansion of the hydrophobic core to include the *e* and *g* positions and restriction of ionisable residues to positions *b, c* and *f*, improved the oligomerization state and thermal stability of the coiled coil [67, 68]. In this stable arrangement the side-chains were positioned along the outside of the coil. The specificity and stability of coiled coils relate to the number of helical strands and the orientation of the helices [66, 69], which are affected by hydrophobic core packing and inter-helical ionic interactions [66]. Holowka *et al.*, produced coiled coil nanovesicles by designing peptides with hydrophilic residues in one part of the peptide chain and hydrophobic residues in another part of the chain (such as K_xL_y where x=20-80 and y=10-30) which allowed for the encapsulation of the model drug (dextran) [70, 71]. The stability and unfolding of coiled coil motifs is dependent on the

temperature, pH and ionic strength which is often used in the design of controlled release delivery systems that respond to a specific stimulus [66]. Furthermore, the unique association-dissociation of coiled coils make them an ideal candidate for physical cross-linkers to form protein-based supramolecular fibrils [1, 72].

2.3. Peptide amphiphiles

Peptide amphiphiles are a class of self-assembled peptides that combine the structural features of amphiphilic surfactants with the functions of bioactive peptides to deliver therapeutics [4]. They contain single or multiple alkyl chains attached to one end of the peptide comprising a beta sheet forming residues, a hydrophilic or charged region with a bioactive head group (Figure 4) [5, 73]. Peptide amphiphiles are known to assemble into nanostructures with cylindrical/fibril geometries to provide a concentrated multivalent display of peptide antigens [5, 74]. This antigen display has been used in effective peptide-based vaccine delivery systems by protecting the antigen from degradation, and enhancing uptake and processing by antigen presenting cells (APCs), thus enabling the induction of a strong immune response [45, 75].
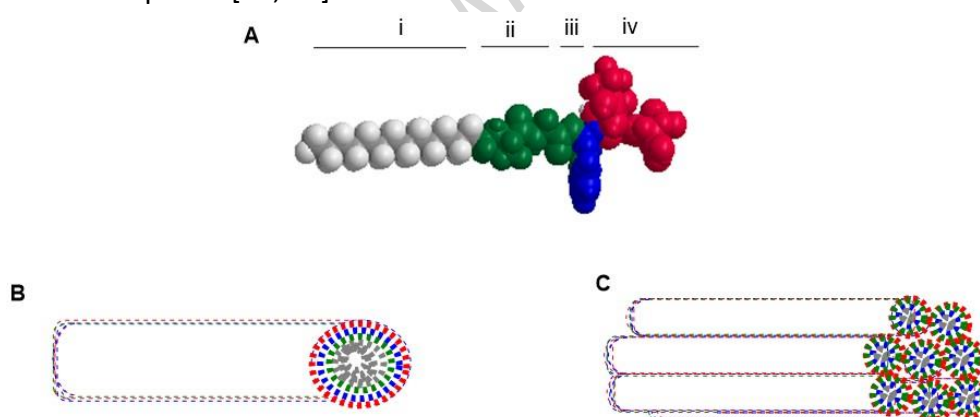

Figure 4. Typical chemical structure of a peptide amphiphile and its self-assembled structure. A: Schematic chemical structure containing (i) lipid tail, (ii) sequence capable of hydrogen bonding or forming beta sheets, (iii) charged peptide region, and (iv) bioactive peptide region. B: representation of a peptide amphiphile assembly in aqueous media forming a cylinder. C: fibril formation from a bundle of cylinders [76]. Reproduced and adapted from open access Elsevier.

The lipid tail length, number of charged sequences, and self-assembly conditions have been shown to influence the length and diameter of the nanofibers. These alterations enabled hydrogen bonding and/or beta sheet formation but did not affect cylindrical shape once a minimum number of hydrophobic residues was reached. Peptide amphiphiles with a branched bioactive sequence were shown to form nanofibers with a low degree of internal order, which is beneficial for presenting bioactive peptide epitopes on the nanofiber surface to interact with protein receptors [77]. Thus the degree of internal order in these self-assembled nanostructures controlled their bioactivity and physical properties. Polypeptide

dendrimers that contained multiple branches could mimic protein structure (due to their nanoscale size), and produced stable micelles that demonstrated different phase behaviours when compared to linear polypeptides. Additionally, their physicochemical properties could be controlled by the functionalization of peripheral groups, which is important for their application, e.g. acting as a drug carrier or targeted drug therapy [78-81].

The nanostructures of peptide amphiphiles have been shown to encapsulate and slowly release both hydrophilic and hydrophobic drugs. Micelles of peptide amphiphiles that bore an alkyl chain (C16) were shown to permeate and internalize in the endocytic vesicles of osteosarcoma cells (SJSA-1 cells) via adsorption-mediated, energy-dependent pathways [79]. Moreover, nanofibers of a peptide amphiphile from a collagen peptide that bore a C16 alkyl chain formed a hydrogel, stimulating collagen production and wound healing [83, 84]. Stupp and co-workers created a biopolymer membrane delivery system by self-assembling negatively charged hyaluronic acid with positively charged peptide amphiphiles C16-VVVAAAKKK-NH (K_3 PA) and C16-VVVAAAGGKLAKLAKKLAKLAK-NH (KLAK PA). The latter, KLAKL PA, was shown to be responsive to the cancerous environment by inducing MB-MDA-231 breast cancer cell death and degradation of the tumor environment. Crosslinking of tyramine-functionalized hyaluronic acid using horse radish peroxidase and H_2O_2 under mild conditions following co-assembly of the peptide amphiphile membrane was shown to stabilize the structure of these peptides against osmotic pressure and ionic degradation in buffered medium. In addition, the morphology of the membrane was shown to be a determinant in its functionality as a sustained release vehicle or localized surface cytotoxic delivery system. Here, the KLAK PA and K_3 PA mixed in a 50:50 ratio formed spherical particles and presented a sustained release reservoir of the cytotoxic peptide following enzymatic degradation by hyaluronidase over a 2 day period. However, when KLAK PA and K_3 PA were mixed in a 20:80 ratio over the course of 2 h, formation of a fibril membrane with localized surface cytotoxicity was observed. In addition, this construct has less cytotoxicity than the 50:50 ratio construct. The introduced biopolymer membrane has the potential to be used as a localized adjuvant therapy post lumpectomy [85]. Peptide amphiphile nanofibers have also been shown to perform as a molecular transporter in brain delivery (discussed in further detail in Section 4.1) [86].

2.4. Amino acid pairing

Another method for designing self-assembled peptides is using an amino acid pairing peptide (AAPP) strategy. This model used combinations of amino acid pairings that self-assembled through weak interactions, including hydrogen bonds, hydrophobic interactions and ionic bonds. For example, a peptide that contained the hydrogen bonding pair (Q-N), one ionic-complementary pair (E-K) and two hydrophobic residue pairs (F-F) [87]. The charge distribution along the backbone of this ionic-complementary peptide was a determining factor in its self-assembly conformation and nanostructured stability. Three types of charged distributions were studied extensively: type I (-+ or +-), type II (--++ or ++--) and type III (----++++ or ++++----) where - and + refer to negatively and positively charged amino acids respectively [6]. EAKA16-I (AEAKAEAKAEAKAEAK, type I, -+) and RADA 16-II

(NH$_2$-RARADADARARADADA-COOH, type II, ++--) are well-known ionic-complementary self-assembled peptides that have been used in drug delivery applications [6, 88-90]. Here, the hydrophobic residues encapsulated and stabilised hydrophobic drugs and enhanced self-association of the peptide. Additionally, hydrogen bonds were found to stabilise peptide assemblies with charged residues, increasing the solubility of the peptide [87]. It has been shown that EAK16-II (AEAEAKAKAEAEAKAK) and EAK16-IV (AEAEAEAEAKAKAKAK) have different charge distributions creating stabilized hydrophobic compounds in aqueous solutions and releasing them into the cell in a controlled manner. Additionally, they have been shown to self-assemble into stable beta sheet fibrils over a wide pH range (1.5-11) in the presence of proteases and denaturing agents without an adverse immune response in mice, rabbits, or goats [9]. This shows their potential as a drug delivery vehicle to maintain therapeutic drug concentrations throughout the body or at a specific location in a cell. Saadatmousavi *et al.*, designed a peptide that contained FEFQFNFK and demonstrated concentration-dependent peptide self-assembly that stabilised ellipticin, an antineoplastic agent. The applications and characteristics of this delivery system are explained in more detail in Section 4.5 [6]. Figure 5 shows representative ionic pairing peptide hydrogels.
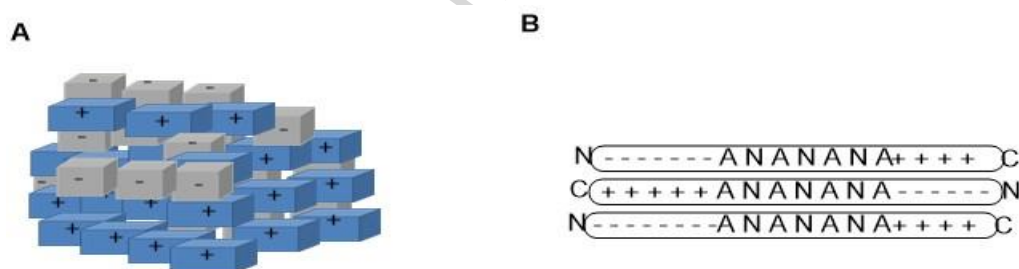

Figure 5. Schematic representation of ionic pairing in a peptide assembly. A: Hydrogel scaffold with ordered aggregates of ionic-complementary self-assembled peptides, B: Hydrogel scaffold composed of alternating hydrophilic and hydrophobic residues and clusters of negative and positive charges on the N- and C-terminus, respectively. Adapted from [87].

2.5. Elastin-like polypeptides (ELPs)

Elastin-like polypeptides (ELPs) are a group of biopolymers that self-assemble under physiological conditions because of their behaviour at their lower critical solution temperature (LCST) [91]. ELPs are protein polymers with [VPGXG]$_n$ amino acid repeats (where X is a variable amino acid) derived from a highly conserved repeat sequence in mammalian tropoelastin. These biopolymers display temperature dependent phase behaviour and form secondary structure ranging from random coil to cylindrical micelles made from beta sheets depending on the polarity of the residue [1, 92]. ELPs are characterised by rubber-like elasticity, large extensibility before rupture, flexible deformation without loss of energy, high resilience upon stretching, thermo-responsiveness, and biodegradability. Elastin and resilin, highly cross-linked proteins, are examples of two elastomeric biopolymers and recombinant polypeptides. Synthetic polypeptides that mimic

elastin composition have been applied as macromolecular and nano-carriers in the form of controlled release gels and drug-eluting films [93]. ELP-based carriers can be categorised into soluble (transition temperature (T_t) above body temperature) and insoluble ELPs (T_t below body temperature) [88]. Conjugation of doxorubicin with ELP[$V_1A_8G_7$-160], which has a T_t above 37 ^{o}C, self-assembled into 40 nm micelles and was shown to reduce tumor size in a mouse model. This conjugate was five times more effective than free doxorubicin when tested against E0771 murine breast tumors [94, 95]. Raucher and coworkers developed a technique to fuse a cell penetrating peptide to the ELP carrier to facilitate cellular uptake of doxorubicin [95]. This technique resulted in an effective cancer therapy *in vitro*, however, to date no *in vivo* studies have been reported [91]. ELPs can also be used for the local delivery of drugs by triggering coacervation in response to changes in body temperature, providing a depot for prolonged release of the drug [96-97]. Here, fusion genes that contained a hydrophilic, high T_t ELP[$V_1A_8G_7$-n] gene at the N-terminus and a hydrophobic, low T_t ELP[V5-n] gene was created to encode a diblock (contains ELP[$V_1A_8G_7$-n] and ELP[V5-n]) ELP. This ELP self-assembled into spherical micelles at 40 °C and presented multiple copies of the targeting moiety attached to the N-terminal ELP and entrapped the drug within the core of the micelle (Figure 6).
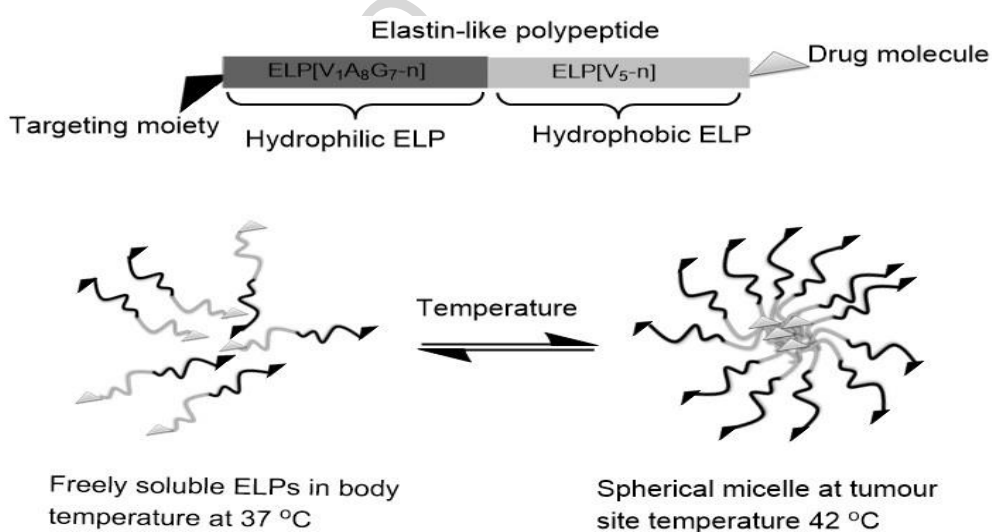
Figure 6. Schematic illustration of depot micelle formation at the tumor site in response to coacervation of an elastin-like polypeptide with a T_t above 37 °C. Reprinted with permission from [98]. Copyright {2008} American Chemical Society.

Ghandehari and co-workers applied silk ELP polypeptide hydrogels to the intra-tumoral injection of adenoviruses in a head and neck cancer model in an attempt to overcome the challenges of cancer gene therapy. They showed a ten-fold increase in β-galactosidase gene expression in a head and neck cancer model, indicating a more efficient and localized transfection compared to gene therapy with viral vectors (adenovirus carrying the β-galactosidase gene) without the biopolymer [91, 99, 100]. ELP nanocarriers produced via

genetic engineering techniques were shown to have no acute systemic toxicity or immunogenicity in mice following intraperitoneal, intravenous and subcutaneous administration, and no systemic antigenicity in guinea pigs following intravenous administration [101]. However, further tests are required to demonstrate the safety of these engineered nanocarriers *in vivo* [101]. Additionally, drug molecules with high hydrophobicity and/or a large number of hydrogen-bond donors and acceptors showed high ELP encapsulation efficiency. Therefore, drugs that are currently challenging to formulate using more conventional delivery vehicles might be good candidates for delivery by genetically engineered ELPs [92].

2.6. Cyclic peptides

Peptide cyclisation has been employed to impose rigidity to allow the molecule to adopt a number of conformations that are not available to linear peptides. Increasing the rigidity of the structure has been shown to enhance receptor binding affinity [102]. Cyclic peptides have been shown to adopt flat conformations or stack via hydrogen bonding to form self-assembled peptide nanotubes where the amino acid side chains of the peptide ring are oriented outward (Figure 7) [103]. The diameter of the nanotube was controlled by the size of the unit ring, and its external surface provided specific conformational properties by modifying the interaction of the side chains. These nanostructures can be used in artificial photosystems, biosensors, antimicrobials, electronic devices, photoresponsive materials, selective transmembrane transport channels, and drug delivery [103-105]. A novel drug delivery system comprised of alternating tryptophan and arginine in a cyclic octapeptide [WR]4 was introduced by Parang and co-workers [102]. They showed that the optimal balance between electrostatic and hydrophobic interactions of the cyclic peptides (drug carrier) and phosphopeptides (transporters which give on/off signals to many enzymes through interactions with protein kinases), led to the formation of circular vesicle-like nanostructures (25-60 nm in diameter) with improved intracellular phosphopeptide delivery. Compared to its linear counterpart this delivery system had higher enzymatic stability, bypassed endosomal uptake, improved cell permeability, and allowed the nuclear targeting and cellular delivery of impermeable phosphopeptides [102-106]. Furthermore, in an aqueous solution of chloroaurate this cyclic peptide formed gold-capped nanoparticles through the reducing activity of the tryptophan residue and attraction of chloroaurate anions towards the positively charged arginine residues. This gold-capped cyclic peptide delivery system was loaded with hydrophobic drugs (including doxorubicin, lamivudine, emtricitabine, and stavudine) in an equal molar ratio and showed improved cellular uptake and retention when used as a molecular transporter [107]. Additionally, cyclic peptide nanotubes composed of (W-$_D$-L)4-Q-$_D$-L and applied to the delivery of the antitumor drug 5-fluorouracil (5-FU) rapidly reached a high level of penetration into tumor cells where the drug effect was strengthened as the dosage of the cyclic peptide increased. This increase was associated with the cyclic peptide improving the transport of the drug into the target cell [108].
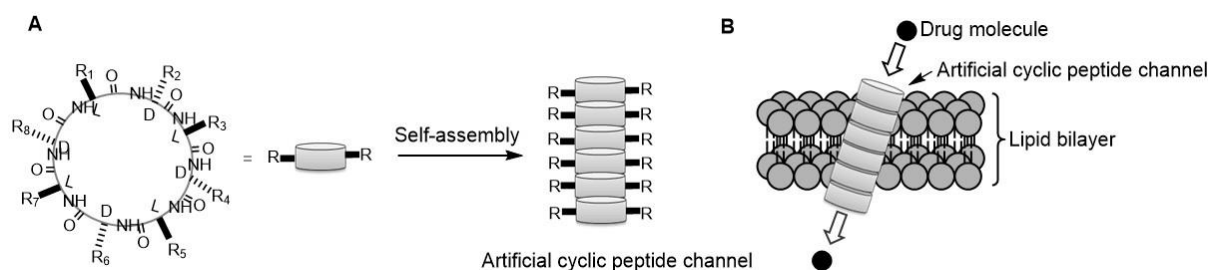
Figure 7. Schematic structure and function of cyclic peptides. A: schematic representation of nanotube formation from a cyclic peptide, B: schematic illustration of an artificial channel formed by stacked cyclic peptides passing through the lipid bilayer membrane to facilitate drug transport into the cell. Reproduced from [103] with permission from The Royal Society of Chemistry.

2.7. Short peptides

Short peptides are comprised of two or three amino acids [46, 109, 110]. Gupta and co-workers showed that, when heated and cooled in a 0.8 M sodium acetate buffer at pH 7, a F-ΔF dipeptide (H-F-α,β-dehydrophenylalanine, Figure 8) self-assembled into a highly ordered, dense nano-tubular hydrogel that responded to changing pH and salt concentration [110]. Here, the gel showed disassembly at low (pH=2) and high (pH=10) pH. The double bond between the C_α and C_β atoms of phenylalanine induced conformational constraints and proteolytic stability. Additionally, the free amino and carboxyl groups at the N- and C-terminus offered the ability to control self-assembly through electrostatic interactions, compared to the F-F dipeptide which exhibited no gelation under similar reaction conditions.
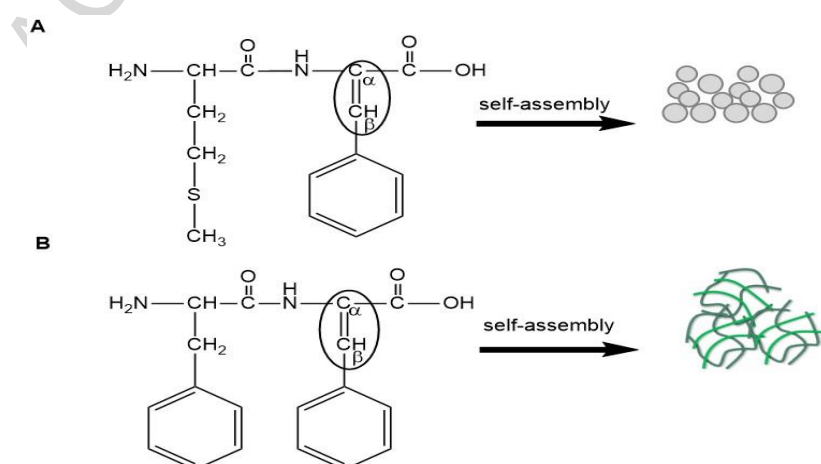

Figure 8. Molecular structure and nanostructures of ΔF (α,β dehydrophenylalanine) dipeptides. A: M-ΔF self-assembled into nanovesicles. B: F-ΔF self-assembled into a hydrogel.

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The F-ΔF hydrogel showed elastic behaviour and a high mechanical strength with a greater storage modulus (stored energy representing elasticity) than its loss modulus (viscous properties of a material). Loading and release of drug molecules from this gel showed increased entrapment and sustained release of all drug molecules tested including: vitamins (ascorbic acid, riboflavin, and vitamin B12), antibiotics (ampicillin and chloramphenicol), insulin, and antimalarial (amodiaquin), anticancer (fludarabine, mitoxantrone) and anti-tuberculosis (L-cycloserine and isoniazid) drugs. Among these, drugs with higher molecular weights, increased hydrophilicity and a higher percentage of negative charges showed a lower diffusion release coefficient, a correlate of improved entrapment [111]. Chauhan and co-workers developed a library of ΔF dipeptide nanostructures for drug delivery applications, including F-ΔF, R-ΔF, L-ΔF, E-ΔF and M-ΔF. These nanovesicles encapsulated drugs such as riboflavin, niacin, amodiaquine, mitoxantrone, and ampicillin with varying entrapment efficiencies. Among them, M-ΔF showed a maximal loading capacity of 35% for hydrophobic drugs and sustained release characteristics. Additionally, R-ΔF nanostructures were shown to escape from the reticuloendothelial organs and were present in the peripheral circulation for 1 h. This indicated that a rapid removal from blood compartment had been avoided [112, 113]. In another study, a tri-peptide amphiphilic hydrogel composed of Boc-aminoundecanoic acid-F-F-COOH exhibited thixotropic properties following heating in 50 nM PBS at pH 7.4. Here, the required temperature for hydrogelation was 32-70 ^{o}C and was shown to be concentration dependent (0.3-0.9% w/v). This delivery system was employed to separately encapsulate an antibiotic (vancomycin) or vitamin B12 and was shown to release these drugs over a two day period at physiological pH and temperature [114]. Banergi and coworkers developed stable nanovesicles (320 ± 50 nm) over a wide pH range (pH 2-12) and responsive to Ca^{+2} ions from dipeptides that contained a glutamic acid residue located at C-terminus and C4 lipoamino acid at N-terminus. The vesicles encapsulated fluorescent dye and doxorubicin and released them in the presence of calcium ions [115]. Modification of short peptides containing phenylalanine to constrain their conformation ensured sustained released from hydrogel delivery systems with improved mechanical properties. The π-π stacking mechanism between aromatic hydrophobic residues enhanced self-assembly of the peptides into hydrogel formation.

2.8. Fluorenylmethoxycarbonyl (Fmoc) peptides

The addition of an aromatic group, such as Fmoc to their N-terminus allowed peptides to self-assemble into stable hydrogels when exposed to changes in pH or solvent polarity [1]. Figure 9 depicts the molecular structure and interactions between Fmoc-F peptides as an example of this type of peptide design. Fmoc peptide hydrogels (such as Fmoc-FG, Fmoc-RGDF and Fmoc-FF) were shown to have similar physical properties to those of natural ECM. Mechanistically, Fmoc hydrogels underwent beta sheet formation and fibrillization through the π-π stacking of their aromatic groups. The peptides formed an anti-parallel arrangement of beta sheets with the Fmoc groups acting like a zipper to bring neighbouring sheets

together to create a cylindrical structure [116]. This approach allowed the use of a much shorter peptide sequence compared to those used in other areas of peptide self-assembly [117]. Sutton *et al.*, showed that Fmoc-F and Fmoc-Y formed strong hydrogels triggered by the pH adjustment using glucono-δ-lactone [118]. Both hydrogels released hydrophilic drug models of different radius of gyration (dye molecules) under Fickian diffusion control the viscoelastic properties. Of these two hydrogels, Fmoc-Y had a higher storage modulus that retained different sizes of dye molecules tighter than Fmoc-F [118]. It was found that differences in charge distribution and chemical structure of phenylalanine and tyrosine were attributed to the difference in the hydrogels rheological properties. Here, release of dye from Fmoc-Y and Fmoc-F hydrogels was controlled by altering the mesh size of the gel network and dynamics of the gel, in terms of the time scale of breaking and re-forming, respectively [118]. To improve the stability of Fmoc peptide hydrogels under physiological conditions, a hydrogel consisting of 10% wt Fmoc-RGD in water was developed. The 10% wt Fmoc-RGD hydrogel formed a beta sheet fibril network and, when used as a slow release vehicle for the delivery of model hydrophilic drugs, was stable in water for nearly 40 days [119]. The self-assembly of Fmoc-FF in a polysaccharide solution, such as konjac glucomannan (KGM), improved the stability and mechanical properties of the Fmoc peptide hydrogel through enhanced hydrogen bonding and the formation of a stable three dimensional gel network, in addition to creating a weak hydrogel at high pH and non-gelling flat ribbons at an intermediate pH [120]. This hybrid hydrogel delivery system is promising for sustained drug release in colon-targeted drug delivery where KGM has been shown to only be degraded by beta-glycosidase which is found in a high concentration in the colon [44].
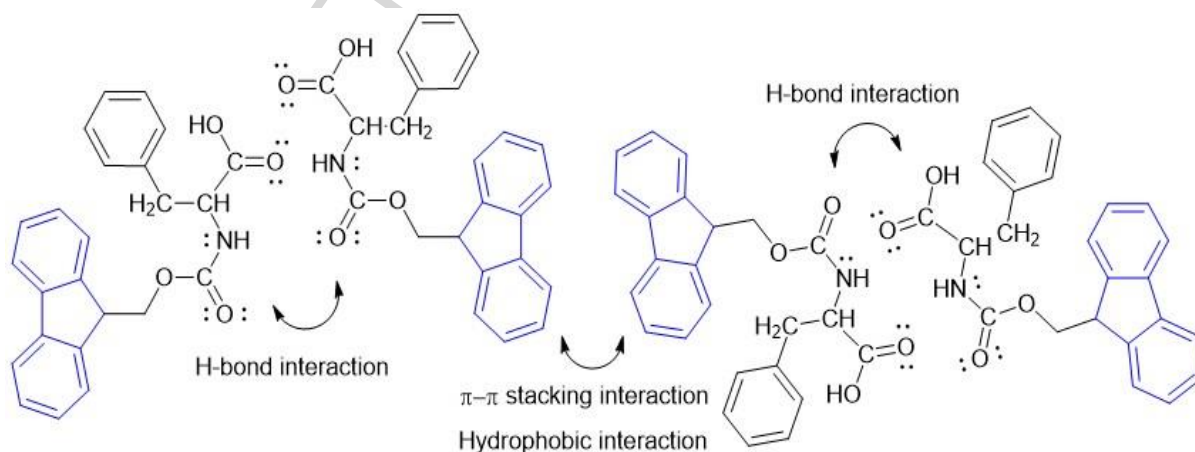

Figure 9. Molecular structure and possible interactions between Fmoc-F peptides to form hydrogels. Adapted from [120, 121].

3. Self-assembled peptide hydrogel characteristics and release kinetics

3.1. Peptide hydrogel characteristics

An ideal hydrogel system should have good biocompatibility, biosafety, high stability, optimal mechanical strength, and readily allow incorporation of bioactive ingredients with controlled release at the required biological sites [122]. Altering the nano-architecture of the peptide hydrogel network modulates hydrogel stiffness and porosity, effect the viscosity and overall drug release profile [4].

In peptide hydrogel delivery systems, water soluble peptides undergo a transition from liquid to a gel-like state (which is more commonly referred to as sol-gel transition) at the target site, in response to changes in the ionic strength, temperature or pH of the medium [44]. Physical gelation under biological conditions (pH-, ionic strength and thermo-responsiveness) allows for the three dimensional, homogeneous encapsulation of desired molecules and/or cells [48]. Moreover, peptide hydrogels exhibit mechano-responsive properties. Thixotropic hydrogels induced a gel-sol transition by mechanical shaking and quickly recovered into a gel-state after the stress was removed [123]. This self-healing property is essential when designing a delivery system that has the ability to shear-thin within a syringe needle and transform back to the gel after expulsion from the syringe. Thixotropic hydrogels allow the drug to be injected directly into the targeted site without surgical implantation [114]. For example, Schneider and co-workers developed a peptide hydrogel comprised of 20 amino acids (VKVKVKVKV$_D$P$_L$PTKVKVKVKV-NH$_2$) with sol-gel transformation triggered by an increase in ionic strength in the presence of Dulbecco's Modified Eagle's medium buffer. This gel-forming construct showed a shear-thin recovery after injection to the targeted site, without syringe-clogging [48].

Veerman et al., showed that assembly time (tg) of the beta hairpin peptide VKVKVKVKV$_D$P$_L$PTKVKVKVKV-NH$_2$ decreased as the peptide concentration increased, from approximately 30 min for 0.05 wt % to 11 min for 0.15 wt %. They also demonstrated that the relationship between gelation time and concentration followed power-law equation 1 where tg is gelation time and c is peptide concentration in weight percent [124, 125].

$$tg = kc^{-1} \qquad \text{Eq.1}$$

Examples of self-assembled peptide hydrogels are shown in Table 2.

Table 2. Examples of self-assembled peptide-based drug delivery systems

Self-assembled peptide	Triggering agent	Active ingredient	Features	*In-vitro*/in-vivo experimental model	Ref.
FEFQFNFK	Water	Ellipticine	Beta sheet fibril, sustained release	MCF-7 and A549 cells	[6]
Palmitic acid-GTAGLIGQRGDS	Drug	Cisplatin	Nanofiber, bioresponsive hydrogel with burst release		[26]
KRRASVAGK[C12]-NH$_2$	CaCl$_2$	Doxorubicin	Nanofibril, protein kinase A stimuli responsive,	MDA-MB-231	[47]
Ac-I$_3$K-NH$_2$	pH change	Anionic hydrophilic methyl orange	Nanotube, pH responsive release and sustained release at pH=7		[64]
F$_D$-F$_D$-Y$_D$	Water, sonication, heating	Naproxen	Hydrogel, sustained release over 25 h, selective inhibition of cyclooxygenase-2		[57]
Ac-A$_6$K-CONH$_2$ and Ac-A$_6$D-COOH (mixed)	Probe sonicate PBS (pH 7.4)	Carboxyfluorescein and nile red	Nanovesicle 100-200 nm, negatively charged for i.v. administration, sustained release		[78]
Cyclic peptide (WRWRWRWR)	Water	Phosphopeptides, doxorubicin, lamivudine, emtricitabine, and stavudine	Vesicle-like nanostructures	BT-20, CCRF-CEM, SK-OV-3, and HCT-116 cells	[102, 106, 107]
Palmitoyl-GGGAAAR and palmitoyl-GGAAAKRK	Probe sonicate in 5% dextrose	Nile red	Nanofiber, molecular transporter to the brain	Brain delivery in rat model	[86]
F-ΔF (Phe-α, β dehydrophenylalanine)	Heating/cooling of 0.2 % peptide in 0.8 M sodium acetate buffer (pH=7)	Ascorbic acid, riboflavin, vitamin B12, ampicillin, chloramphenicol, amodiaquin, fludarabine, insulin, mitoxantrone, and L-cycloserine	Stable hydrogel, sustained release, responsive to salt concentration and pH		[46, 124]
M-ΔF	Aqueous 50 % methanol	Curcumin	Nanovesicles , sustained release	HeLa, MCF-7, HuH-7 cell lines; Balb/c mice bearing a B16F10 melanoma tumor	[112]

Table 2. Examples of self-assembled peptide-based drug delivery systems (continued)

Self-assembled peptide	Triggering agent	Active ingredient	Features	*In-vitro*/in-vivo experimental model	Ref.
F-ΔF	Water/DMSO/ isopropanol at 37 °C	Pazopanib	Nanotube, sustained release, high drug levels in the vitreous, retina and choroid l pigments	Epithelial cells; rat model	[124]
Boc-aminoundecanoic acid-FF-COOH	Heating in 50 mM PBS (pH 7.4)	Vancomycine, vitamin B12	Nanofibrilar hydrogel, shear thinning properties	MCF7 cell line	[114]
Fmoc-F	Glucono-δ-lactone (GdL)	Direct red, napthol yellow, FITC dextran, 4K, 10K, and 20K	Strong viscose hydrogel, sustained release		[118]
Fmoc-Y	GdL	Direct red, napthol yellow, FITC dextran	Viscoelastic hydrogel, sustained release		[118]
Fmoc-RGD	Cooling-heating/ ultrasonic process	Hydrophilic drug model/salcilic acid	Sustained release, beta sheet fiber network		[119]
Fmoc-FF in polysaccharide KGM	Polysaccharide	Docetaxel	Fiber (10-30 nm in diameter), antiparallel-beta sheet arrangement, sustained release (Fickian diffusion)		[44]
Cyclic peptide (DWL)4-QDL	Proton-triggered in water	5-FU	Nanotube, self-associate into a lipid bilayer facilitating drug transmembrane transport		[108, 125]
C16-βAH	Water	L-carnosine	Bilayer nanotape, antioxidant, cosmetic applications		[126]

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Table 2. Examples of self-assembled peptide-based drug delivery systems (continued)

Self-assembled peptide	Triggering agent	Active ingredient	Features	*In-vitro*/in-vivo experimental model	Ref.
RADA16 (RADARADARADARADA)	PBS	Pindolol, quinine, timolol maleate	Beta sheet nanofibril hydrogel, sustained release		[127]
Ac-(RADA)$_4$-CONH$_2$	PBS (pH 7.4)	Lysozyme, trypsin inhibitor, BSA and IgG	Sustained release, biphasic diffusion kinetics		[128]
EAK16-II, EFK-II, EFK16-II	Mechanical stirring	Ellipticine, pyrene	Beta sheet fibril, colloidal suspension, sustained release	MCF-7 and A549 cells	[9, 131, 132]
Palmitoyl dalargine (pal-Y$_D$AGFLR)	Probe sonication	Dalargin	Beta sheet fibril, sustained release for brain delivery	Mice	[133]
Ac-(AF)$_6$H$_5$K$_{15}$-NH$_2$	Water	Co-delivery of doxorubicin and luciferase reporter gene	Micelles of cationic core-shell nanostructures, sustained release	HepG2 cells	[134]
VKVKVKVKV$_D$PPTKVEVKVKV-NH$_2$ and VKVKVKVKV$_D$P$_L$PTKVEVKVKV-NH$_2$	Dulbecco's Modified Eagle's medium	Curcumin	Sustained release, beta sheet hairpin gel, shear-thin, porous	Medulloblastoma cells	[41, 48]
VKVKVKVKV$_D$P$_L$PTKVEVKVKV-NH$_2$	Bist-Tris propan buffer (100 mM, pH 7.4) and 100 mM NaCl	Dextran-FITC, Lactoferrin, nerve growth factor, brain-derived neurotrophic factor	Semi flexible hydrogel, mesh size 18-49 nm, controlled release over one month, no activation of pro-inflammatory cytokines, shear-thin	J774 mouse peritoneal macrophages, PC12 pheochromocytoma cells	[135-137]
Fmoc-FFRGDF	Water	5-FU	Fibril beta sheet hydrogel, sustained release for seven days, biocompatible for implantable ocular delivery system	Rabbit eyes	[138-139]

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Table 2. Examples of self-assembled peptide-based drug delivery systems (continued)

Self-assembled peptide	Triggering agent	Active ingredient	Features	*In-vitro*/in-vivo experimental model	Ref.
C16-$V_2A_2E_2$	$CaCl_2$	Dexamethasone	Beta sheet nanofiberil, hydrogel network, sustained release over 32 days (zero-order), Sustained localized anti-inflammatory effect and reduced oxidative stress.	THP1 Human monocyte cell line, H9c2 cardiomyocyte/ hairless SKH1-E mice	[140]
palmitoyl-$A_4G_3E_3$	Solvent evaporation hydration in water	Camptothecin	Bet sheet nanofibril, sustained release over 1 week, increased cellular uptake and anti-tumor activity	BT-474, MCF-7, and SKBR-3 human breast cancer cells/ BT-474 orthotopic xenograft model of breast cancer in athymic nude mice	[141]
C16-$V_3A_3E_3$	Solvent evaporation hydration in PBS	Naproxen	Beta sheet nanofibril, controlled drug release over 24h by enzymatic degradation	Mouse mesenchymal stem cells	[142]
C16$V_3A_3E_3$K(βD)* *Aspartic acid was attached through its side chain	$CaCl_2$	Carbon monoxide (conjugated [Ru(CO)3Cl2]2)	Beta sheet nanofiber, slow release over 2 h,	H9c2 cardiomyocytes	[143]
C16$V_3A_3K_3$K(folate)-NH_2	Sonication	Doxorubicin	Beta sheet nanofiber, slow release over 5 days	MDA-MB-231 breast cancer cells	[144]

Table 2. Examples of self-assembled peptide-based drug delivery systems (continued)

Self-assembled peptide	Triggering agent	Active ingredient	Features	*In-vitro*/in-vivo experimental model	Ref.
oligo(ethylene glycol)-C16H6	1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) evaporation technique	Camptothecin	Fibril shape, pH-dependent delivery system. 50% release at pH 7.5 over 7 days and 80% release at pH 6.0, encapsulation efficiency 60%,	MDA-MB-231 human breast cancer cells,	[145]
$C16V_2A_2E_2$	$CaCl_2$	fluorescent dye molecule, Prodan, coavalently hydrazone bond to PA	Nanofibril, zero-order sustained release kinetics over 40 h,		[146, 147]

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3.2. Hydrogel release kinetics

Drug release through a peptide hydrogel matrix can be controlled by many factors, including the network mesh size, surface area to volume ratio, the properties and concentration of the gelator, salt concentration, pH, and interactions between the matrix and the entrapped molecules [118]. Briuglia *et al.*, showed that the drug release kinetics were dependent on the chemical properties of the drug (Log P, pKa, isoelectric point, presence of aromatic rings, and steric hindrance) and the medium chosen for the release study. For instance, controlled release of small hydrophobic drug molecules (quinine and pindolol) from the RADA16 peptide hydrogel caused specific binding between the aromatic groups and peptide matrix, or sterically hindered the drug molecule, which reduced the drugs diffusion capacity [129]. This system enabled release of the drug over a seven day period without changes to the drug morphology [129].

The release kinetics of proteins from peptide hydrogels was predominantly dependent on the density of the peptide hydrogel, protein size and charge [46, 111, 118]. In a study by Nagai *et al.*, the release of four proteins (lysozyme, trypsin inhibitor, BSA and IgG) from the peptide hydrogel (Ac-(RADA)4-CONH$_2$) were investigated separately. All four proteins released from this delivery system showed a biphasic diffusion with burst release in the first hour (related to the escape of the proteins located in the solvent-hydrogel interface which had a larger pore size, Figure 10A), and obeyed Fickian diffusion (Figure 10B). The remaining protein drug was released in a hyperbolic manner up to 100% over 30-50 hours. Small pore size or other obstacles to diffusion restricted the release of these proteins to a non-Fickian, anomalous diffusion (Figure 10). Increased peptide hydrogel density and increased protein drugs size were associated with a decrease in the release rate. Drug release from Fmoc-amino acid hydrogels was controlled by the dynamics of the gel network and followed a Fickian diffusion model [118].

Another factor that affected the kinetics of protein-release from the nanofiber hydrogel was the protein charge under physiological conditions [46, 111, 148]. Here, the conformational properties and functionality of the proteins did not change before or after release from this system, indicating that minimal interactions occurred between the folded proteins and the nanofiber hydrogel [130]. The effect of a peptide hydrogels pore size, density and electrostatic interactions between the drug and peptide(s) upon drug release from the beta hairpin, VKVKVKVKV$_D$P$_L$PTKVEVKVKV-NH$_2$ was also studied by Branco et al. [135, 137, 148].

Another feature of peptide hydrogels is that they do not swell after formation, even when exposed to bodily fluids, supporting consistent diffusion characteristics [41]. Moreover, the shape of the peptide hydrogel can be maintained during the release process without shrinkage [111, 119].
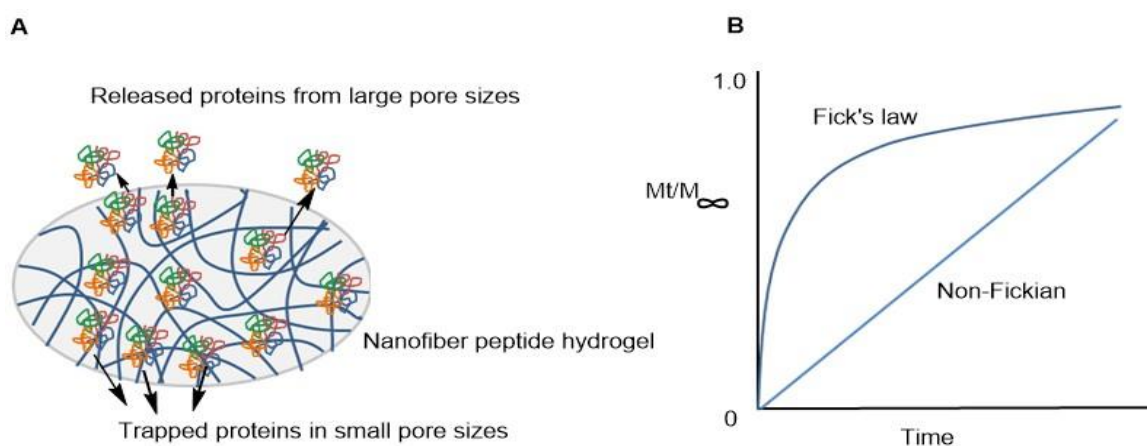
Figure 10. Schematic release mechanism of proteins from nanostructures of self-assembled peptide hydrogels. A: proteins released rapidly from large pore sizes at the solvent-hydrogel interface, and proteins trapped by small pore sizes are released slowly, B: a Fickian (Fick's law) and non-Fickian release kinetic [111]. Reproduced and adapted with permission from Elsevier.

4. Drug delivery applications of self-assembled peptides

4.1. Delivering drugs to the central nervous system

Delivery of therapeutic peptides and large hydrophilic molecules to the central nervous system (CNS) remains a challenge. Dalargin (Tyr-$_D$A-G-F-L-R) is an opioid receptor (μ receptor) agonist that is normally effluxed from the CNS and metabolized in the blood. An amphiphilic dalargin derivative that had been modified by attaching a palmitoyl moiety to the tyrosine hydroxyl group via an ester linkage self-assembled into beta sheet fibrils when sonicated in water. This modification overcame the main challenges that peptides must endure when delivered to the CNS by increasing the stability of the drug in the plasma, promoting endocytosis transportation across the blood-brain barrier. The dalargin derivative had improved uptake, pharmacodynamic effects, and reduced clearance from the brain parenchyma. In this instance, the prolonged activity of palmitoyl-dalargin nanofibers was associated with the slow dissociation of the drug from agonist receptors, and slow conversion of the prodrug to dalargine by plasma esterases [133]. Mazza *et al.*, showed that nanofibers of palmitoyl-GGGAAAR and palmitoyl-GGGAAAKRK in 5% dextrose could be used to transport molecules to the brain. Here, these peptide nanofibers were found to internalize in the neuron cells where the peptide was degraded over time by plasma enzymes, limiting accumulation of the delivery system in the brain, thus reducing potential cellular toxicity [83].

4.2. Intra-ocular drug delivery

Ocular drug delivery is restricted by efflux pumps and many static (different layers of cornea, sclera, and retina, including blood-aqueous and blood-retinal barriers) and dynamic (choroidal and conjunctival blood flow, lymphatic clearance, and dilution by tears) barriers

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[149]. Ocular targeting and maintaining therapeutic drug levels over time present major delivery challenges [150]. Nanotechnology-based formulations, such as nanomicelles, liposomes and poly-lysine dendrimers (200-2000 nm), have been developed to overcome these barriers and deliver drugs to the posterior segment of the eye with a long term release profile [150, 151]. Recent studies found that the Fmoc-FFRGDF peptide self-assembled into beta sheet fibrils with a width of 20 nm. This transparent hydrogel exhibited biocompatibility in rabbit eyes as an implantable delivery system for the treatment of ocular anterior diseases such as glaucoma and keratopathy [139]. When loaded with 5-fluorouracil (5-FU, 5% wt), an anti-proliferative agent, this hydrogel demonstrated a sustained release profile without swelling, shrinking or erosion of the gel in the aqueous media. Additionally, no burst release was observed. Here, the peptide hydrogel released the drug with a Fickian diffusion controlled mechanism of (Figure 10B) [152]. This implantable delivery system significantly lowered the intraocular pressure of the rabbits eyes within the 28 day postoperative period [138]. Furthermore, Panda *et al.*, used nanotubes generated from a F-ΔF dipeptide for the intravitreal delivery of pazopanib to treat choroidal neovascularisation. An *in vitro* study showed 25% drug loading, 55% loading efficiency and a profile of sustained release over 35 days. Moreover high drug levels were detected in vitreous, retina and choroidal pigment epithelial cells, compared to the plain drug, with a sustained release over 15 days after intravitreal injection in an *in vivo* rat model [124].

4.3. Cardiovascular drug delivery

Growth factors (GFs), hormones, and other proteins can promote myocardial regeneration and enhance the survival of grafted cells. To successfully deliver these drugs to the myocardial tissue a novel and promising strategy that combined self-assembling peptides with functional motifs to design noncytotoxic, biodegradable, porous, permeable, and flexible scaffolds. Here, prolonged cardiogenesis was stimulated at the myocardial scars by the sustained delivery of multiple GFs with distinct release kinetics [153]. The peptide amphiphile RADA16 was found to adsorb GFs through noncovalent interactions and induced angiogenesis with sustained release over a 14 day period [153]. In another study, a peptide scaffold was designed by attaching the heparin-binding sequence domain LRKKLGKA to the RADA16 peptide, which self-assembled into nanofibers under physiological conditions. This delivery system provided sustained release of a vascular endothelial growth factor (VEGF) for at least one month after transplantation when delivered by ventricular myocardial injection in a murine model. Here, improvement of cardiac function and reduction of scar size and collagen deposition was observed [154]. Moreover, a nitric oxide (NO) releasing peptide amphiphile that mimicked the native endothelial ECM was designed to coat cardiovascular implants. Two different lipidated peptide amphiphiles were mixed with deionized water (9:1 molar ratio) forming nanofibrous scaffolds and then subsequently reacted with pure NO under high pressure. Peptide amphiphiles contained either an endothelial cell-adhesive ligand (GTAGLIGQ-YIGSR) or a poly-lysine (GTAGLIGQ-KKKKK) NO donor. Burst release of NO was detected within 48 hours followed by 30 days of sustained

release. The self-assembled nanofiber matrix coating significantly enhanced the initial adhesion and proliferation of endothelial cells, but limited proliferation of smooth muscle cells. Additionally, the matrix limited adhesion of platelets, used to correlate the risk of thrombosis, was 150-fold less than the standard, a collagen-coated surface [155].

4.4. Bone drug delivery

Targeting drugs to bone tissue for disorders such as osteoporosis or osteosarcoma is very challenging due to the complex mineralized micro- and nano-structure of bone [156]. Short repetitive peptide sequences of aspartic acid were shown to bind to hydroxyapatite *in vitro* and *in vivo,* promoting accumulation of small drug molecules in the bone [156, 157]. Hydroxyapatite is a naturally occurring mineral form of calcium apatite [$Ca_5(PO_4)_3$] found in bone and dentin. Proteins that naturally bind calcium phosphate are rich in phosphorylated serine [157, 158]. Stupp and co-workers showed that hydroxyapatite nucleated on the surface of peptide amphiphile nanofibers that contained phosphorylated serine [158, 160]. Self-assembled peptide hydrogels have been modified by adding phosphoserine or RGD residues to improve mineralisation and cell adhesion, respectively. Peptide sequences P(S-PO_4-F)$_5$-S-PO_4-P, $C_{15}H_{31}C(O)$-C_4G_3SARGD-COOH and P(Y-PO_4-F)$_5$-Y-PO_4-P are examples of self-assembled peptide hydrogels used in bone tissue drug delivery [118, 161]. These peptide sequences can be formulated into hydrogels, membranes, solid matrices and mineral-peptide composites, to deliver therapeutic agents, such as anti-resorptive drugs, to bone or adjacent tissues [118, 162]. Peptide hydrogels composed of PD(FD)$_5$P and loaded with tricalcium phosphate increased alkaline phosphatase activity (an early osteogenic marker) and bone regeneration in an *in vivo* rat bone-defect model [162]. Figure 11. Shows a schematic representation of peptide assembly for applications in biomineralization.
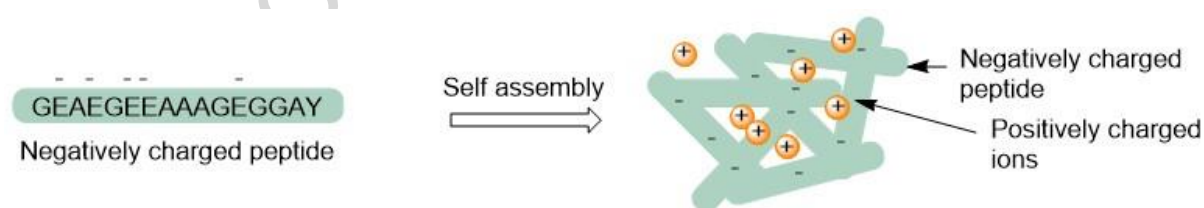

Figure 11. Schematic peptide hydrogel for biomineralization application. Negatively charged self-assembled peptide attract positively charged ions. Adapted from [162].

4.5. Anticancer drug delivery

The use of peptide ligands to target cancer chemotherapeutics has gained great attention over the last decade because of the low immunogenicity, high biodegradability of peptides and the ability to manipulate nanoparticle size by changing the composition of the peptide [163]. For instance, applications of cyclic RGD peptides, cell-surface hormone receptors (LHRH receptor), and tumor vasculature antigens in chemotherapeutic delivery systems have shown promising results [163, 164]. Intensive research into the development of self-

assembled peptide delivery vehicles for active or passive targeting of chemotherapeutics has accelerated due to the peptides' desirable physicochemical properties and potential for tailoring to suit specific biological applications [26]. Successful applications include: design of nanodelivery systems for targeted therapy, enhancing the efficiency of existing delivery systems, and optimizing peptide-drug formulations to increase stability, loading efficiency and control the release of the chemotherapeutic cargo [6]. The RGD sequence in the peptide amphiphile is known to have cell adhesion properties, the ability to mimic characteristics of the ECM, and is able to specifically bind to the upregulated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (transmembrane glycoproteins) during tumor growth and metastasis, thus gaining entry into the cells and enabling endocytosis [165]. This makes RGD an important sequence in the design of peptide amphiphile for targeted anticancer drug delivery that aim to release clinically significant levels of cytotoxic drugs into the localized tumor region. To this end, a bioresponsive gel delivery system formed by the self-assembly of cisplatin (*cis*-dichlorodiamine platinum(II)) in a 4% solution of palmitic acid-GTAGLIGQRGDS in water at 37 ^{o}C was designed [158]. This delivery system assembled into a nanofiber gel via the formation of complexes between platinium and the carboxylic acid groups on the adjacent nanofiber. The resultant gel displayed RGD ligands on the surface, which were directed at the integrin receptors that are overexpressed on cancer cells. Upon increasing concentration of matrix metalloproteinase-2 (MMP2) and biodegradation of the gel by MMP2, the drug was released at the tumor site [26].

Stupp and co-workers developed a protein kinase A (PKA, an extracellular biomarker for cancer) stimuli responsive peptide hydrogel to deliver doxorubicin to cancer cells. The peptide amphiphile KRRASVAGK[C12]-NH$_2$ contained the specific consensus substrate (RRXSO, X: any residue, O: hydrophobic) for PKA that formed nanofibril structures. This substrate was phosphorylated and dephosphorylated at the serine position by PKA and alkaline phosphatase (AP), respectively. Treatment of the nanofibrils using PKA diminished fibril morphology while subsequent treatment with AP restored fibril morphology. This substrate was specific for PKA rather than other protein kinases. Loading of doxorubicin into this delivery system showed a 40% release rate over 7 hours. Treatment of the breast cancer cell line MDA-MB-231 with this drug loaded peptide amphiphile resulted in the death of all cancer cells [47]. Delivery of therapeutics using dynamic assemblies is a promising method for targeted drug delivery.

Fung *et al.*, showed that EAK16-II and EAK16-IV formed a colloidal suspension in the presence of ellipticine in an aqueous environment. The *in vitro* anticancer activity of these formulations was dependent on the molecular state (protonated, crystal or neutral) of ellipticine that was present during formulation [132]. Interestingly, this complex was formed in a time-dependent manner by mechanically stirring (not sonication or shaking) the solution, and was independent of peptide concentration over the tested range (0.01-0.5 mg/ml) [9]. When the peptide concentration was close to its critical aggregation concentration (CAC, 0.1 mg/ml), the equilibration time was minimal (5 hours). Peptide concentrations higher than the CAC conserved the protonated ellipticine for a longer time

40 hours [131]. Nevertheless, the rate at which the drug was released from this system was dependent on the peptide concentration, and the loading capacity was dependent on the drug concentration [9]. *In vitro* toxicity assays indicated that the complexes (>5:1 ratio of peptide:drug) formed with protonated ellipticine were effective at killing both human breast cancer cells (MCF-7) and adenocarcinomic human alveolar basal epithelial cells (A549), although their toxicity decreased significantly at lower concentrations [131]. Further, increasing the hydrophobicity of the EFK16-II peptide increased hydrophobic interactions between the drug and peptide, significantly increasing the stability of the formulation. The original desired cytotoxic effect of the EFK16-II peptide against cancer cells was low due to the formation of complexes with the crystal state of ellipticine [132]. Saadatmousavi *et al.*, showed that the complementary peptide AC8 (FEFQFNFK) self-assembled in aqueous solution with a CMC of 10-15 μM (0.01-0.015 mg/ml) and could encapsulate ellipticine (0.05 mg/ml) in its neutral molecular state in aqueous media at 0.1 mg/ml (above CMC) to form 100 nm particles. This complex (>2:1 ratio of peptide:drug) with neutral ellipticine was effective at killing MCF-7 and A-549 cell lines after 48 hours [6]. The peptide sequence affects properties of peptide-drug assemblies and their cellular toxicities, however, these peptide-drug formulations need to be optimized to enhance the therapeutic effect and the efficacy of the delivery system.

Haines-Butterick *et al.*, designed a curcumin delivery peptide hydrogel (VKVKVKVKV$_D$P$_L$PTKVKVKVKV-NH$_2$) that gelated in response to an increase in the ionic strength of the medium. Curcumin is a hydrophobic polyphenol drug derived from the turmeric root and has been shown to have antioxidant, anti-inflammatory and anti-tumorigenic properties. In a low strength ionic buffer at pH 7.4, electrostatic repulsion between the positively charged lysine residues prevented the folding of the peptide. The lysine-associated charges were neutralised by adding Dulbecco's Modified Eagle's medium containing 160 mM of inorganic salts. This induced self-assembly of the peptide, both laterally (intermolecular H-bond and van der Waals attractions) and facially (hydrophobic face of the peptide), which led to a beta sheet hairpin structure (Figure 2) [48]. This peptide hydrogel succeeded as a reservoir for the sustained release of curcumin (4 mM) over a two-week period. Incorporating the drug in a peptide scaffold stiffened the gels. This peptide hydrogel showed shear-thinning properties, increased drug stability under physiologic conditions, and retained its bioactivity [41]. Liu *et al.*, encapsulated curcumin in a tumor-targeting self-assembled peptide nanofiber formed by Nap-GFFYGRGD with a 32% encapsulation efficiency (0.5 mM curcumin encapsulated by the peptide at 5mg/mL). This delivery system released 60% of the drug in a sustained manner over two days with high cellular uptake in $\alpha_v\beta_3$ integrin-positive HepG2 liver carcinoma cells and was found to accumulate in tumor cells after intravenous administration in a mouse model [166].

Panda and co-workers created nanovesicles that permitted the sustained release of cucurmin over a 36 hour period. The ~40 nm nanovesicles were generated from the self-assembly of M-ΔF in methanol followed by methanol evaporation resulting in a drug loading of 30% and a loading efficiency of 92%. The high drug loading may be related to the π-π

stacking interaction between curcumin and ΔF. The cytotoxic effect of the cucurmin nanovesicle system was higher than native cucurmin when tested in different cancerous cell lines including human cervical cancer cell line (HeLa), MCF-7 and human hepatocarcinoma cell line (HuH-7). Moreover, this formulation inhibited tumor growth in Balb/c mice that bore a B16F10 melanoma tumor [112]. Future work for self-assembled anti-cancer delivery systems requires the formulation of stable complexes and investigation into their immunogenicity and *in vivo* therapeutic properties.

5. Vaccine engineering

The engineering of immunomodulatory materials to act as delivery vehicles and gain a better understanding of the immune system is a challenging field. Self-assembled nanomaterials can be designed to induce or prevent complement activation and can be used as an adjuvant or adjuvant carrier. Also, these nanomaterials provide microenvironments that may aid interaction with antigen presenting cells (APCs), thus contributing to both prophylactic and immunotherapeutic vaccines [167].

5.1. Self-assembled peptides for vaccine design

Self-assembled peptides have shown various effects on the immune system. Many peptides stimulate no detectable antibody responses while others elicit potent responses without the addition of supplementary adjuvants. Strategies for avoiding or inducing an immune response using these materials has not been well characterised [167]. Developing peptide-based vaccines has been hampered by the need to display antigens in their native conformation and the use of immuno-adjuvants that only exhibit adjuvanting properties under specific circumstances [168]. Recently, several patents have been registered for the development of self-adjuvanting vaccines from self-assembled peptides [164, 165]. These materials provide scaffolds that can accommodate multivalency and molecular specificity, and enable anable control over epitope position [169].

Self-assembled nanomaterials were shown to display high densities of antigens on their surface and elicited high-titre, high affinity, and neutralizing IgG responses as well as CD8^{+} T cell mediated protection [172, 173]. Nanomaterials have an important role in vaccine delivery due to their intrinsic adjuvanticity influenced by the properties of particle, enhancing the immune response [174, 175]. Most attempts at engineering a vaccine that use this class of biomaterials include peptides that form beta sheets and lipidated peptide amphiphiles (Sections 5.1.1 and 5.1.2). Another approach used repeats of the immunodominant peptide that self-assembled into nanoparticles. Kaba *et al.* designed a self-assembled peptide nanovaccine composed of approximately 25 nm nanoparticles with high antigen display on their surface. Here, a nanovaccine comprised of the tandem repeats of the B cell immunodominant malaria parasite, *Plasmodium berghei* circumsporozite protein [(DPPPPNPN)$_2$D], stimulated a long-lived protective immune response without an additional adjuvant. Mice that received the vaccine candidates (three times at two weeks intervals) were protected 6 months after the last immunization and up to 15 months after

the second challenge. The resulting CD4$^+$ T cell dependent antibodies had higher avidity, correlated with improved protection, than antibodies produced when using the adjuvant Montanide ISA-720 [176].

5.1.1. Vaccine design using peptides that form beta sheets

Peptides that form beta sheets were able to display functional amino acids on the surface of their self-assembled nanofibers. Here, one strategy used fibrilized peptides to design a self-adjuvanting vaccine delivery system by covalently conjugating the non-immunogenic fibrilizing domain Q11 [(Ac-QQKFQFQFEQQ-Am) or KFE8 (FKFEFKFE)] to a model peptide antigen (OVA$_{323-339}$, ISQAVHAAHAEINEAGR) to form beta sheet nanoparticles. This elicited robust and long-lived antibody responses (for at least one year) in the absence of any adjuvant, which was comparable to the peptide epitope delivered with complete Freund's adjuvant (CFA). However, physical mixtures of Q11 and OVA$_{323-339}$ did not increase any OVA-specific IgG indicating the Q11 adjuvanting properties were dependent on covalent bonding to the peptide [10, 168]. Moreover, the antibody response generated was dependent on the presence of a T cell helper epitope and fibrilization. The immunogenicity of these constructs was shown to be modulated by inclusion of a CD4$^+$ T cell epitope where attachment of an additional non-immunogenic fibrilizing peptide, such as KFE8, to the same epitope induced high antibody responses, diminishing the fibrilizing domain attenuated immunogenicity of the peptide [10]. Conjugation of a multi-branched peptide epitope from *Plasmodium falciparum* circumsporozoite protein, NANP, to the Q11 peptide using a SGSG linker [(NANP)$_3$-SGSG-Q11] formed nanofibrils that elicited durable (40 weeks), T cell dependent antibody responses in a C57BL/6 mice model, with one boost at 28 days at 50% primary immunization dose. Additionally, co-assembly of the different epitope-bearing peptides, such as an immunogenic OVA-Q11 and the non-immunogenic peptide RGD-Q11, could be applied to develop self-adjuvanting multi-antigenic vaccines without compromising strength or duration of antibody response against either epitope [10]. When Q11 was conjugated to the OVA CD8$^+$ T cell SIINFEKL epitope the peptide self-assembled into nanofibers that generated a robust CD8$^+$ T cell response and protected against influenza virus challenge [172]. The ability to self-assemble into nanofiber adjuvants to stimulate a cytotoxic T lymphocyte response can be applied to the design of vaccines against cancer and infectious diseases.

Another challenge in vaccine design is balancing immunogenicity with inflammation, particularly for subunit vaccines that require pro-inflammatory adjuvants such as Alum. Aluminium hydroxide (or aluminium phosphate microparticles, Alum) is the only approved vaccine adjuvant by the US Food and Drug Administration and has resulted in local and systemic reactions followed by the production of IgE-mediated allergies towards the vaccine antigen [177, 178]. One promising method for avoiding inflammation is the use of self-adjuvanting vaccines that omit the toll-like receptor (TLR)-2 ligands that are associated with

inflammatory signals [179]. Chen *et al.*, demonstrated that nanofibers of the self-assembled OVA$_{323-339}$-Q11 vaccine did not increase inflammatory cytokines [173]. Furthermore, a construct formed by two palmitic acid chains (C16) conjugated to the N-terminus of a peptide that contained a cytotoxic T cell model antigen OVA$_{253-266}$ (EQLESIINFEKLTE) self-assembled into alpha helical cylindrical micelles of 8.0 ± 2.3 nm in diameter. These micelles enhanced uptake by APCs without TLR activation. The *in vivo* protection from tumors by stimulating OVA-specific cytotoxic T cells without the addition of an adjuvant has also been shown [45].

The effectiveness of fibrils as a self-adjuvanting delivery system for subunit vaccines encouraged researchers to design a potential vaccine candidate which was able to self-assemble into fibrils at physiological pH via a chemical intramolecular reaction such as O-N isopeptide acyl migration reaction (Figure 12) [180]. Skwarczynski *et al.* presented O-acyl isopeptides as a novel method to form self-adjuvanting subunit-peptide vaccine that formed long beta sheet fibrils from non-fibrilizing precursors. This delivery system was introduced as a vaccine prodrug to overcome over-aggregation and changes in fibril characteristics of the vaccine candidate upon storage. Here, a native amide bond at the threonine residue was isomerized to form an ester bond between the threonine and valine amino acids forming an isopeptide unit (Figure 12). The stable isopeptide showed high aqueous solubility and released the native peptide at physiological pH, triggering an O-N intramolecular acyl migration reaction. The characteristics of the isopeptide solid changed drastically after O-N acyl isomerization into beta sheet fibril formation. This method has also been used for the synthesis of peptides containing difficult amino acid sequences [180].
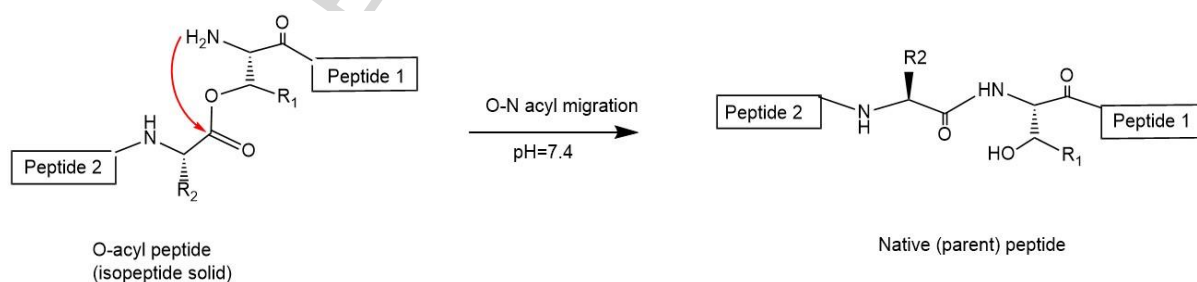

 Figure 12. O-N isopeptide acyl migration reaction [175]. Reproduced from Herbert open access Journal.

5.1.2. Vaccine design using lipidated peptide amphiphiles

The covalent conjugation of lipids (self-adjuvanting moiety) to poorly immunogenic peptides have been shown to act as potentially safe adjuvants [181, 182]. Over the past few decades, Toth *et al.*, developed a unique lipid core peptide (LCP) delivery approach that featured a lipopeptide moiety (inbuilt adjuvant) where unnatural lipids (lipoamino acids) were conjugated to epitopes via a poly-lysine or sugar branching unit (carrier). This was found to

induce self-assembly and provide self-adjuvanting properties to vaccine constructs [183, 184]. Toth *et al.*, tested the LCP vaccine delivery system by developing vaccine candidates that contained antigens from Group A Streptococcus (GAS) strains that were endemic to northern Australia. The native alpha helical secondary structure of the minimal B cell epitope from a GAS membrane protein (M protein) was maintained by flanking the antigen with regions of the yeast GCN4 protein to create the J14 sequence (KQAEDKVK-ASREAKKQVEKALE-QLEDKVK). Molecules that contained multiple copies of J14 conjugated to the LCP delivery system self-assembled into large nanoparticles (200-1000 nm in diameter) and stimulated J14-specific IgG antibody response in a B10.Br (H-2K) mice model more immunogenic than the negative control PBS. This study showed attachment of J14 epitope from its N- or C-terminus to the LCP (Figure 13A) system produced similar physicochemical properties and antibody response [185].

The J14-like epitope dJ14i, contained two repeats of the minimal B cell epitope and a triple-alanine spacer (AAA-ASREAKKQVEKALE-ASREAKKQVEKALE), and self-assembled into smaller nanoparticles (15-20 nm) than J14 when used in an LCP system (Figure 13A) while retaining a helical secondary structure. The dJ14i nanoparticles induced a similar immune response to the J14 epitope when administered subcutaneously with CFA in a mouse model. However, the antibodies did not recognize the native GAS M protein epitope, p145 post second boost [186]. A further study investigated the efficacy of the self-adjuvanting lipopeptide-based GAS vaccine, LP-88/30-J14, which contained the conserved C-terminal J14 epitope, the N-terminal GAS epitope (88/30, DNGKAIYERARERALQELGP), a universal T cell helper peptide epitope (P25, KLIPNASLIENCTKAEL), and two lipoamino acids (C16). Here, LP-88/30-J14 (Figure 13B) self-assembled into nanoparticles of 5 nm, enhanced APC uptake, maturation and antibody response without additional adjuvant. Furthermore, incorporation of N-terminus 88/30 epitope into the construct did not diminish J14-specific antibody response. The IgG antibodies were capable of binding to endemic GAS strains in a immunofluorescence microscopy assay [187]. This study found that the immune response correlated with particle size and the structural arrangement of the vaccine components in the chimeric molecule. Azmi *et al.* designed vaccine constructs where J14 was conjugated to the fibrilizing peptide Q11 (Figure 13C) with or without lipoamino acids were characterized and tested in a B10.BR mice model. The Q11 peptide failed to induce fibril formation when conjugated to J14, instead resulting in polydisperse nanoparticles of 20-250 nm. These nanoparticles failed to stimulate a high antibody titre, a result that was inconsistent with the earlier findings of Rudra *et al.* [168, 188]. Azmi *et al.*, hypothesized that the poor immune response may have resulted from disruption of fibril formation or administering one tenth of the peptide dose compared to the high immunogenicity results previously obtained from Q11 peptide conjugates[10, 189]. Nevertheless, the J14-Q11-lipoamino acid constructs (10-250 nm in diameter) induced a high antibody response in a non-size dependent manner, emphasising the importance of lipoamino acids as adjuvanting moieties in this delivery system [188].
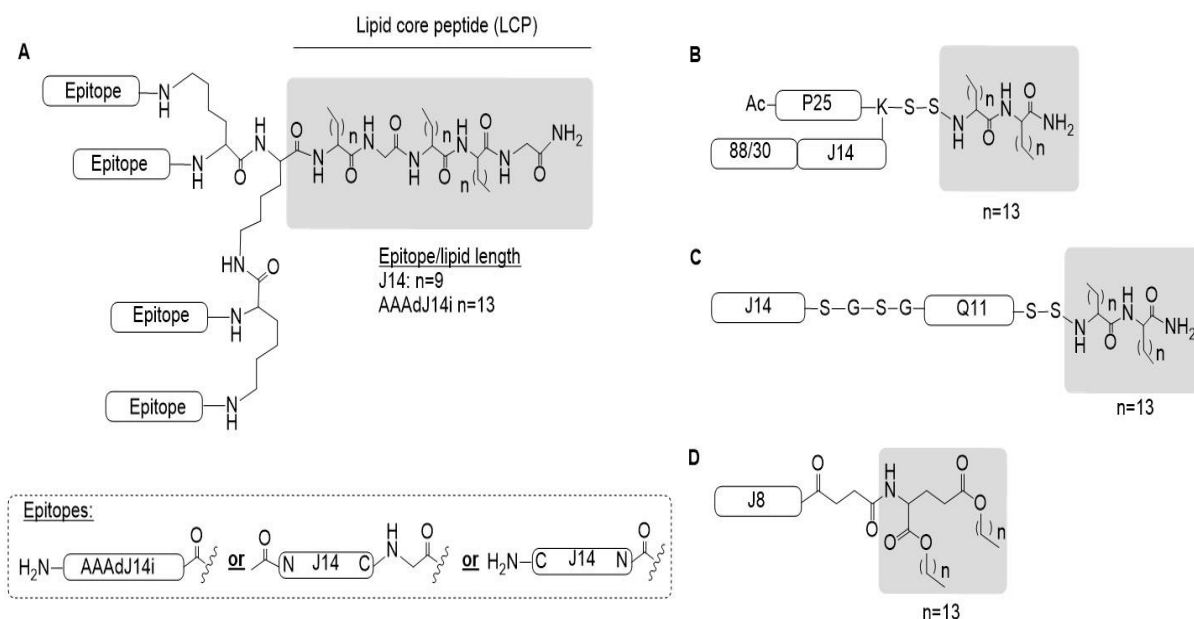
Figure 13. Self-assembled lipopeptide constructs studied for the development of a Group A Streptococcus vaccine. A: Lipid core peptide (LCP) containing peptide epitope of C-terminally or N-terminally conjugated J14 or AAAdJ14i peptide epitopes [180, 181]. Reproduced with permission from Bentham Science, B: vaccine candidate containing Group A Streptococcus J14, universal T helper P25 and 88/30-J14 epitopes [182]. Reproduced with permission from Future Medicine, C: vaccine construct containing Group A Streptococcus J14 and Q11, a fibrilizing peptide [183]. Reproduced with permission from Elsevier, D: vaccine candidate containing Group A Streptococcus J8 epitope [185]. Reproduced with permission from Springer.

Tirrell and co-workers developed a self-assembled, self-adjuvanting lipopeptide vaccine against GAS by conjugating a di-alkyl chain (di-C16) at the N-terminus of the GAS B cell antigen J8 (QAEDKVKQSREAKKQVEKALKQLEDKVQK), which was derived from the C-terminal domain of the GAS M1 protein. They showed that attachment of two C16 lipids at this position (Figure 13D) induced self-assembly into short cylindrical micelles, approximately 5–15 nm in diameter and 25-125 nm in length, with high alpha helicity. They annealed the micelles at 70 °C to form stable long fibrils while preserving the alpha helical secondary structure. These micelles induced high IgG1 antibody titres without the assistance of TLR-2 receptors in BALB/c mice and HEK-293 cells expressing the TLR-2 receptor [190]. This study demonstrated that micellization of the peptide amphiphiles enhanced the delivery of antigens in their correct conformation.

An important feature of vaccine design centres on exploiting differences in specific modifications of antigenic proteins to support a selective immunological response [191]. For example, a lipid adjuvant conjugated into a vaccine construct containing MUC1, a transmembrane glycoprotein highly overexpressed in tumors, enhanced immune responses to the glycopeptide derived from antigenic glycoprotein [191].

In 2007, Boons and co-workers developed a MUC1-based tricomponent therapeutic vaccine comprised of (i) a decapeptide fragment of the variable-number tandem repeat (VNTR) of the tumor-associated mucin 1 (MUC1) glycopeptide, (ii) a promiscuous T cell helper epitope, and (iii) the immunoadjuvant $Pam_3CysSer(Lys)_4$. High humoral and cellular immune responses in a BALB/c mice model, and enhanced cellular binding and uptake in MUC1-expressing MCF7 human breast cancer and HEK 293T cells lines was observed [191]. This concept led Apostolopoulos and co-workers to synthesise a self-assembled and self-adjuvanting therautic peptide cancer vaccine candidate with isotropic particles of uniform size 20 nm [186]. Apostolopoulos vaccine consisted of a complete copy of the VNTR eptiope from the MUC1 glycopeptide, the universal T cell helper peptide epitope (PADRE), and the lipidic immunoadjuvant, $(Pam_3CysSer)$. Here, strong IgG1 antibodies in C57BL/6 mice was elicited and were found to bind tumor cells over-expressing the MUC1 glycoprotein, including MCF7 and melanoma B16.MUC1 [192]. This study showed suitable design features required for self-assembly of the molecularly defined vaccine into nanostructures to generate a high antibody response. Robinson and co-workers designed lipopeptide vaccine constructs by conjugation of a phospholipid to the N-terminus of a coiled coil peptide sequence from the heptad repeat region 1 of respiratory syncytial virus and used for antigen attachment at its C-terminus via a cysteine linker. This delivery system self-assembled into homogenous 20 nm nanoparticles which displayed multiple copies of the antigen, inducing a high antigen-specific antibody titre in New Zealand white rabbits without the need for an additional adjuvant [193]. This study highlighted promising results for the development of self-adjuvanting vaccine constructs using self-assembled peptides. Vaccine engineering using these materials is seen as a way to systematically explore the impact of structure and particle characterization on immune response.

The importance of particle size and mono dispersity has previously been reviewed and plays an important role in vaccine development because it affects the mechanism by which APCs take up the vaccine candidate. However, there is no consensus on the optimal particle size to achieve this goal [194-196]. The higher immunogenicity of smaller particles (20-200 nm) is thought to result from increased facile transport into the lymphatic system and improved uptake by dendritic cells. Other particle characteristics such as shape, stability and the ability to display multiple antigens on their surfaces have shown an important impact on the immunogenicity of nanoparticles. Nevertheless, a more detailed understanding of the fundamental effect of the physical properties of a nanoparticle on cellular interaction and biodistribution within diverse tissues is required. Rational design and synthesis of nanovaccines in combination with the development of reproducible nanoparticle synthesis methods will provide vaccines with desired immunity properties.

6. Conclusion

There is increasing demand to design efficient and biodegradable delivery systems to target and control the release of therapeutics and decrease drug side effects. Supramolecular self-assembled peptide nanostructures offer a promising alternative to existing delivery vehicles due to their low toxicity, high biocompatibility and ability to target therapeutic small drug molecules and proteins. These materials are able to improve cellular permeability and regulate biological action of other pharmaceutical agents [197]. Peptide hydrogels also have favourable mechanical properties: shear-thinning for convenient delivery and long release profiles to enhance drug uptake and minimize the total drug dose. Self-assembled peptide nanostructures have shown promising outcomes when used to deliver therapeutics to the brain, eye, cardiovascular system, bone tissue, and cancer cells. However, these delivery systems require further investigation before they will be suitable candidates for clinical studies.

Several challenges remain in the design, functionalization and development of peptide nanostructures as drug delivery vehicles. To successfully transition to clinical use, peptide nanostructures must have increased stability across a broader pH range and the ability to be manipulated to produce nanoparticles of a specific size. The relationship between the morphology of the self-assembled peptides, their physicochemical properties, and biological activity must also be explored in more detail [4]. Future experiments that employ rational systematic design will provide a deeper understanding of peptide self-assembly and allow the production of stable self-assembled peptides for drug encapsulation. This class of materials has shown a varying impact on the immune system, ranging from non-immunogenic (which is beneficial for drug delivery designs) to self-adjuvanting (which is favourable for vaccine delivery). Therefore, the immunogenicity of the self-assembled nanostructures designed for drug delivery needs to be investigated more thoroughly *in vivo* [4]. Peptide designs that generated nanofibrils and lipidated peptide amphiphiles conjugated to antigenic epitopes have shown promising results in the development of subunit vaccines. Peptides play a valuable role in the delivery and targeting of future drug and vaccine candidates. Further research is needed before the potential of this growing field can be translated to clinical application.

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