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Factors Affecting Secondary and Supramolecular Structures of Self-Assembling Peptide Nanocarriers

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Self-assembling peptides are a popular vector for therapeutic cargo delivery due to their versatility, tunability, and biocompatibility. Accurately predicting secondary and supramolecular structures of self-assembling peptides is essential for de novo peptide design. However, computational modeling of such assemblies is not yet able to accurately predict structure formation for many peptide sequences. This review identifies patterns in literature between secondary and supramolecular structures, primary sequences, and applications to provide a guide for informed peptide design. An overview of peptide structures, their applications as nanocarriers, and analytical methods for characterizing secondary and supramolecular structure is examined. A top-down approach is then used to identify trends between peptide sequence and assembly structure from the current literature, including an analysis of the drivers at work, such as local and nonlocal sequence effects and solution conditions.

1. Introduction

Self-assembling materials have been studied for potential biomedical uses since as early as the 1960s, following Richard Feynman's 1959 lecture proposing atom-scale material manipulation.[1,2] The idea of self-assembly was inspired by nature, as many cellular and molecular structures are formed via self-assembly, such as proteins, nucleic acids, and lipid membranes.[3] With the ability to assemble into uniform, reproducible nano- and microstructures, self-assembling materials have since been investigated for a wide variety of biomedical uses, such as drug delivery, regenerative medicine, and 3D cell culture.[4]

Self-assembly has become a term used for many processes in which individual components come together to form a supramolecular structure.[5] It is mediated by the formation of non-covalent secondary bonds and can occur between diverse types of monomers, including peptides, proteins, polymers, and even genetic material such as DNA.[6] While natural assembly processes sometimes require the assistance of chaperones or helper molecules, research over the last few decades has explored some materials that self-assemble independently, such as short peptides that can form supramolecular structures without helper molecules.[7] **Figure 1** illustrates a few types of self-assembling molecules and their assembly processes, including DNA origami particles, proteins, and peptide structures. Peptide-based self-assembling materials are especially promising for applications where biocompatible and biodegradable materials are necessary, since their primary building blocks are amino acids, making them naturally biocompatible. Because amino acids are bound by peptide bonds, they can be easily degraded via hydrolysis.[8] As peptides and proteins degrade, amino acids are recognized and used as metabolic fuel, and thus do not elicit an immune response.[9,10] This is an advantage over many polymers, which can be difficult to degrade and are often less biocompatible upon degradation.[11]

In addition to their biocompatibility, the ability of self-assembling nano- and micro-sized peptide structures to control and/or sustain drug release makes them ideal for use in drug delivery applications.[9] The highly tunable nature of peptide sequences enables loading of many classes of therapeutics via hydrophobic association or charge interactions. The tunability of the peptide sequence also allows the designer to adjust the drug release profile dependent on the rate of peptide degradation.[12] For example, enzymatic degradation can be used to control degradation rate of peptide assemblies. Specific amino acid sequences, or linkers, are recognized and degraded by enzymes such as Cathepsin B, and can be included in peptide sequences to trigger release of conjugated cargo.[13,14] Furthermore, multiple functionalities that aid in drug delivery can be combined into one peptide sequence, including targeting moieties, cell-penetrating sequences, or stimuli-responsive groups.[11,15,16] Depending on the sequence and desired cargo, self-assembling peptides can be applied in a wide range of systemic or local drug delivery applications.

Predicting peptide assembly structure is an important step in designing self-assembling peptide nanocarriers. However, many factors affect peptide self-assembly, including amino acid sequence, charge distribution, peptide concentration, solution pH, presence of salts, and thermal or mechanical stimulation.[17]

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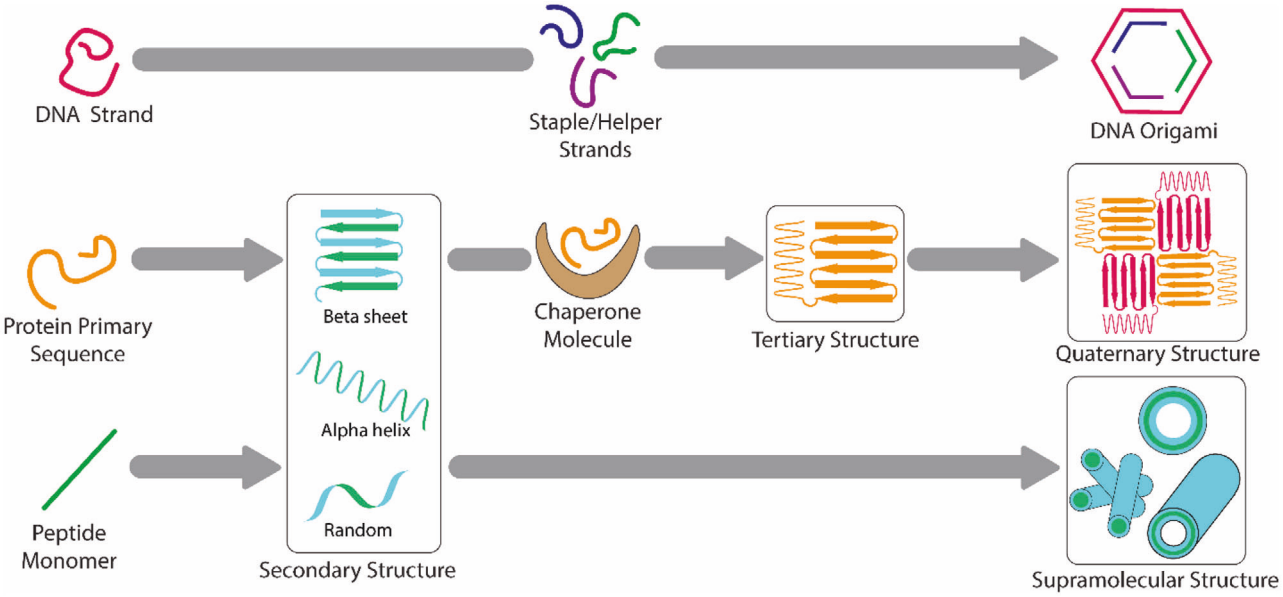

Figure 1. Self-assembly processes for DNA origami, proteins, and peptide assemblies. DNA strands assemble via association with staple or helper strands to form origami structures. Protein primary sequences assemble into secondary structures, then fold into tertiary and quaternary structures with the help of chaperone molecules. Peptide monomers self-assemble into secondary and supramolecular structures without the use of additional molecules.

Therefore, considering all of these factors, predicting peptide assembly using a bottom-up approach can be difficult. This review will first discuss common structures formed by self-assembling peptides and their applications as nanocarriers, then use a top-down approach to identify key trends between assembly factors and resulting supramolecular peptide structures. Identifying these patterns in the current literature is invaluable in expediting effective peptide design.

2. Primary Sequences

A peptide's primary sequence is analogous to the primary sequence of a protein, consisting of amino acids bound in a chain by peptide bonds.[18] Peptide sequences are typically reported as a string of one-letter amino acid abbreviations capped by end modifications. Additionally, alterations to amino acids such as L or D chirality are specified in the written sequence. Since amino acids have varying charge, hydrophobicity, size, and shape, altering the primary sequence of amino acids affects the folding of proteins and peptides into both their secondary structures and supramolecular assemblies.[19] While primary peptide sequence can consist of any order of amino acids, some specific patterns such as alternating charge or hydrophobicity and amphiphilic sequences are especially beneficial for promoting self-assembly and loading cargo for delivery.

2.1. Alternating Amino Acids

Alternating amino acid sequences consist of alternating hydrophobic and hydrophilic amino acids for much or all of the peptide sequence. These sequences are frequently used to create two planes of differing hydrophobicity that readily fold into

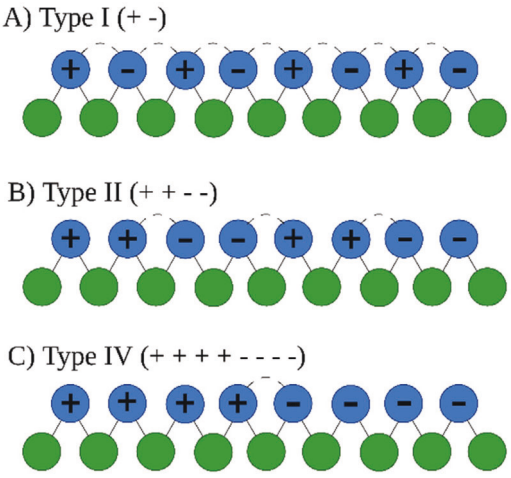

Figure 2. Peptides with alternating side groups. Blue indicates hydrophilic, charged side chains, and green indicates hydrophobic side chains. Peptide bonds are denoted with solid lines and electrostatic interactions are denoted with dotted lines. Created with BioRender.com.

beta sheet secondary structures. The hydrophilic side chains can also form complementary ionic bonds if the hydrophilic amino acids alternate positive and negative charges, stabilizing beta sheet formation.[17] **Figure 2** depicts several examples of alternating amino acid peptide sequences with differing charge patterns on the hydrophilic side chains. Types I, II, and IV correspond to one, two, or four hydrophilic amino acids of matching charge per block. Peptides with alternating amino acids, especially with alternating charges, typically form beta sheet or beta turn secondary structures by folding to align amino acid residues with matching hydrophobicity.[20] Beta sheets then stabilize via

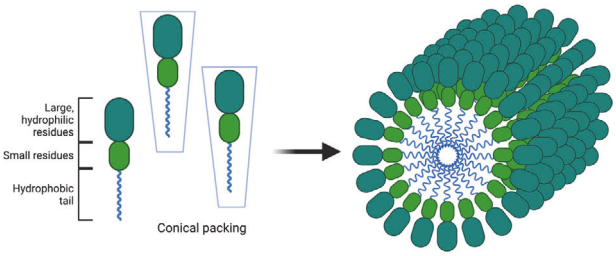

Figure 3. Amphiphilic peptides mimic lipid structures and consist of a hydrophilic head and a hydrophobic tail. Amphiphilic peptides assemble in aqueous solution to bury hydrophobic tails. Created with BioRender.com.

association between the hydrophobic peptide regions and electrostatic bonds between hydrophilic amino acids of opposing charge. Hydrophobic molecules can be loaded into the hydrophobic regions and protected during delivery, making these alternating amino acid structures especially ideal for hydrophobic drug delivery. For example, nanoribbons and hydrogels created using alternating amino acid peptide sequences have been demonstrated to load Nile Red dye and curcumin, both of which are fluorescent hydrophobic molecules used to model hydrophobic drugs.[21,22]

One of the most commonly used alternating peptide sequences is the RADA16 peptide. This sequence consists of a Type I alternating pattern of arginine, alanine, and aspartic acid repeated four times for a total length of 16 amino acids.[23] The RADA16 peptide assembles into beta sheet nanofiber hydrogels in aqueous solution and has exhibited sustained release of several different molecules and can be used as a scaffold for cell growth.[12,24,25] Three variants of the RADA16 peptide, RADA16-I, RADA16-DGE, and RADA16-PFS, with net neutral, negative, and positive charge, respectively, all self-assemble into nanofiber networks.[12] Each peptide hydrogel demonstrates sustained release of active cytokines for up to 2–3 weeks, with negative cytokines exhibiting more release from positive peptides and vice versa.[12] Additionally, a RADA16 fiber network functions similarly to Matrigel as an effective 3D cell culture scaffold, and enhances neural cell survival when functionalized with bone marrow homing motifs.[25] The extensive use of the RADA16 peptide for various applications shows the versatility and easily tunable nature of alternating peptide sequences.

2.2. Amphiphiles

Amphiphilic peptides are peptide sequences designed to have a hydrophobic and a hydrophilic end using blocks of amino acids. Similarly, peptide amphiphiles (PAs) are molecules produced with a hydrophilic peptide block attached to a hydrophobic molecule, such as an alkyl chain.[9,26] Both amphiphilic peptides and PAs are designed to mimic the structure of a lipid and are therefore well-suited to form lipid-like vesicles and micelles. PAs often exhibit alpha helix secondary structures that assemble into conical or cylindrical shapes, since the hydrophobic and hydrophilic ends do not interact, as shown in **Figure 3**. This conical structure enables the formation of vesicles, lipopeptides, or nanotubes.[27] Amphiphilic peptides are known as surfactant-like peptides and generally share similar features, including 1–2 charged amino acids in the hydrophilic head and four or more hydrophobic amino acids in the tail.[28] Peptides with this primary sequence consistently form vesicles and tubes with an average diameter between 30–50 nm.[28] The ratio of hydrophobic to hydrophilic groups in amphiphilic peptides is essential for determining how these peptides will self-assemble, but an ideal ratio has not yet been determined.[28,29] Short amphiphilic peptides are demonstrated to have increased stability, mechanical strength, and biocompatibility compared to longer peptide sequences.[30,31] Therefore, short amphiphilic peptide assemblies are better able to protect loaded cargo, making them especially beneficial as nanocarriers. Furthermore, due to the inclusion of both hydrophobic and hydrophilic regions, carriers formed from amphiphilic peptides are well-suited for co-delivery of multiple molecule types.

The ability to concurrently deliver multiple therapies is particularly advantageous in cancer treatment, where combination drug and/or gene therapy has been proven to reduce the effects of multidrug resistance.[32,33] For example, the amphiphilic peptide sequence R3V6 (RRRVVVVVV) has been used to create self-assembling peptide micelles.[34] These micelles were coloaded with bis-chloroethylnitrosourea (BCNU) in the hydrophobic core and small interfering RNA targeting vascular endothelial growth factor (VEGF-siRNA) was electrostatically bound to the positively charged arginine shell.[34] The peptide micelles were able to deliver both BCNU and VEGF-siRNA more effectively than either molecule alone.[34] These findings exhibit the potential of amphiphilic peptide assemblies for efficient co-delivery applications.

3. Secondary Structures

Peptide secondary structure refers to the local interactions between amino acids in the peptide chain primary structure. Weak, non-ionic interactions between amino acids cause peptides to fold into unique combinations of secondary structures. Among all the potential non-ionic interactions, the hydrophobic effect generally dominates.[35] The hydrophobic effect results in a favorable increase in entropy, which is the major thermodynamic driving force for the organization of hydrophobic groups in aqueous solution. When a hydrophobic molecule is introduced into water, the optimal arrangement of hydrogen bonds results in grouping of hydrophobic amino acids in the interior, forming a hydrophobic core with a hydrophilic exterior.[35] Additionally, side chains must be positioned in a manner that minimizes their steric interference, leading to distinct R group orientation for each secondary structure.[36] Alpha helix and beta sheet peptide secondary structures dominate under physiological conditions because they are the most thermodynamically stable.[35,36] When an alpha helix or beta sheet pattern is not identified, the secondary structure is referred to as a random coil or irregular structure.[35,36] These turns and loops can create irregular or random secondary structure and typically are the links between alpha helices and beta sheets.[35] Most proteins consist of a combination of regular and irregular secondary structures.[36]

3.1. Alpha Helices

Alpha helices are comprised of a peptide backbone tightly wound around an imaginary axis with the R groups of the amino

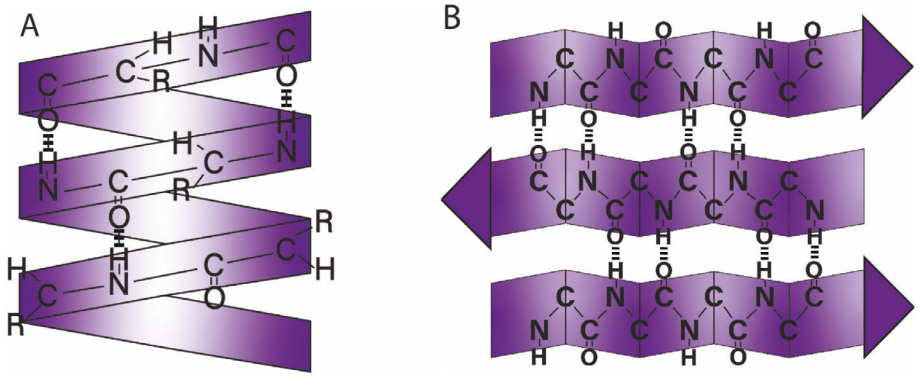

Figure 4. A) Alpha helix and B) beta sheet secondary structures.

acid residues protruding outward from the helical backbone, as shown in **Figure 4**A. There are 3.6 amino acids per turn, and most alpha helices are about 12 amino acids long.[35–37] Hydrogen bonds between the carbonyl oxygen atom and the hydrogen atom in the amine group stabilize the structure.[35] Coiled coils are composed of two or more alpha helices intertwined in a supercoil arrangement.[35] This structure is commonly found in tissues and cells and has many different roles.[38] Additionally, the majority of coiled coils possess a repetitive sequence pattern known as a heptad repeat. A heptad repeat is composed of seven amino acids denoted *abcdefg*, where hydrophobic or nonpolar residues are located at the "*a*" and "*d*" positions, leading to an (*NPPNPPP*)n pattern.[37] Coiled coils have been implemented in the design of self-assembling fibers, hydrogels, and nanoparticles for use in tissue engineering, drug delivery, and antigen display.[37] Conversely, alpha helix bundles consist of multiple helices folded parallel or antiparallel to one another but do not fulfill the packing and sequence criteria of coiled coils.[35] However, helix bundles have structural flexibility that makes them good candidates for the binding of small molecules.[37]

The 20 amino acid anionic peptide sequence GLFEALLELLESLWELLLEA is an example of a sequence that folds into alpha helix secondary structures in aqueous solution.[37] When added to a solution of the cationic K16 peptide, the mixture of both peptides assembles into nanoparticles.[39] The stable nanoparticles have potential for targeted delivery of single or multiple therapeutics into the cytosol via endocytosis.[37] Many surface proteins of pathogens have coiled coil secondary structure. A 64-amino acid peptide sequence was developed to mimic an antigen display pattern by replicating this structure.[38] The peptide folds into coiled coils with cysteine residues at the *f* position of heptad repeats.[38] The cysteine residues form a disulfide bond to stabilize the structure, which then assembles into polyhedral nanoparticles.[40] The coils can be engineered to display immunogenic sequences, making these nanoparticles ideal for immunization technologies.

3.2. Beta Sheets

A beta sheet (or beta-pleated sheet) is formed by arranging several beta strands side by side.[35] Beta strands are created by folding the backbone of a peptide chain into a zigzag. The beta strands can then be arranged in two ways: in a parallel beta sheet, neighboring strands run in the same direction, and in an antiparallel beta sheet, neighboring strands run in opposite directions, as shown in Figure 4B.[35] Each amino acid forms two hydrogen bonds with a neighboring strand so that hydrogen bonds stabilize the entire secondary structure.[36] The R groups of adjacent amino acids protrude from the zigzag structure in opposite directions. Beta sheet structured peptides commonly self-assemble into fibers, hydrogels, and nanoparticles that have been used in tissue engineering and drug delivery.[37]

Two examples of sequences that self-assemble into beta sheets are RADA16 and CG3RTAT.[22,39] RADA16 peptides fold into beta sheets and subsequently form nanofibers and fibrillar hydrogels. The stable beta sheet structure makes RADA16 a great candidate to form hydrogels, and the structure has shown efficient controlled release of hydrophobic drugs.[22] The CG3R6TAT peptide sequence assembles into beta-sheet nanoparticles.[39] CG3R6TAT is amphiphilic and can cross the blood brain barrier.[39] It has also been shown to be an effective antimicrobial agent.[39]

4. Supramolecular Structures

The supramolecular structure of peptide assemblies is comparable to the quaternary structure of proteins. Groups of secondary structures associate based on charge and hydrophobic effect, creating the final peptide assembly which can load drugs and other cargoes. Common supramolecular structures are depicted in **Figure 5**. There are advantages and disadvantages of each supramolecular structure depending on the nanocarrier application. Therefore, understanding characteristics of various supramolecular structures and their corresponding applications is essential for designing self-assembling peptide nanocarrier systems.

4.1. Nanospheres

Nanospheres or nanoparticles are highly versatile and can be used to deliver a wide variety of cargo such as drugs, contrast agents, or genetic molecules.[41] Peptide nanospheres can take the form of vesicles or micelles as shown in Figure 5A.[11,20] Vesicles are formed by self-assembly of amphiphilic molecules

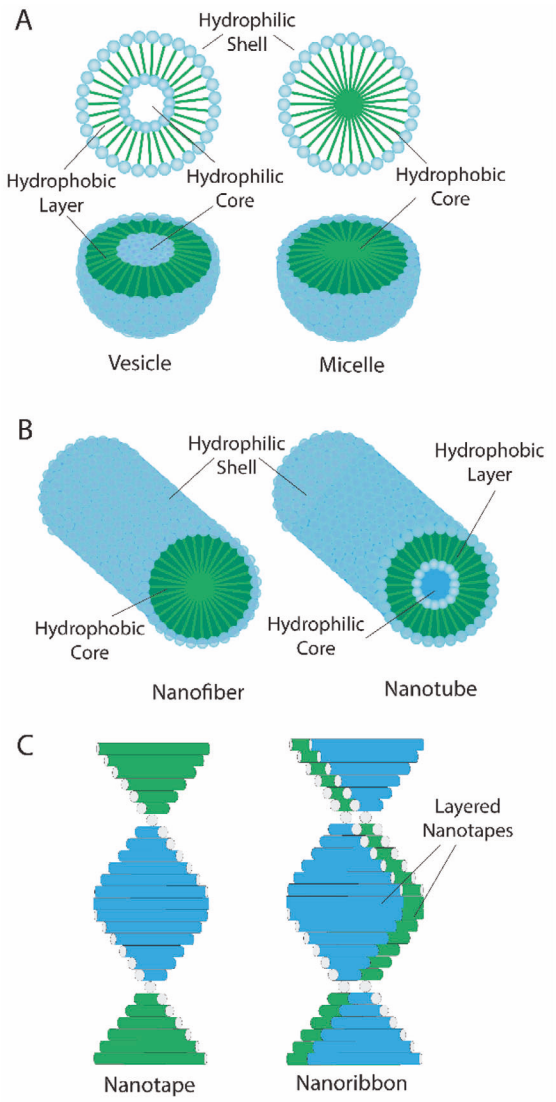

Figure 5. Common supramolecular structures formed by self-assembling peptides. A) Depictions of vesicles and micelles, B) nanofibers and nanotubes, and C) nanotapes and nanoribbons formed with nonspecific peptide monomers. Green depicts hydrophobic regions and blue depicts hydrophilic regions.

into a bilayer, encapsulating aqueous solutions in the core and providing a hydrophobic loading region within the bilayer. Micelles are created with a monolayer of amphiphiles, encapsulating a hydrophobic loading region. These structures are typically formed with PAs or amphiphilic peptides, as these sequences are driven to bury the hydrophobic blocks internally during self-assembly.[28] Peptide nanospheres can range in size from less than ten nanometers to a few hundred nanometers in diameter.[42,43] The potential to load and deliver drugs using self-assembling vesicles and micelles is extensive; vesicles can encapsulate hydrophilic drugs or genetic material in the aqueous core and load hydrophobic drugs in the bilayer, while micelles can load hydrophobic drugs in the core and potentially immobilize charged cargoes on the outer shell. Additionally, these nanoparticle structures can be functionalized by binding aptamers, antibodies, or peptides with targeting, cell-penetrating, stimuli-responsive, or other capabilities to the nanoparticle's outer shell. Functionalization can decrease off-target effects and increase drug accumulation in target sites for therapeutic applications.[44]

Because of their small size and versatility for loading and functionalization, peptide nanoparticles are especially advantageous for systemic delivery of molecules such as chemotherapy or tumor imaging contrast agents. The short peptide NH_2-Leu-Aib-Tyr-COONa can be functionalized to gold nanoparticles, enabling pH-sensitive self-assembly at pH = 4.[45] These nanoparticle assemblies can be used for pH-sensitive in vivo imaging. Additionally, nanoparticles are capable of co-loading multiple agents/therapeutics into regions of differing hydrophobicity. For example, H3SSgT is an amphiphilic peptide that self-assembles into uniform nanoparticles in water.[33] These nanoparticles can co-load and efficiently deliver a model drug and oligonucleotide.[33] Nanoparticles are therefore an effective supramolecular structure for delivery of single or multiple cargoes systemically or locally.

4.2. Nanofibers and Nanotubes

Nanofibers and nanotubes as well as nanofiber matrices, networks, and scaffolds, have been used for drug delivery, tissue regeneration, and 3D cell culture development.[25,46,47] A nanofiber is an extended micelle, while a nanotube is an extension of a vesicle,[11] and both assemblies often exhibit beta sheet secondary structures.[23,24,48–50] Fibers and tubes often form at higher peptide concentrations when it is energetically favorable for monomers to add to micelles or vesicles in a nucleation-expansion kinetic pattern.[51] Additionally, some fibers and networks exhibit dynamic reassembly over time in an aqueous solution after being broken apart using sonication.[4] Instead of forming single fibers, nanofibers, and nanotubes often crosslink electrostatically to create networks, scaffolds, or gels.[52] Nanofibers are some of the most common supramolecular structures for self-assembling peptides. Various peptide sequences that form beta sheets have been demonstrated to assemble into fibers; therefore, it is difficult to precisely determine which peptide sequence properties may prompt a fiber structure.[11]

Peptide fibers, tubes, and networks exhibit potential for drug encapsulation and delivery. In particular, self-assembled TAT nanofibers efficiently load and deliver paclitaxel by binding the drug in the inner hydrophobic regions of the assemblies in aqueous solutions.[46] Nanotubes can be formed using enantiomeric pairs of cyclic peptides, in which case the diameter of the tube can be controlled by the peptide length.[50] These tubes can be used for artificial channel formation but also show promise for encapsulating drugs into their core.

4.3. Nanoribbons

Nanoribbons are less common self-assembling structures with temperature stable properties and applications in hydrophobic drug delivery and targeting.[21,53] Ribbons consist of two layered nanotapes, comprising beta sheet-forming peptides stacked together and twisted according to amino acid chirality,[11] as shown

in Figure 5C. Peptides with alternating amino acid primary structure can form layered ribbons stabilized by the hydrophobic interactions between sheets.[21] Hydrophobic molecules can be loaded into the hydrophobic regions inside the ribbons and kept stable during delivery, making the assembled structures ideal for delivery of hydrophobic drugs. The TβP peptide consists of three regions: a TAT block, a flexible linker, and a beta-sheet assembly block.[21] In a salt solution, these peptides assemble into nanoribbons which are capable of loading hydrophobic molecules pyrene and nile red.[21] Although nanoribbons are less common, they exhibit potential for effective delivery of hydrophobic cargo and could be explored further to determine benefits or drawbacks compared to other supramolecular structures.

4.4. Hydrogels

Self-assembling peptide hydrogels can be nano-, micro-, or even macroscale and are used in drug delivery, 3D cultures, regenerative medicine, and tissue engineering. Often, self-assembled hydrogels are composed of a combination of secondary structures, including alpha helices and beta sheets.[54] Hydrogels can also consist of other supramolecular structures, such as nanofibers or nanotubes.[49] Electrostatic interactions or hydrogen bonds between fibers or tubes can result in the formation of micro- or macro-sized matrix hydrogels. Self-assembling hydrogels have unique benefits compared to crosslinked synthetic polymer hydrogels; due to their amino acid-based structure and hydrolytic degradability, peptide hydrogels are more biocompatible than polymer hydrogels. For example, self-assembling hydrogels can be safely injected without the need for crosslinking agents,[55] which can be harmful to surrounding tissue due to toxicity of unreacted monomers or radiation damage from photopolymerization.[22] Additionally, the tunable nature of peptide sequences allows for easy manipulation of hydrogel properties such as charge, stiffness, and degradation rate.

Peptide hydrogels can vary widely in characteristics such as secondary structure, size, and loading capabilities. For example, the MAX8 peptide forms a nanofiber network hydrogel capable of loading the hydrophobic anticancer molecule curcumin.[22] Alternatively, nanogels can be formed with the Fmoc-FF dipeptide using an inverse emulsion method.[41] These nanogels can load gold nanoparticles, doxorubicin, and 5-flourouracil.[41] Furthermore, the addition of an aromatic group to various pentapeptide sequences often initiated the assembly of a nanofiber-based hydrogel.[49] Because there is so much variation in hydrogel structure, there is virtually limitless potential for loading and functionality.

5. Methods for Determining Peptide Structure

The structural characteristics of peptide nanocarriers directly relate to their function and efficacy. Characterization of self-assembling peptides includes analysis of secondary structure, supramolecular structure, size, and surface charge. Understanding the information gained from these characterization methods can provide insight into how nanocarriers interact with cargo and their target environment, allowing for the precise design of peptide assemblies for their intended application.

5.1. Circular Dichroism

Circular dichroism (CD) is commonly used to determine the secondary structure of peptides and proteins. CD is an absorption spectroscopic technique that uses left- and right-handed polarized light to interact with chiral molecules.[56] Because different conformations of proteins and peptides absorb left- and right-handed circularly polarized light differently, CD can be used to determine the secondary structure of these molecules.[57] Most laboratory-based CD instruments work in the lower UV wavelengths, between 190 and 310 nm, which are capable of identifying secondary and some supramolecular structures.[56,57] Alpha-helical proteins typically exhibit a positive band at 193 nm and negative bands at 222 and 208 nm.[58] Beta sheets are characterized by a positive band at 195 nm and a negative band at 218 nm.[58] Proteins with random or disordered secondary structures display low ellipticity above 210 nm and a negative band near 195 nm.[58]

Given that the CD spectra for a molecule is a linear combination of the individual secondary structures, CD data can be analyzed using a variety of empirical methods and algorithms that quantify secondary structure.[59] Several algorithms, including CONTINILL, CDSSTRA, and SELCON3 are available for public download; however, to achieve the most accurate determination of secondary structure, all three algorithms must be compared using performance indices.[57,59] For this reason, web-based software such as DICHROWEB, DicroMatch, and Capito have been developed to provide more comprehensive CD analysis platforms.[57,60,61]

5.2. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is categorized as a low-resolution spectroscopy method similar to CD that provides information about the secondary structure of proteins but cannot locate three-dimensional structural elements.[62] FT-IR is a non-destructive method that can determine secondary structure based on the molecule's vibrational frequencies.[63] There are nine characteristic group frequencies resulting from polypeptide structures (amides A, B, and I–VII), with amide I ($\approx$1650 cm^{-1}) bands being the most sensitive.[63,64] Alpha helices can be observed at frequencies in the region from 1650–1660 cm^{-1}, and beta sheets in the frequency range from 1630–1640 cm^{-1}.[65] In order to quantitatively determine the presence of secondary structures, FT-IR instrument manufacturers use curve fitting of the deconvoluted or second derivative spectra to identify the secondary components of the protein.[63] Similar to CD, the secondary structure is reported as a percentage found in the analyzed sample as a whole. A distinct advantage of FT-IR is its ability to rapidly acquire high-quality spectra from aqueous polypeptide samples of less than 100 μg.[62,65] Thus, for analysis of small concentrations of peptides not suitable for CD, FT-IR is a more appropriate analysis method to determine the secondary structure.

5.3. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy provides information about the atomic structure of molecules based on the

magnetic spin of certain atomic nuclei, such as ^{1}H and ^{13}C.[66] When the core of a sample is perturbed, it absorbs electromagnetic radiation at different frequencies, called chemical shifts, that are compared to a standard.[66] Recent advances have allowed NMR chemical shifts to be used for identifying the secondary structures of protein sequences of up to 130 amino acids when in solution or a solid state.[67] Using the chemical shift index (CSI) analysis, alpha-helices, random coils, and beta strands are assigned the values of −1, 0, and 1, respectively.[67] Another technique, known as the secondary structure propensity (SSP) method, assigns alpha helices a value of 1 and beta sheets a value of −1, with fractions indicating a partially formed alpha helix or beta sheet structure.[68] Chemical shifts can also provide information about the supramolecular structure of a protein when used with other NMR probes that report interproton distances and the orientations of nuclei in a protein structure.[69] NMR can be used to characterize a wide variety of peptide assemblies and has even been used to develop a structural model to better understand how specific peptide sequences form nanofibers.[23]

5.4. Dynamic Light Scattering

Unlike microscopy techniques that measure single-particle sizes, dynamic light scattering (DLS) is a non-destructive method able to measure the distribution of particle sizes in a sample. A laser is directed at the peptide solution, and the light scatters as it interacts with particles. The scatter angles are recorded by a detector and can be used to determine the size and homogeneity of peptide assemblies at a specific temperature and viscosity.[70] Certain restrictions limit the applicability of DLS, such as the need for optimized concentrations as well as transparent and monodisperse samples. The low resolution of DLS cannot distinguish between very similar particles, making this method unsuitable for multi-assembly or polydisperse solutions.[70] DLS has been used for characterization of a wide variety of peptide nanostructures, including peptide-carbon nanotube hybrids,[71] nanofiber scaffolds and hydrogels,[24,25,49] and nanoparticles.[42]

5.5. Microscopy

Multiple microscopy methods can be used to characterize self-assembling structures, including atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). AFM is used to study the surface topography and morphology of structures. A probing tip is attached to a cantilever spring, and images are produced by scanning the sample with the probing tip.[72] The deflection in the z-direction of piezoelectric material is then digitized as a function of the x and y positions.[72] AFM can be used for individual particle characterization but also is able to resolve complex particle size distributions.[73] AFM is commonly used to measure the height of nanostructures, especially nanoribbons and nanofibers, that are 10 nm or less in thickness.[23,74] Compared to techniques such as SEM and TEM, AFM is more cost-effective and offers comparable resolution.[73]

SEM is a type of electron microscopy that generates images using a high-energy beam of electrons directed at a sample's surface.[75] When these electrons interact with a sample, a signal is produced, providing a description of the surface topography and morphological characteristics of the sample.[75] Structures imaged with SEM must be deposited on a film or a silicon wafer, and samples that are not inherently conductive must be coated with a conductive metal such as gold, palladium, or platinum.[76] SEM is best suited for imaging nanoparticles or nanofibers in the 10 to 1000 nm diameter range.[76] Additionally, SEM can be used to examine surface morphology and shape of hydrogels.[77]

TEM offers a higher resolution than SEM and can image smaller nanostructures between 1 and 1000 nm, making it suitable for characterizing internal structures of nanoparticles.[76] Particle and powder samples are usually deposited and imaged on TEM grids available with different thicknesses and mesh sizes made out of various metals, including copper, nickel, and gold.[78] Cryo-TEM can be used for imaging soft nanoparticle structures and nanostructured liquids.[79] This TEM method is especially beneficial for samples with diverse morphologies, sizes, and complex internal structures but requires additional preparation and analysis that contributes to its higher cost.[79,80] Electron microscopy techniques can be used to determine the size and morphology of common nanocarrier supramolecular structures including peptide nanoparticles, nanoribbons, and hydrogels.

Confocal laser scanning microscopy (CLSM) is another microscopy technique used to visualize secondary and supramolecular peptide assemblies.[81] Unlike the previous described microscopy techniques, CLSM relies on the addition of a fluorescent molecule into the structure of interest for visualization and does not require a conductive coating or costly sample preparation. Fluorophores can either be conjugated directly to the peptide monomer sequence, added as a fluorescent dye during the assembly process, or used to stain the structure post-assembly.[82,83] Recently, based on the principle that self-assembling structures form a hydrophobic core, confocal microscopy has been used to screen the ability of different peptide sequences to self-assemble by using the fluorophore nitro-1,2,3-benzoxadiazole, which fluoresces in hydrophobic environments.[82] Although CLSM has a higher resolution compared to traditional epifluorescence or wide field fluorescence microscopy, super resolution microscopy (SRM) techniques have been developed with a spatial resolution of less than 100 nm. The main disadvantage of SRM is that fluorophores must have "photo switching" ability that allows them to switch from "off" to "on" states in the presence of light, so not all fluorophores compatible with traditional CLSM can be used with SRM.[84,85] New technologies such as the Zeiss Airyscan detector provide an increased signal-to-noise ratio without the use of specific dyes to achieve super resolution, making this a popular technique to image self-assembling structures.[86–88]

6. Effect of Peptide Sequence on Structure

Defining the amino acid sequence is commonly the first step when designing peptide assemblies for any application. Local effects, nonlocal effects, charge distribution, and aromatic residues are important factors to consider when determining peptide self-assembly behavior. Local effects are the amino acid

Table 1. Examples of peptide sequences that form specified supramolecular and secondary structures. For charge and aromatic distributions, abbreviations are defined as follows: P = polar uncharged, N = nonpolar, + = positively charged, − = negatively charged, R = aromatic structure.

Supramolecular structure	Secondary structure	Peptide sequences	Charge and aromatic distribution	Ref.
Amyloid fibers	50% parallel beta sheets, 20–40% disordered or random	NYNYNYN	PRPRPRP	[48]
		QYQYQYQ	PRPRPRP	
		SYSYSYS	PRPRPRP	
		GYGYGYG	PRPRPRP	
		NYNNYN	PRPPRP	
		QYQQYQ	PRPPRP	
		SYSSYS	PRPPRP	
Nanofibers	Antiparallel beta sheet	Ac-RADARADARADARADA-$CONH_2$	+N-N+N-N+N-N+N-N (Alternating Type I)	[24]
	Parallel beta sheet	$COCH_3$-RADARADARADARADA-$CONH_2$	+N-N+N-N+N-N+N-N (Alternating Type I)	[23]
	Beta sheet (direction unspecified)	RADARADARADARADA	+N-N+N-N+N-N+N-N (Alternating Type I)	[25,90]
		AEAEAKAKAEAEAKAK	N-N-N+N+N-N-N+N+ (Alternating Type II)	
		AEAEAEAEAKAKAKAK	N-N-N-N-N+N+N+N+ (Alternating Type IV)	
Fibrous hydrogel	Beta sheet (direction unspecified)	VKVKVKVKVDPPTKVEVKVKV-NH_2	N+N+N+N+N-RRP+N-N+N+N	[49]
		GAGASF	PNPNPR	
		GAGASP	PNPNPR	
		YGFGGF	RPRPPR	
	Beta turn	VTEEIF	NP–NR	
	Alpha helix	VTEEIP	NP–NR	
Ribbons	Random coil and beta turn	GVPVPF	PNRNRR	
Nanotubes	Antiparallel beta sheet	cyclo[-(L-Gln-D-Tle-L-Glu-D- Tle)$_2$-] plus cyclo[-(D-Gln-L-Tle-D-Glu-L-Tle)$_2$-]		[50]
Nanoparticles	Beta sheet	CGGGRRRRRRYGRKKRRQRRR	PPPP++++++RP+++++P+++	[42]
	Alpha helix	GLFEALLELLESLWELLLEA	PNR-NNN-NN-PNR-NNN-N	[39]
	Coiled coils	Ac-DEMLRELQETNAALQDVRELLRQQVKQIT FLKCLLMGGRLLCRLEELERRLEELERRLEELERR-NH_2	–NN+-NP-PPNNNP-N+-NN+PPN+PNPRN+PNNNPP+NNP+(N–N-++)$_3$	[40]

residues' intrinsic tendency to self-assemble into specific secondary structures.[89] For example, inclusion of amino acids with aromatic groups can lead to stabilizing interactions during peptide assembly that affect supramolecular structure.[48,49] In comparison, nonlocal effects are the positioning of the individual amino acids in the sequence and the impact of attractive forces between amino acids allowing for precise chain folding.[89] A significant contribution to nonlocal effects is the periodicity of polar and nonpolar residues.[89] It was shown that the periodicity of a sequence is more important than the intrinsic tendencies of individual residues to form alpha helices or beta strands in self-assembling oligomeric peptides.[89] Secondary and supramolecular structures can also be modified through rearranging residues in the sequence to produce different charge distribution patterns,[90] and sequences can be designed to include reactive side chains, which allow for crosslinking and chemical modification.[91] The structure formation trends due to local and nonlocal effects of peptide sequence are described in this section and summarized in **Table 1**, comparing peptide charge and aromatic distribution, secondary structure, and supramolecular structure.

6.1. Local Effects

Peptide sequence local effects determine secondary structure based on the partiality of each individual amino acid to form particular structures. The predisposition of an amino acid to form specific structures can be determined by analyzing structures of natural proteins containing the residue of interest; for example, leucine, glutamic acid, and lysine have been determined to exhibit intrinsic alpha helical properties.[89] Therefore, amino acid sequences can be chosen specifically for the individual residues' intrinsic properties to produce desired structures.

The amino acid sequence of designer peptide RADA16[23–25] was selected due to its reliable reproducibility of beta sheet nanofibers. The sequence has alternating charged and hydrophobic residues, which promotes beta sheet formation. The RADA16

nanofibers are composed of two stacked beta sheets comprised of parallel beta strands, and the structure is stabilized by positive arginine residues staggered with negatively charged aspartic acid residues.[23] The nonpolar alanine residues form the hydrophobic interior, while the polar arginine and aspartic acid residues form the fiber's surface.[24] The RADA16 peptide is used to create nanofibrous networks for tissue regeneration, three-dimensional cell culture, and drug delivery.[23,24] The MAX8 peptide also forms a solid hydrogel under physiological conditions to be used as an injectable delivery vehicle for curcumin.[20] The four residue sequence $V^{D}PPT$ is repeated to create a stable 20 amino acid peptide and adopts a type II beta turn secondary structure.[22] The MAX8 peptide is stabilized by intrastrand hydrogen bonding and consists of hydrophobic valine and hydrophilic lysine residues on opposite faces of the beta hairpin. Fibril formation is caused by hydrophobic side chain interactions and lateral hydrogen bonding along the fibril axis, which produces the mechanical rigidity of the hydrogel.[22] Additionally, studies have shown that small changes in the amino acid sequence can affect the formation of the supramolecular structure. One study demonstrated that a difference of as little as two amino acids could change the assembly of the X4-2-6 peptide, a transmembrane antagonist of the CXCR4 chemokine receptor, which forms nanoparticles. After the removal of two *N*-terminal leucine residues, the peptides assembled into fibrils instead.[92] The sequence was then modified with polyethylene glycol extensions at the C-terminus, successfully preventing aggregation without interfering with self-assembly.[92] The X4-2-6 self-assembled nanoparticles inhibited CXCR4 function in vitro, which hampered CXCR4- dependent tumor metastasis in vivo, and enabled encapsulation of hydrophobic drugs, providing a dual-action delivery system.[92]

When designing de novo peptide sequences it can be useful to consider local effects of individual amino acids by selecting amino acids with a higher propensity for the desired structure. However, it is difficult to accurately predict secondary or supramolecular structures based solely on local effects. Aromatic groups are one exception to this statement.

6.2. Aromatic Groups

Aromatic groups are amino acids with ring structures on their side chain and are commonly used to form hydrogels since aromatic-aromatic interactions are stronger than forces between alkyl chains.[49] Intermolecular aromatic-aromatic interactions improve hydrogel self-assembly in aqueous solution; the addition of aromatic groups to a peptide sequence results in hydrogelation of nanofiber networks, providing a stabilizing force and resulting in stable hydrogels.[49] Aromatic groups have also been found to contribute to the self-assembly of amyloid fibers.[48] Amyloid fibers are protein assemblies formed naturally in the body. Polar and uncharged amino acids such as glutamine, asparagine, tyrosine, serine, and glycine, known as prion domains (PrDs), are necessary for forming these amyloid fiber structures and contribute to both inter- and intrasheet stabilization.[48] Aromatic interactions using tyrosine have been successfully exploited to form tailored prion-inspired nanostructures.[48] These prion-inspired nanostructures have been used in tissue engineering, drug delivery, adhesive materials, biodegradable nanocomposites, nanowires, and biosensors.[93]

Including aromatic groups in peptide sequences lends a stabilizing force to secondary and supramolecular structures.[49] The pattern of aromatic groups in a peptide sequence may induce different supramolecular structures. Using many aromatic residues in an alternating pattern exhibits beta sheet amyloid fiber formation,[48] including only one or two aromatic residues at the end of a sequence leads to hydrogel formation,[49] and using a few aromatic residues near the center of a long sequence may encourage nanoparticle formation.[39,40,42] Secondary structures in hydrogels and nanoparticles with aromatic groups include beta sheets, alpha helices, and coiled coils. Therefore, the use of aromatic groups in self-assembling peptide sequences should be considered for amyloid fiber, hydrogel, or nanoparticle formation dependent on the number and pattern of aromatic residues.

6.3. Nonlocal Effects

While each individual amino acid has an intrinsic tendency to affect the peptide structure, the placement of the residues in a sequence dominates the forces surrounding the self-assembly of peptide structures. The order of amino acids determines the class of the peptide sequence, such as alternating sequences or amphiphilic peptides. Hydrogen bonding is a primary driver for the assembly of peptide sequences, creating a buried hydrophobic core and outer hydrophilic layer in aqueous solutions. This phenomenon is known as the hydrophobic effect; when introduced into water, peptides with both polar and nonpolar regions have the natural tendency to self-assemble via hydrophobic interactions.[94] Therefore, the pattern of polar and nonpolar amino acids largely affects the peptide folding and supramolecular assembly. Additionally, electrostatic interactions between charged residues contribute to determination of the secondary structure and stabilization, especially in beta sheet structures.[95]

For example, the 16-carbon sequence with the motif VEVE ($C_{16}H_{31}$OVEVE) self-assembles into flat nanobelts to deliver epitopes to cells for immunotherapies.[94] VEVE adopts a beta strand structure, but the hydrophilic and hydrophobic side chains are flipped to the opposite sides of the peptide backbone, causing a loss of curvature and lateral growth.[94] When the nonpolar valine and charged polar glutamic acid residues are rearranged to create a new motif with the sequence VVEE, the nanostructures gain curvature, forming cylindrical nanofibers under the same conditions.[94] Furthermore, a peptide sequence comprised only of amino acids with intrinsic propensities for alpha helix formation but arranged in a beta-sheet favoring pattern will form a beta sheet.[89] The peptide sequence EAK16 (AEAEAKAKAEAEAKAK) is made from alpha-helix favoring residues but reliably forms beta sheet secondary structures because of the alternating charge and polarity pattern.[17,90,96] Depending on salt concentration, EAK16 can associate into a macroscopic membrane composed of interwoven filaments or nanofibers that can be used as biological scaffolds in tissue engineering applications.[90,96]

Trends in the literature suggest that including regions of alternating amino acids predisposes peptides to form beta sheet secondary structures and nanofiber supramolecular

structures. Amino acids with differing polarity, charge, and aromatic structures can be alternated to achieve beta sheet interaction, and charge alternation is most effective to create nanofiber assemblies.[23–25,48,49,90,96] Predicting alpha helix secondary structure based on local effects is less clear; sequences that form alpha helical or coiled coil secondary structure typically consist of a complex pattern of nonpolar and aromatic groups, with charged or polar groups included in varying amounts.[39,40,49] Self-assembling peptides with alpha helical secondary structures are often derived from naturally-occurring peptides with similar structure instead of being designed de novo.[39] Therefore, due to the lack of a clear trend between primary sequence, local effects, and alpha helical secondary structure, sequences derived and altered from nature may be the most effective method to design functional alpha helical sequences.

6.4. Charge Distributions

Charge distributions are essential to consider when predicting the secondary and supramolecular structures of self-assembling peptides because they supersede intrinsic tendencies to mediate peptide folding. Positive and negative charges control which amino acid blocks are drawn together through electrostatic forces and can also determine for which applications sequences can be utilized. For example, highly cationic sequences can create cytotoxicity, limiting in vivo applications. One way to control the charge of the sequence is through *N*-terminal acetylation and C-terminal amidation at the ends of a peptide. Both are used to neutralize charges and mimic proteins to prevent degradation caused by exopeptidases in vivo.[48,91] Nanospheres, in comparison to nanofibers, form when there is a greater imbalance of charge; additional negative charges on the *N*-terminal help peptides self-assemble into spherical nanoparticles with reproducibility.[92]

The most common way to affect charge distribution is by changing the pattern of charged peptides throughout the sequence. EAK16 commonly has a Type II charge distribution (–++–++) and forms fibrillar structures. If this sequence is altered to become EAK16-IV, with a Type IV charge distribution (—-++++), the fiber structure is no longer observed; instead, the sequence forms globular assemblies.[90] The TAT (YGRKKRRQRRR) sequence is a cell-penetrating peptide that allows membrane translocation and was first derived from the transactivator of transcription protein (TAT) in the human immunodeficiency virus (HIV).[42] The peptide amphiphile CG3R6TAT is comprised of the hydrophilic TAT sequence, six arginine residues that increase the cationic charge to improve membrane translocation, and a hydrophobic block of cholesterol to promote self-assembly.[42] The cationic charge and hydrophobic cholesterol block drive assembly of the peptides into nanoparticles with the TAT sequence organized on the outer surface.[42] The CG3R6TAT peptide nanoparticles were used as an antimicrobial agent in the brain due to their ability to cross the blood-brain barrier.[42]

When considering charge in de novo peptide design, it is important to consider end terminal modifications and location of charged amino acids in the sequence. To minimize degradation in vivo, both ends should be neutralized via *N*-terminal acetylation and *C*-terminal amidation.[48,91] Addition of negative charge on the *N*-terminal of a peptide sequence can induce nanoparticle formation, and alternating positive and negative charges can prompt beta sheet nanofiber formation.[23–25,90,92]

7. Effect of Peptide Assembly Conditions on Structure

Methods for creating peptide self-assemblies vary widely. Similar peptides under different conditions can form very different assemblies, including alpha helix and beta sheet secondary structures and nanofiber, nanoparticle, and nanotube supramolecular structures.[24,42,48,50,89,92] Considerations for solution preparation such as the addition of salts or buffers, manipulation of pH and temperature, and physical processing have been shown to affect peptide assembly patterns and can therefore be used as another tool to predict and modulate peptide assembly structures.[20] The following section and **Table 2** summarize methods commonly used for peptide assembly and how each of these conditions affects peptide structure.

7.1. Salts and Buffers

Salts or buffers are often added to peptide mixtures to initiate or improve peptide assembly via the Hofmeister effect. The Hofmeister effect states that high concentrations of anions and cations in a solution can impact the hydrophobic interactions that drive peptide and protein folding;[98] these ions are known as kosmotropes and chaotropes, and can be intentionally added to solutions to improve or disrupt peptide and protein interactions. Kosmotropes are anions that are highly water-soluble and enhance hydrophobic interactions, improving peptide assembly. In contrast, chaotropes interfere with hydrophobic interactions in an aqueous environment and destabilize peptide assemblies.[99] Phosphate buffered saline (PBS) is a kosmotrope commonly added to peptide solutions to trigger assembly.[23,39,48,96] While peptide secondary structure is largely unaffected by the addition of salts, the inclusion of different salts has been shown to alter the supramolecular structure of peptide assemblies, as illustrated in **Figure 6**.

An aromatic dipeptide, FmocYL, was examined in three different salt solutions. Phosphate acts as a kosmotrope, increasing the order of water, thiocyanate is a chaotrope, disrupting the order of water an, and chloride is a borderline salt.[100] The kosmotropic salt, phosphate, encourages unidirectional fiber formation while the chaotrope, thiocyanate, disrupts fiber formation. As an intermediate salt, chloride allows unordered fiber formation.[100] Additionally, the 20 amino acid anionic fusogenic peptide (GLFEALLELLESLWELLLEA) mixed with the K_{16} peptide in PBS forms a uniform population of spherical nanoparticles.[39] These nanoparticles exhibit salt-dependent growth, increasing in hydrodynamic diameter as salt concentration increases to 150×10^{-3} M NaCl. The combination nanoparticles exhibit the potential to deliver hydrophobic molecules to the cytosol via endocytosis.[39]

Most peptide nanocarriers are formed in solution by adding the raw peptides to a solvent such as deionized (DI) water. However, some peptides must be dissolved in an organic solvent and dialyzed against an aqueous solution to assemble.[50] For

Table 2. Examples of peptides which form the specified structures under varying salt, pH, and temperature conditions.

Supramolecular structure	Secondary structure	Sequence	Salt	pH	Temperature	Ref
Amyloid nanofibers	>50% Parallel beta sheets	NYNYNYN	100×10^{-3} M KH_2PO_4	6	RT	[48]
		QYQYQYQ	100×10^{-3} M KH_2PO_4	7		
		SYSYSYS GYGYGYG	150×10^{-3} M NaCl, 100×10^{-3} M KH_2PO_4	6		
			150×10^{-3} M NaCl, 100×10^{-3} M KH_2PO_4	7		
		NYNNYN	100×10^{-3} M KH_2PO_4	7	25C	
		QYQQYQ	100×10^{-3} M KH_2PO_4			
		SYSSYS	100×10^{-3} M KH_2PO_4			
Nanofibers	Parallel beta sheets	$COCH_3$-RADA16-$CONH_2$	10×10^{-3} M phosphate buffer	4.85	RT	[23]
	Beta sheets	RADA16	–	–	37C	[25]
		AEK16-II	40×10^{-3} M NaH_2PO_4/Na_2HPO_4	7	RT	[90]
		AEK16-IV		<6.5, >7.5		
Nanoparticles	Beta turn			6.5–7.5		
	Beta sheets	CG3R6TAT	–	7	RT	[42]
	Alpha helix	GLFEALLELLESLWELLLEA	PBS	7.4	RT	[39]
Nanofibrous Hydrogel	Anti-parallel beta sheets	Ac-RADA16-$CONH_2$	NaCl	7	RT	[24]
	beta sheets	MAX8	≈161×10^{-3} M (equivalent ionic strength)	7.4	RT	[22]
		MAX1	10×10^{-3} M NaCl	9	>15C	[97]
				9.7	>25C	
		GAGASF	–	2.7	65C	[49]
		GAGASP		2.5	88C	
		YGFGGF		7.8	55C	
	Beta turn	VTEEIF		6.1	90C	
	Alpha helix	VTEEIP		5.4	80C	
	Random coil and beta sheet	VYGGGF		4.3	58C	
Ribbons	Random coil and beta turn	GVPVPF	–	4.8	–	
Filaments	Beta sheets	(AEAEAKAK)2	150×10^{-3} M NaCl, 10×10^{-3} M sodium phosphate	7.4	–	[96]

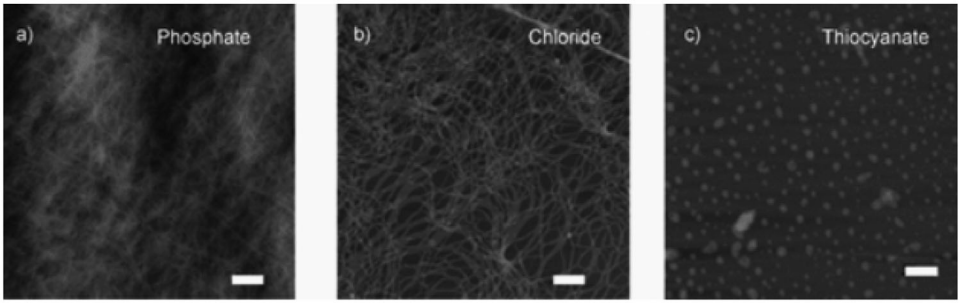

Figure 6. AFM images of self-assembled peptide gels in the presence of A) phosphate, B) chloride, and C) thiocyanate salts of sodium. Scale bar represents 500 nm. Reproduced with permission.[100] Copyright 2012, WILEY-VCH.

example, the peptide sequence *cyclo*[-(L-Gln-D-Tle-L-Glu-DTle)$_2$-] and its enantiomeric pair self-assemble into beta sheet-based nanotubes in a 33% (v/v) acetonitrile-water solution.[50] Another example of organic solvents for peptide assembly is the mixture of KS5-DEAP2-A488 and KS5-DEAP2-BHQ-1, which were designed such that three primary amino groups on the lysine residues could be used for conjugation to each other.[91] Both KS5 variants were mixed in dimethylformamide before being dialyzed against a Tris-HCl buffer, leading to the formation of vesicle-like pH-sensitive nanoparticles.[91] Each of the two variants was conjugated with a different fluorescent dye, and when mixed in solution, the two building blocks alternate, forming a

bilayer that folds into a spherical nanoparticle.[91] The fluorescent dye changes intensity when exposed to an acidic tumor microenvironment, allowing the nanoparticle to be used as a tumor probe for both intratumoral and intravenous tumor imaging.[91] Both of these assemblies were made with dual-peptide systems, indicating that an organic solvent may be helpful for the formation of some multi-peptide assemblies.

Salt concentration and buffer solutions are important considerations when designing self-assembling peptides. By adding kosmotropes such as phosphate or sodium salts to a peptide solution, the order of water increases, and further assembly is encouraged, such as the extension of nanoparticles into fibers or increase in nanoparticle diameter.[39,100] Organic solvents should also be considered; especially in dual-peptide formulas, organic solvents dialyzed against aqueous solvents encourage uniform self-assembly.[50,91]

7.2. pH and Temperature

The environment in which peptide assembly occurs can affect the degree and nature of peptide assembly. Therefore, chemical or physical environmental factors such as pH and temperature can be used to control peptide assembly.[101] Changing these parameters can trigger different degrees of peptide folding and assembly. For example, buffering peptides in solution to a particular pH can lead to controlled and reliable assembly of peptides into desired structures.[45,49] Most peptides designed for biomedical and drug delivery applications are terminated with carboxyl and amine groups, which become deprotonated or protonated in solutions above or below their pK values, respectively.[101] Additionally, amino acids with side chains terminating in carboxyl or amine groups can also be included in the peptide sequence to increase the effect of protonation or deprotonation, enhancing charge-specific sensitivity. This principle can be used to design pH-sensitive peptides for which self-assembly can be mediated by pH, or pH adjustment can alter the type of assembly. For instance, an 11 amino acid sequence, P11, was substituted with either one or two residues in each variant (P_{11}-2, P_{11}-3, P_{11}-4, P_{11}-5) and examined in aqueous solutions of varying pH. Each peptide variant formed hydrogels with various phases of assembly across pH ranging from 2–12, as shown in **Figure 7**. Each of the P_{11} peptide hydrogel variants exhibited contrasting phase characteristics within the tested pH scale despite having very similar primary sequences.[101] This experiment demonstrates that pH dependence of peptide assemblies is heavily reliant on the intrinsic properties of the primary sequence.

Temperature can also be used to manipulate peptide folding and assembly. It has been demonstrated that the hydrophobic effect in protein folding is dependent on temperature.[102] The hydrophobic effect results in hydrophobic residues being buried in the center of proteins to avoid contact with an aqueous solution. This occurs because liquid hydrocarbons are poorly soluble in aqueous solutions. Therefore, it is energetically favorable for them to shift to the organic phase in the center of the protein.[103] The hydrophobic effect is temperature-sensitive because the amount of thermal energy required to trigger assembly is dependent on electrostatic interactions and associations unique to each sequence. As temperature increases, hydrophobic amino acid residues are concealed, driving peptide folding and assembly.[97] Several studies have confirmed that peptide assembly is partly dependent on temperature.[97,100,104,105]

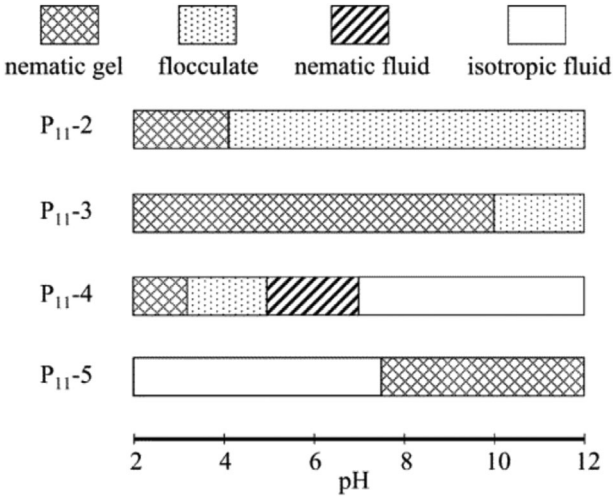

Figure 7. pH- dependent assembly of peptides in aqueous solution into various phases. Reproduced with permission.[101] Copyright 2003, American Chemical Society.

Notably, it is often observed that the interplay between pH, temperature, and ionic concentration can determine how peptides fold and assemble. One study showed that the temperature-induced assembly of the MAX1 peptide is pH-sensitive.[97] The MAX1 peptide has several lysine side chains which become deprotonated at increased pH.[97] In a solution with a pH of 9.7, most of the lysines are deprotonated, and not much thermal energy is required to trigger assembly into beta sheet fibrillar hydrogels.[97] At a pH of 9, only a few lysines are deprotonated, and therefore a higher temperature is necessary to induce self-assembly.[97] Finally, at a pH of 8, no lysines are deprotonated, and assembly is not observed at any temperature.[97] In another study, four different peptide sequences were each incubated in four solutions of varying pH and NaCl concentrations. These peptides were designed to form amyloid fiber structures and were analyzed using amyloid-specific dyes. The number of amyloid structures identified in each combination was found to be dependent on both the heptad repeats and tyrosine in the peptide sequence as well as a PBS solution of pH 7.[48] Therefore, it is important to consider all aspects of solution conditions when predicting peptide assembly.

7.3. Sample Processing During Characterization

Additional steps involving physical manipulation of peptide samples are sometimes required to accomplish ideal peptide assembly, such as filtering, sonication, or centrifugation.[25,91,92] These steps may help achieve the desired structure, size, or loading capacity of the assembled peptides. However, some analysis methods, such as electron microscopy, require alternative or additional sample preparation.[23] A potential concern is that the alternative processing steps required for a particular analysis method may change the assembled peptide structure and properties, leading to inconsistent results.[106] To minimize differences in structure

across characterization experiments, sample preparation methods should be kept as consistent as possible across various analyses. Alternatively, if the assembly method must be differentially manipulated for some analysis methods, the assembled structure can be compared with multiple characterization methods to confirm minimal change in assembly structure due to differences in preparation steps.

8. Conclusion

Self-assembling peptides are versatile materials that can be used for a variety of applications in cargo delivery. Their highly tunable nature allows the amino acid sequence of each peptide to be acutely designed to create different structures with various loading capabilities. Supramolecular assemblies such as nanoparticles, nanotubes, nanofibers, nanoribbons, and hydrogels are effective as nanocarriers for delivery of chemotherapeutic drugs, genetic material, contrast agents, or other cargo. Peptide sequences can be defined by class, the most common of which are alternating sequences and amphiphilic molecules. Peptides fold primarily into beta sheet and alpha helical secondary structures, as well as some less common structures, such as coiled coils, which then assemble into supramolecular structures. Nanoparticles, fibers, ribbons, and hydrogels have each demonstrated utility as peptide nanocarriers.

Predicting the supramolecular assembled structure is paramount to designing peptides for specific applications. As bottom-up prediction models are insufficient for predicting de novo peptide assembly accurately, a top-down approach was used in this review to identify key patterns in self-assembled nanocarrier structure. Methods used to determine secondary structure in current literature include CD, FTIR, and NMR, while DLS, AFM, and electron microscopy are the most common methods for determining the size and morphology of supramolecular structures. Using a combination of analysis methods is the best way to ensure accurate peptide secondary and supramolecular structure characterization.

Various patterns of peptides were reviewed across all secondary and supramolecular structure classes to understand how peptide sequence itself affects peptide assembly. Trends in the literature indicate that nonlocal effects, or the pattern of a peptide sequence, are more important than the local effects, or structural propensity of individual amino acids. To this end, alternating sequence patterns are demonstrated to induce beta sheet formation. Alternating sequences can be created with peptides of differing charge, polarity, or aromatic groups. Conversely, amphiphilic peptide sequences often give rise to tubes and vesicles.

Finally, solution conditions can also alter peptide assembly patterns. Salts, buffers, pH, and temperature are all important considerations that influence formation of the supramolecular structure. Salts can act as either kosmotropes, increasing the order of water and promoting self-assembly and growth into uniform nanofibers or other extended structures, or chaotropes, which disrupt self-assembly and lead to globular or nanoparticle formation. For largely hydrophobic sequences, peptides in organic solvents can be dialyzed against an aqueous solution to induce assembly. Changing the pH or temperature of a peptide solution can lead to phase changes in hydrogel formation or trigger assembly of some stimuli-responsive sequences. Charged peptide sequences in particular, are likely to exhibit different assembly behaviors at varying pH, creating fibers at pH values close to the peptide's isoelectric point, where the peptide remains zwitterionic, and nanoparticles or globular assemblies at pH values causing significant deprotonation or protonation of side chains.

When designing self-assembling peptides for nanocarrier applications, there are many aspects to consider, especially the ideal secondary and supramolecular structures dependent on the cargo. However, current molecular modeling simulations are not advanced enough to accurately predict assembly structures of de novo peptide sequences. Therefore, the top-down analysis provided here takes into account the local and nonlocal effects of the peptide sequence, solution conditions, including salts, pH, and temperature, and examines common structural analysis methods used across current literature to identify key trends in peptide assembly. The patterns identified in this review provide a new resource for prediction of self-assembled peptide structures. Therefore, these trends can be used to inform intentional design of de novo self-assembling peptides for nanocarrier applications.

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Conflict of Interest

The authors declare no conflict of interest.

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