100th Anniversary of Macromolecular Science Viewpoint: User's Guide to Supramolecular Peptide-Polymer Conjugates

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Abstract

This perspective highlights the design principles and development of peptide-based supramolecular polymers. Here we delve deep into the practicalities of synthesising and characterising these macromolecular structures and provide a thorough overview of the benefits and challenges that come with these systems. This article emphasises to beginners and experts alike, the importance of understanding the fundamental behaviour and self-assembly processes when designing these complex and dynamic functional materials.

Introduction

This viewpoint celebrates the 100-year anniversary of Staudinger's seminal paper proposing the concept of polymers.[1] This notion of forming large macromolecular structures by covalently linking short repeat units has transformed the world we live in today. Notable examples of polymers that are naturally occurring are DNA and proteins, which are made up of small subunits (nucleic acids and amino acids respectively) covalently bound together. Supramolecular polymers refer to the self-assembly of molecules bonded through non-covalent interactions: such as hydrogen bonding, π-π stacking, hydrophobic interactions, van der Waals forces, metal coordination, and electrostatics.[2, 3] The prefix 'supra' means 'beyond the limits of' or 'outside of', and from this, the term 'supramolecular' is used to denote the interactions outside the limits of the molecule. The reversibility afforded by these interactions has inspired countless applications in the area of responsive materials.[3-9] The distinction between 'intra' and 'inter' molecular is made by differentiating how the molecules are bonded together, either covalently or non-covalently.

Supramolecular polymers have been shown to form a range of different morphologies on the nanoscale, such as nanofibers,[10-14] nanoribbons,[15] and nanotubes.[16] Examples of these systems can also be found in Nature, such as actin[17, 18] and tubulin.[19, 20] Making synthetic

supramolecular polymers via a ‘bottom-up’ approach is a very appealing proposition for chemists. Using well-established organic chemistry, the functionality of these unimers can be manipulated to systematically promote assembly, introduce secondary interactions, and provide attachment sites for post-modification. This gives us endless possibilities to design and tailor these materials for a wide range of applications.

In classical/conventional polymerisation monomers are covalently linked together to form a long polymer chain; in supramolecular polymerisation the long chain of unimers are linked by non-covalent interactions. The terms ‘monomers’ and ‘unimers’ are used interchangeably in both types of polymerisation. Just for this article *to avoid any confusion, we will use monomers for classical/covalently bound units and unimers for supramolecular/non-covalently bound units (which here are peptide-polymer conjugates).*[21]

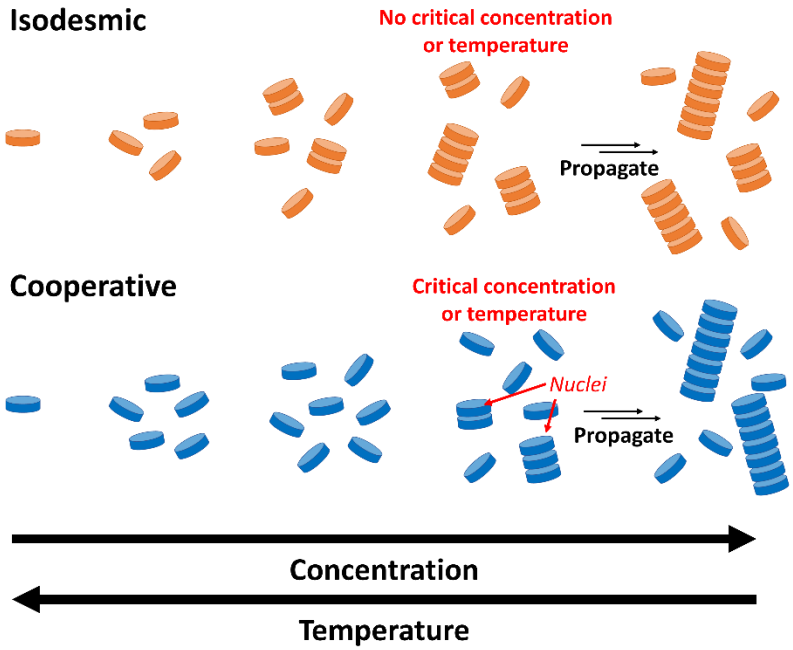
Almost all important physiological functions in Nature rely on the combination of covalent (conventional) and non-covalent (supramolecular) polymers. The formation of proteins is reliant on the covalent sequence defined chain of amino acids that make up the peptide, which in turn dictates the non-covalent intermolecular interactions within the chain that leads to their hierarchical structures and functionality. Inspired by Nature, scientists have long attempted to replicate this synthetically. One of the most promising areas of supramolecular polymers is the field of peptide-mediated self-assembly. This viewpoint will highlight some key examples, benefits, and challenges of peptide-based self-assemblies and how they may play a role in the next generation of biological therapeutics. Here we hope that by shining a light on these complex systems and providing practical advice for their synthesis, we inspire and help others to develop more sophisticated supramolecular polymers in the future.

Supramolecular polymers

Under the umbrella of supramolecular assemblies, which can be formed by any supramolecular interactions, supramolecular polymers often refer to systems that adopt specific bonding arrangements, which are multivalence and directional. Multivalency is crucial for the continued growth of the polymer past two units. Additionally, the bond strength scales with the number of bonds between the two monomers. It is also important to understand that the molecular structure of these unimers can dramatically affect their ability to aggregate with one another and the morphology they adopt. This article will quickly recap some key aspects of supramolecular polymers, which we will build upon in the later sections. We hope that this short introductory section may also encourage you to delve deeper into the intricacies of this area. Here are some great reviews that cover the field of supramolecular polymer chemistry more extensively.[2, 3, 21-23]

Mechanism of growth

Depending on the unimer, the self-assembly of the supramolecular polymerisation should proceed via one of two prominent mechanisms: isodesmic or cooperative.[3] See Scheme 1.



Scheme 1. The self-assembly process in an isodesmic and cooperative mechanism.

In an isodesmic polymerization the reactivity of the unimer and the growing chain is equal; as a result, the polymerisation occurs in a manner comparable to step-growth polymerization. This happens when there are no neighbouring group effects. The decrease in free energy with each successive monomer attachment to the growing polymer remains constant throughout the polymerisation. The aggregation number is dependent on temperature and concentration, increasing the temperature or decreasing the concentration will lead to disassembly and vice versa. Most notably, there is no critical concentration and temperature.

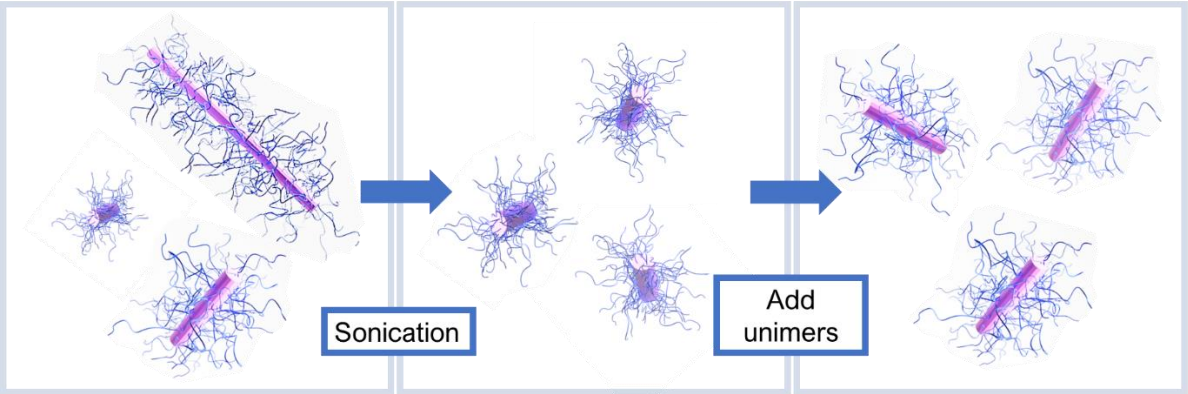
When proceeding by a cooperative mechanism, the polymerisation occurs in a non-linear fashion in two stages, a nucleation stage followed by an elongation stage. For the formation of a nucleus, a critical concentration/temperature must be reached (nucleation stage). After this point, further increases in concentration or decreases in temperature lead to the growth of the polymer chain from the nucleus (elongation stage). Notably, the change in the rate of polymerisation between the nucleation and elongation stages can be represented by two different rate constants. In a cooperative mechanism, the rate constant for nucleation is smaller than that of elongation. Conversely, when the rate constant of nucleation is larger than that of elongation, the mechanism is referred to as anti-cooperative. This chain-growth polymerisation method, whereby the monomers can only react with the ‘active’ growing chain,

makes it possible to control the dispersity of the polymer via living polymerisation techniques. A detailed breakdown of the equations and methods to determine which mechanism a supramolecular polymerisation proceeds by can be found in a review by Hartlieb *et al.*[24]

Living supramolecular polymers

Nature produces mono-disperse polymers, such as proteins, to carry out vital living processes. However, achieving this for synthetic polymers has been far more challenging. Dispersity is the measure of the heterogeneity of polymers, the larger the length distribution of chains, the higher the dispersity. Conventional polymers, where the monomers are linked covalently, have used living polymerisation techniques to better control the length and distribution of polymers. During polymerisation, an initiator can react with another monomer to generate a new active centre on the monomer. With each successive monomer addition, the polymer propagates and the last monomer to react importantly retain the active chain end. In a living system, where the initiation is faster than propagation and there are no side reactions via termination or chain transfer, all the active chain ends can be nucleated before growing uniformly – this results in a much lower dispersity (controlled) polymer. Controlling the dispersity of the polymers is vital as the degree of polymerisation (DP), i.e., the polymer chain length, can drastically affect the properties of the material. Lower dispersity polymers will enable us to better target, tune, and reproduce the polymers for their desired applications. Using the concept of 'living' supramolecular polymerization we can synthesise uniform self-assembled structures.

The first approach leading to a living supramolecular polymerization, which was established and developed by Manners, Winnik and coworkers, utilises crystallisation-driven self-assembly (CDSA), see Scheme 2.[25, 26] In CDSA, a block co-polymer first self-assembles in a solution as one block is solvophobic and the other is solvophillic. Importantly the solvophobic block, most commonly polyferrocenylsilane (PFS), forms a semi-crystalline core.[27] This crystallinity generates directionality and kinetically traps the self-assembled structures preventing them from dynamically disassembling and re-assembling. Upon sonication, these self-assembled structures can be broken up down to uniform 'seed micelles' which do not recombine with one another. In the presence of additional unimers, the seed micelles behave as nuclei from which they can propagate to produce uniform elongated supramolecular polymers. Since their discovery, this technique has been used to create a wide range of morphologies from multiblock[28-30] to 2D-platelets.[31, 32] More recently, systems that are increasingly compatible with aqueous environments have been pioneered, in particular in systems that use polylactides as their core-forming block.[33, 34]



Scheme 2. Simplified route to uniform cylindrical micelles via crystallization driven self-assembly. First, the block co-polymer unimers are self-assembled in an uncontrolled fashion (left), with the crystallisable core-forming block (pink) and the solvophillic corona (blue). When sonicated, they form short stabilised 'seed micelles' (middle), which upon addition of further unimers can grow to produce uniform supramolecular polymers (right).

Aida and coworkers also developed a method to form living supramolecular polymers using metastable cage-like structures.[35] The initiator and monomer species consist of a concave structure of aromatic rings functionalised with a range of different R-groups on the periphery. Once assembled, the phenyl groups take part in π-π stacking, but crucially, the R-groups contain amide bonds which can hydrogen bond to each other. The carboxylic acid group on the initiator can be used to spring open the metastable cage monomer; this hydrogen bond reorganisation initiates the polymerisation, and the resultant initiator-monomer complex can further propagate with more monomers during the chain growth stage. The subsequent polymers were shown to be well controlled via size exclusion chromatography (SEC) and atomic force microscopy (AFM). In addition, both SEC and AFM also showed that the size and length of the polymers could be tuned by increasing the monomer to initiator ratio, as expected in a living polymerisation.

Subsequently, a third way to produce defined supramolecular polymers was developed by Sugiyasu, Takeushi, and coworkers, using the formation of stabilised aggregates of zinc or copper complexed porphyrin derivatives.[36] As the zinc porphyrin-based molecules were heated, they preferential disassembled to its unimeric state. Upon cooling, they stacked to form J-aggregated nanoparticles; however, given a few days to equilibrate, the system could rearrange to form H-aggregated nanofibers. This phenomenon can be seen to take place much faster (in a few hours) if the H-aggregates are put in the presence of some J-aggregates. This suggests the transformation into H-aggregates can be initiated by 'seeding'. By introducing 'nuclei' or 'seeds', the polymerization can proceed via a controlled pathway,

resulting in the formation of uniform fibres. More recently these porphyrins have also been shown to form uniform assemblies *via* the CDSA approach.[37]

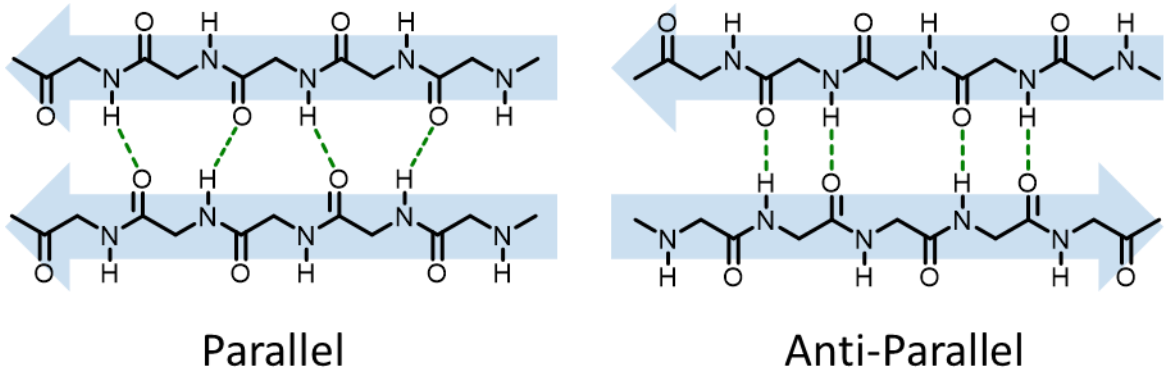
All of these approaches illustrate remarkable control over the supramolecular polymerisation; however, they require highly specialised unimers and conditions. Despite these advances, designing controlled self-assembly systems for biological relevant applications in aqueous conditions have been very challenging.

Self-assembling peptide

Hydrogen bonds can be found everywhere in the natural world. For this reason, when designing supramolecular polymers for biological applications, many systems use hydrogen bonds to recreate their desired nanostructures. In particular, the self-assembling peptides, which can spontaneously aggregate to form long ordered structures have been widely exploited to form β-sheet and α-helix assemblies.[38]

Using amino acids that are naturally occurring in the body, the aim is to minimise their potential biological side effects. These systems have garnered much attention for their potential biological applications in drug delivery[39, 40] and tissue engineering.[41] Additionally, the amino acid building blocks of peptides provide an extensive library of functional groups with their in-built self-assembly motif in the form of amide bonds. The amide bonds on the peptides can take part in hydrogen bonding to form directional β-sheet arrays.

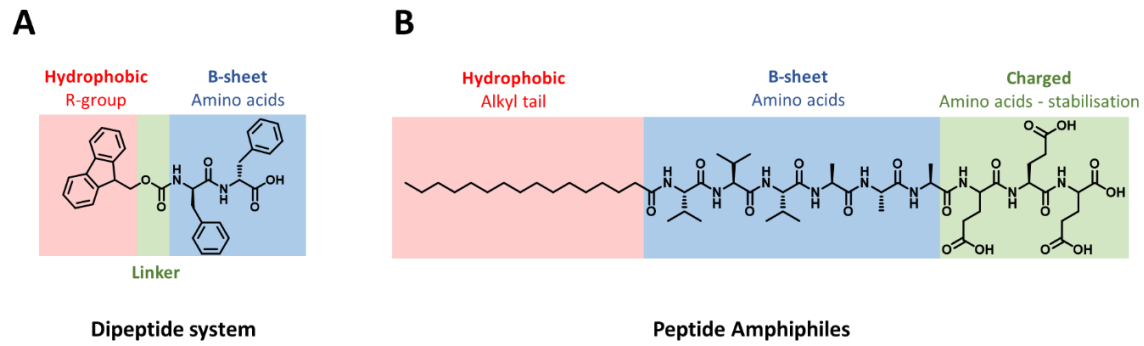
The amide bonds perpendicular to the peptide backbone can take part in hydrogen bonding to form supramolecular structures. As hydrogen bonds are directional, peptides tend to form one-dimensional elongated structures, typically nanofibers. The β-sheet formation of the peptide can occur in a parallel or anti-parallel fashion, scheme 3.



Scheme 3. Parallel and Anti-parallel β–sheet formation.

The simplest example of self-assembling peptides is the dipeptide systems.[42, 43] The general design of these dipeptides consists of two amino acids, typically aromatic, attached to a hydrophobic R-group on the N-terminus *via* a linker (i.e., O-CH_2- or –CH_2-), see Scheme 4a. The interplay of hydrophobicity and hydrophilicity between these exchangeable groups makes this system incredibly versatile. These dipeptides have been optimised to self-assemble into extremely long nanofibers in water, which can in turn entangle to produce gel networks. Due to the simple design and ability to form hydrogels in water, these peptides have been studied for their applications as 3D cell culture supports[41] and energy transfer systems for use in bioelectronics.[44]

Another key example of supramolecular peptide-based polymers are peptide amphiphiles (PA).[14] PAs contain an alkyl hydrophobic tail, β-sheet forming middle segment, and outer charged groups for stabilisation, see Scheme 4b. The hydrophobic tail and hydrophilic charged surface groups allow them to behave as surfactants in aqueous media. However, unlike typical surfactants that self-assemble into spheres to form micelles, the directional hydrogen bonding β-sheet motif forces the peptides into a nanofibrillar conformation. This two-fold self-assembly system has been used to develop bio-mimetic self-assembled nanofibers to aid bone mineralisation[45] and targeted cell-signalling.[46]



Scheme 4. Examples and general design features of self-assembling a) dipeptides and b) peptide amphiphiles

More recently, Besenius, Barz, and coworkers have reported the formation of foldable telechelic polymer conjugates.[47] Peptides with alternating phenylalanine-histidine residues were used to promote the pH switchable beta-sheet folding and, in turn, nanorod formation. The PEG and the polysarcosine, which was formed via *N*-Carboxyanhydride (NCA) polymerization, were conjugated in the centre of the triblock. Upon beta-sheet formation, these polymers can shield the nanorod's peptide core.[48, 49]

Peptide supramolecular self-assembly enables us to utilise many facets of chemistry. Exploiting naturally occurring amino acids can reduce the chances of bio-incompatibility while

also giving us access to a vast library of functional groups. Outside the remit of this tutorial review, non-peptide-based systems that use amide hydrogen bonding have also been used to develop one-dimensional aggregates. Notable examples are the bis-urea,[50] benzene 1,3,5, tricarboxamides (BTA)[51], and ureido-pyrimidinone (UPy)[52-54] motifs. In particular, Meijer and coworkers have pioneered these systems to study and understand the dynamic and fundamental behaviour of supramolecular polymers.[3] In addition, Bouteiller, Columbani, and coworkers have shown how multiple β-sheet urea bonds can also be used to create long unidirectional nanofibrillar structures.[50, 55, 56] Examples of these systems have been discussed in detail in a previous review by Besenius, Rybtchinshi, and coworkers.[22] Another more updated review in this area has recently been published by Brendel and coworkers.[57]

Over the last decade, promising supramolecular polymers have been fast-tracked to address a whole host of different applications. As we move forward in applying these systems, striving to better our fundamental understanding of these ever more complex systems, has never been more important. For this reason, we believe that better understanding these supramolecular polymers, will be a pivotal part of improving the design of these systems in the future.

This tutorial review will focus on the development of self-assembling cyclic peptide-polymer conjugates, which can form supramolecular nanotubular polymer brush-like architectures. We aim to provide guidance for others interested in the fundamental understanding, synthetic approaches and characterisations for these, and similar, supramolecular systems.

Self-assembling cyclic peptides

History and Design

Naturally occurring cyclic peptides have been found to exhibit remarkable toxicity and antimicrobial activity.[58] For this reason, cyclic peptides have been synthetically reproduced in the lab to study their potential as drug therapies and antimicrobials. A great example of this being Cyclosporin A which has become a pharmaceutical success as an immunosuppressive drug.[59]

In 1974, De Santis *et al.* first proposed that cyclic peptides with an alternating conformation could stack on top of each other to form nanotubes.[60] The hypothesis was that a linear peptide with an even number of α alternating D- and L- amino acids could form a flat ring like structure upon cyclisation. With this configuration, the amide bonds on the cyclic peptide would be perpendicular to the ring, enabling them hydrogen bond with each other, see Figure 1a. In 1993, with increasing methods to make and characterise synthetic peptides, Ghadiri and coworkers were able to observe these self-assembling cyclic peptide nanotubes by transmission electron microscopy (TEM).[16]

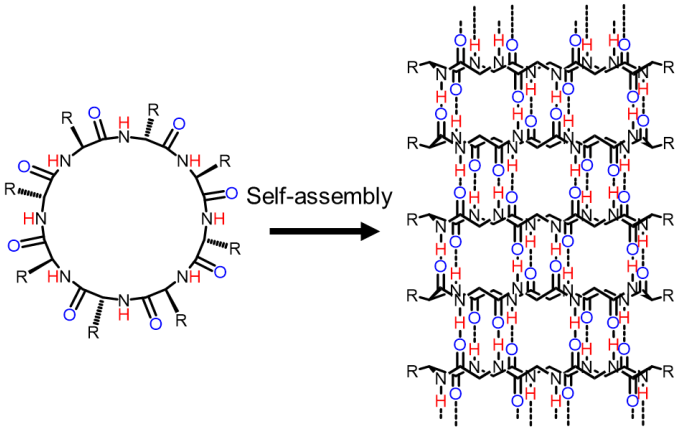


Figure 1. The chemical structure unimeric (left) and self-assembled (right) cyclic peptide nanotubes.

These cyclic peptide nanotubes (CPNT) have several desirable design features. Using a 'bottom up' approach the sequence of the amino acids in the peptide can be altered to provide solubility and site-specific functionality. The functional R group of the amino acid protrude out of the nanotube assembly; therefore, decorating the periphery of the peptide, see Figure 1a. The functional groups on the CPNT are chosen carefully to promote assembly and provide handles for post-modification. Due to the self-assembly process, the peptide can readily precipitate in most solvents, other than trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), and dimethylformamide (DMF), significantly improving the yields during the purification steps, compared to their linear counterparts.[16, 61, 62]

The composition of self-assembling cyclic peptides has been widely studied. An even number of amino acids are required to maintain the alternating configuration of peptide chain which upon cyclisation leads to the flat disc-like structure which can self-assemble.[62-64] Perrier, Joliffe, and coworkers established that the octa-peptide structure provided plenty of nodes for functionality whilst still maintaining the planar ring-structure for self-assembly. Further increases in the ring size lead to floppy ring structures which were not able to stack with one another efficiently. A library of different self-assembling cyclic peptides was extensity studied in a previous review.[63] The internal diameter and distance between two cyclic octa-peptides were found to be about 7.5 Å and 4.5 Å, respectively, determined by X-ray crystallography,[65] electron diffraction[16], and mathematical modelling.[65] Further advances have been made in orthogonal conjugation chemistry, to provide a library of different CPNT conjugates.

Cyclic peptide-polymer conjugates

Improving the solubility of CPNTs

The lateral aggregation of CPNTs, whilst beneficial for purification, makes them highly insoluble in water and, therefore difficult to utilise for biological applications. To dramatically

improve the solubility of these systems, polymers have been introduced to the periphery of these peptides to provide steric hindrance between the nanotubes and prevent their lateral aggregation. In 2006, the Biesalski and Borner groups reported polymers on the periphery of the cyclic peptide.[66, 67] Since then, our group has focused on creating hybrid conjugates which use both peptide self-assembly and tunable controlled polymers for a wide range of biological applications.

Conjugation of polymer to the cyclic peptide

The advent of controlled radical polymerisation (CRP) techniques, such as reversible-deactivation radical polymerisation (RAFT) and atom transfer radical polymerisation (ATRP) have made synthesising a range of different functional polymers with various architectures and low dispersities, more accessible than ever before.[68-72] Recently, supramolecular systems have become ever more complex as many systems adopt both the reversible and responsive Nature of the self-assembling motifs, such as peptides and the versatility and functionality of covalent polymers, like those synthesised through CRP. RAFT polymers, which have been widely used in our group, provide a huge library of the monomers with various functionalities,[73] the ability to form block co-polymer, and most importantly, retain the functionality of the RAFT agent which it can use to attach to the cyclic peptide.[74, 75] The main considerations for conjugation chemistry are orthogonality and efficiency.

When developing a new conjugate for a specific application, one of the first design features to consider is how to attach the polymer to the peptide. There are two main approaches to conjugating, grafting to and grafting from. Previously, Larnaudie *et al.* reported how the same conjugates could be synthesised using these two different approaches.[76] When selecting your method for conjugation, it is important to understand the benefits and drawbacks of each approach.

First, the functionality of the monomer units in the covalent polymer should be considered. If the functional groups on the polymer interfere with the conjugation chemistry intended to attach the polymer to the peptide, using a grafting from approach whereby the RAFT agent is pre-attached to the peptide could be the best option. The RAFT agent conjugated peptide can easily be characterised by methods used for small molecules with high accuracy, such as mass spectrometry and NMR. The major drawback of this method is the amount of peptide and RAFT agent needed for each polymerisation. The small-scale reactions will also make monitoring the kinetics of the polymerisation very difficult, often not possible. Conversely, the grafting to method uses significantly less peptide per reaction. Each peptide and polymer can be fully characterised before conjugation. The conjugation is then often characterised by high-performance liquid chromatography (HPLC) or gel permeation chromatography (GPC) - for

details, *vide infra*. See flowchart in figure 2, for a set-by-step consideration of which approach may be best suited different cyclic peptide-polymer conjugates. Understandably, with the advent of more powerful techniques in the future, the considerations here may be subject to change.

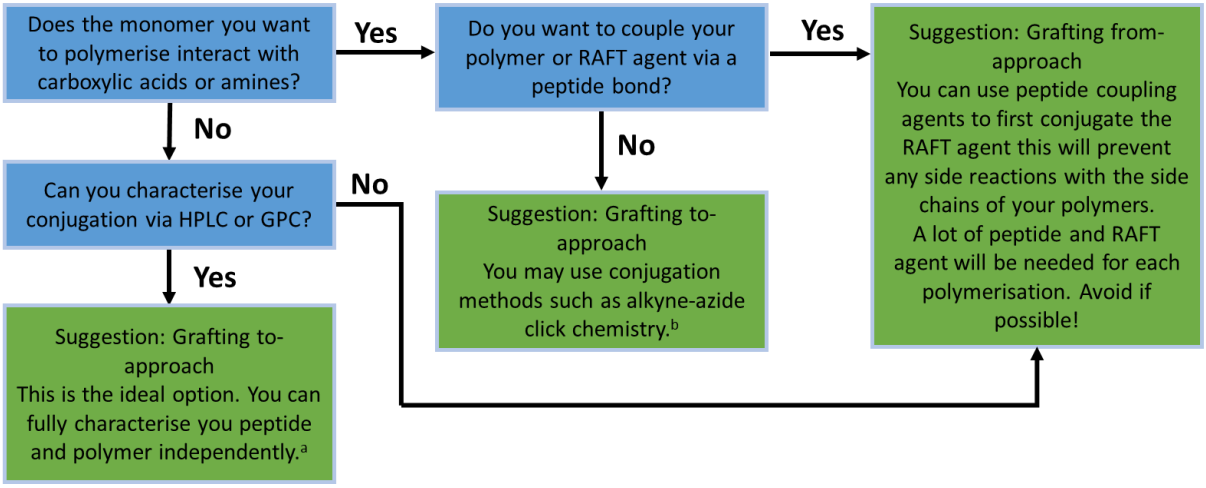
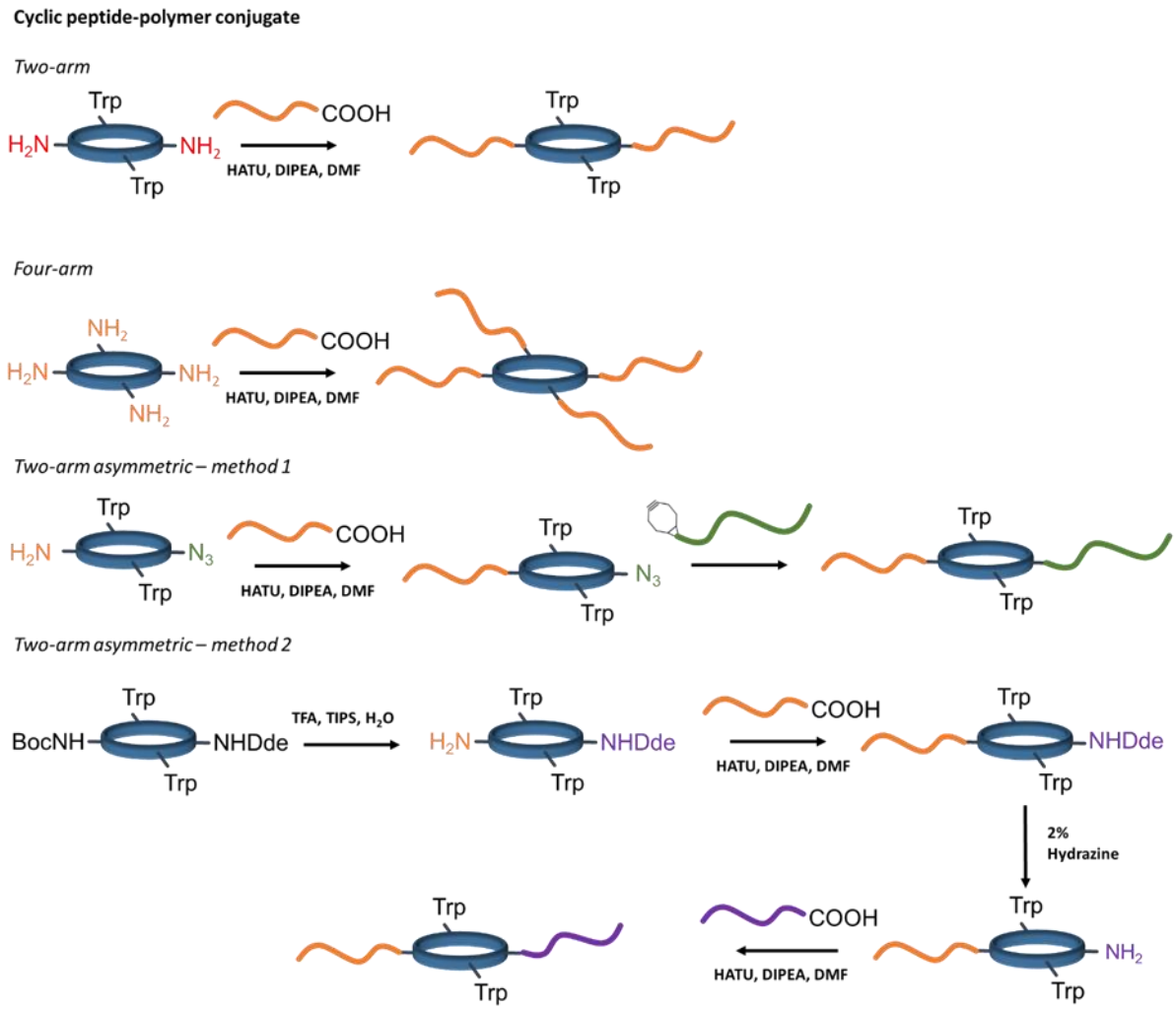


Figure 2. Flowchart to show a step-by-step guide to which conjugation approach would be best suited for different cyclic peptide-polymer conjugates. [a] With clear shifts in HPLC or GPC you can also quantify your excess unreacted peptide or polymer. [b] Both CuAAc and strained alkyne reactions have been shown to work on the cyclic peptide conjugates. For biological applications strained alkyne conjugation can remove the need to purify out the copper catalyst.

Cyclic peptide composition

The composition and sequence of the peptide can be used to direct the attachment of various polymers and small molecules to the periphery of the unimer; see Figure 1a for the general chemical structure. This 'bottom-up' approach, whereby the desired peptide sequence can be grown using solid-phase peptide synthesis (SPPS), gives us an extensive arsenal of amino acids, natural and non-natural, which are commercially available with a library of protecting groups. The functional groups on the peptide unimer can direct where the reactive sites are in relation to one another, i.e. on adjacent or opposite sides. Most commonly, lysine amino acids have been installed on the peptide to provide free primary amines ($-NH_2$), which become attachment sites for polymers and small molecules containing carboxylic acids or pre-functionalised *N*-hydroxysuccinimide groups. Importantly the amino acids are protected using acid-labile protecting groups such as Boc or trityl groups, which are orthogonal to the base-labile Fmoc protecting groups used in the SPPS. Furthermore, multiple polymer arms can be conjugated on the same CP unimer using mono-, bi-, tri-, and quad- amine peptide; see Scheme 5.[77] As expected, an increase in steric hindrance, with increasing polymer arms, leads to a decrease in the nanotubular length of these aggregates.



Scheme 5. Simplified schematic of polymer conjugation to various cyclic peptide unimer.

Orthogonal conjugation methods have also been employed to synthesis multi-component cyclic peptide unimers. Examples of these include systems with two different polymer arms or peptide-polymer-dye conjugated structures.[78] The selection and sequence of the amino acids in the octa-peptide enable us to pre-install the handles for future orthogonal post-functionalisation on the CP. Note that many of these reactions rely on highly efficient coupling chemistries, such as amide bond formation[79, 80] or the (strained or catalysed) 1,3-dipolar cycloaddition reaction.[75, 78] To avoid labour intensive and low yielding column purification, the conjugations are often performed to full conversion with the help of these highly efficient click reactions. Typically, an excess of the polymer is used to make sure all the reaction sites on the peptide are conjugated. This way, only two species remain after conjugation, the excess polymer, and the peptide-polymer conjugate. This makes characterisation and purification much easier and ensures, within the limitations of our characterisation methods, every peptide has the appropriate number of polymers attached. This is of significant importance when the

materials are being designed for kinetic and biotherapeutic studies, which will be mentioned in more detail later *supra infra*.

The conjugation can be followed via HPLC if the coupling leads to a change in polarity and, therefore, a clear shift in the retention time.[78] Though heavily used in peptide chemistry, HPLC is a method that is less commonplace in polymer chemistry. This powerful technique can both quantify the percentage of unconjugated materials and separate them for purification via preparative (prep) HPLC. Liquid chromatography–mass spectrometry (LC-MS) or more simply mass spectrometry analysis of fractions collected through prep-HPLC can be used to identify the species in each peak of the chromatogram. The HPLC methods can be optimised for a wide range of chemistries. The method and gradient of the HPLC run should be adapted to the solubility and polarity of your peptide, polymer, and conjugate. The simplest HPLC setups will have detectors set to specific absorption wavelengths. An appropriate wavelength and detector must be selected based on the compounds. Though most compounds will absorb some light when set to 200 nm, a better indication of the different constituents can be found by looking at specific wavelengths. Other detectors, such as diode arrays, can provide a better overall picture of the absorption profiles of the species within each peak. The cyclic peptides used often contain tryptophan amino acids in the peptide sequence, resulting in an absorption band at 280 nm. The thiocarbonylthio group on RAFT polymers absorb strongly at 309 nm. In addition, fluorescence detectors can also be used to observe the emission band of dyes, which have also been conjugated on these cyclic peptides.

GPC can also be employed if there is a large shift in hydrodynamic volume (related to molecular weight) between your reactants and conjugate.[76] A prime example of this is the double molecular weight shift, upon conjugation of two polymer arms to the cyclic peptide. If there is an excess of polymer, this can also be quantified via the deconvolution of the two distributions, see Figure 3a. The self-assembly of the cyclic peptide can also be used to isolate the conjugate from any remaining free polymer at the end of the reaction. The large disparity in molecular weight between the non-conjugated polymer and assembled peptide-polymer conjugate can be used to separate them via dialysis, see Figure 3a. This method is commonly conducted in water to purify water-soluble conjugates from excess hydrophilic polymers.[77, 81] However, this principle should follow for any system if the molecular weight cut-off of the membrane is appropriate, and all the constituents are fully soluble. Precipitation is also an effective way to remove unconjugated free polymers or small molecules such as dyes.[78] The use of non-polar solvents can help drive the self-assembly of the peptide and precipitation of the conjugates; whilst the polymers or small molecules remain in the supernatant. As previously mentioned, the vast differences in the polarity of the conjugates can also be exploited to separate and isolate the different constituents using Prep-HPLC. Column

chromatography methods are often the last resort due to the low yield obtained from these methods. Previously, conjugates have been successfully purified and separated by size, using Bio-Beads SX-1 resins or Sephadex columns.[75, 82]

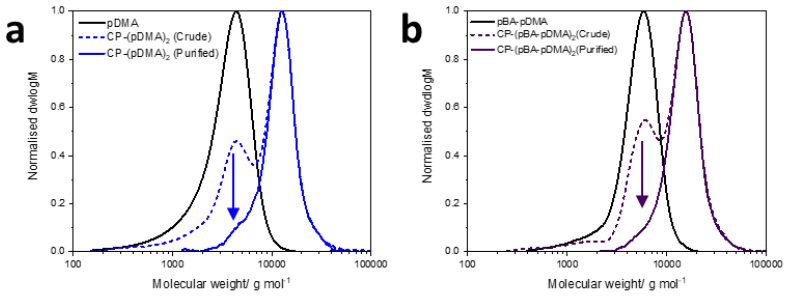


Figure 3. Gel permeation chromatography (GPC) of the attachment of two polymer arms to a di-amine functionalised cyclic peptide. a) The conjugation of hydrophilic poly (dimethyl acrylamide) (pDMA) purified by dialysis. b) The conjugation of an amphiphilic diblock consisting of poly(butyl acrylate) (pBA) and pDMA blocks, purified by fractional precipitation. Reproduced with permission from reference 83. Copyright 2019 Springer Nature.

Another powerful method to analysing these nanotubular aggregates in solution is small-angle neutron scattering (SANS). This technique can be used to obtain both information about the shape and size of the aggregates in solution. Our group has used this technique in conjunction with other light scattering methods, such as static light scattering (SLS)[81] and small-angle X-ray scattering (SAXS), and *ex vivo* characterisation methods such as transmission electron microscopy (TEM) to corroborate the size and shape information obtained by SANS.

When amphiphilic block co-polymers were attached to the periphery of the peptide, it was found that the free polymer could not be differentiated from the self-assembled conjugate. Unfortunately, the free unconjugated block co-polymers also self-assembled into micelles, and for this reason, all attempts to purify these conjugates *via* size, such as dialysis and size exclusion columns, failed. The amphiphilicity of the polymer also made both the polymer and conjugate soluble in many solvents; this, in turn, ruled out purification *via* conventional precipitation.

After numerous solubility test, in a range of solvents and co-solvent mixtures, it became apparent the polymer and conjugates showed different degrees of solubility. Notably, when first sparingly pre-dissolved in a good solvent (DMF, methanol, or ethanol), the polymer did not readily precipitate upon the addition of a bad solvent (diethyl ether). When a small volume of the crude conjugation mixture, which contained the peptide-polymer conjugate and free polymer in DMF, was added to a bad solvent (diethyl ether), precipitates could be seen to form readily. This indicated that the polymer had better solubility than the conjugate when they were

both pre-dissolved in a good solvent. Using this disparity in solubility, the conjugates could be fractionally precipitated out of solution whilst the polymers remained in the supernatant. In the case of a two-arm conjugate, this purification could be confirmed, and followed by GPC. After repeated fractional precipitation, a clear decrease and eventual disappearance of the low molecular weight distribution (related to the free polymer) can be observed, see Figure 2b.[83] This method of utilising the disparity in solubility of self-assembled aggregates, could also be more broadly adopted to remove any undesired starting material for other supramolecular systems.

The dual assembly afforded by the conjugation of diblock polymers to the CP has been shown to improve the peptide assembly and stability of these nanostructures. The hydrogen bond β-sheet stacking of the cyclic peptides provides the primary structure and overall cylindrical morphology of the self-assembled aggregates. More importantly, the hydrophobic interactions and region around the peptide core prevents the solvent/water molecules from competing with the peptide assembly, which in turn stabilises these aggregates. see Figure 3a.[83] This will be explored in more detail below, *vide infra*.

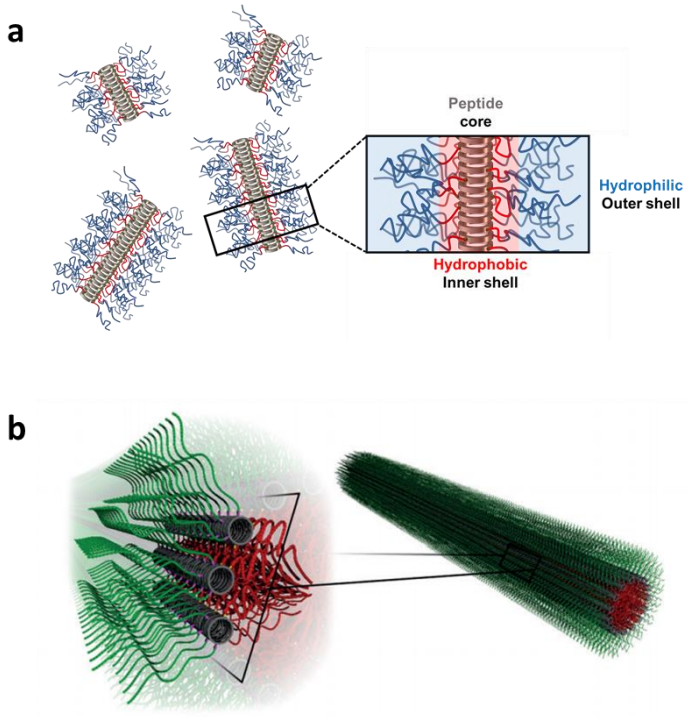
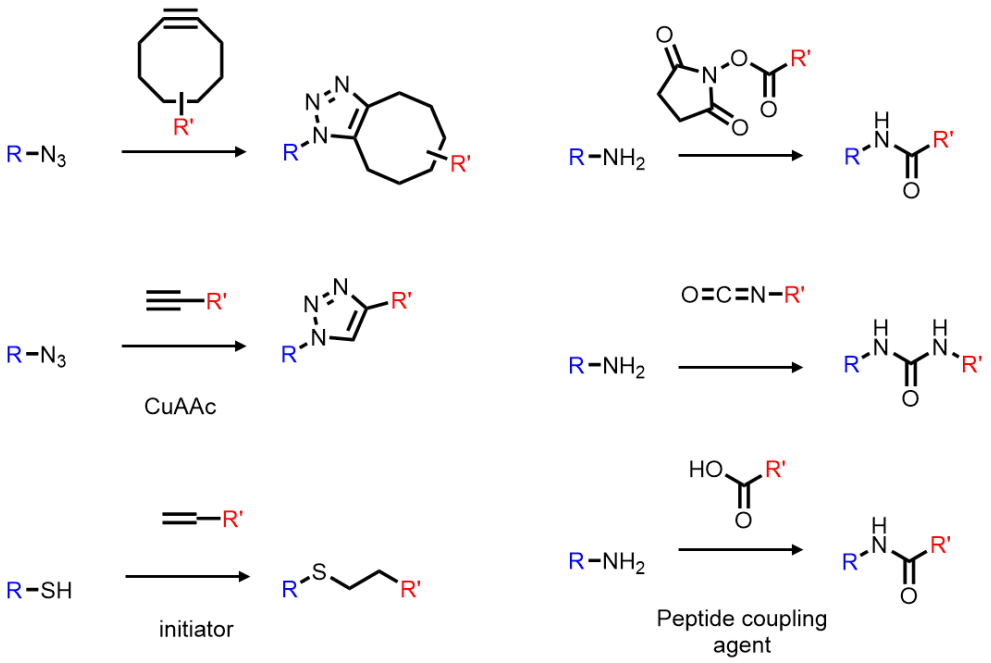


Figure 3. Schematic representation of amphiphilic cyclic peptide-polymer conjugates. a) Amphiphilic block co-polymer (hydrophobic in red, hydrophilic in blue) conjugated cyclic peptide nanotube. Reproduced with permission from reference 83. Copyright 2019 Springer Nature. b) Two different amphiphilic polymers (hydrophobic in red, hydrophilic in green) conjugated orthogonally to the cyclic peptide nanotube, which self-assembly into ‘tubisomes’. Reproduced with permission from reference 84. Copyright 2019 John Wiley and Sons, inc.

The attachment of two different polymer arms to the cyclic peptide can be achieved using orthogonal reactions. Several different orthogonal chemistries have been used to synthesise these types of conjugates: for example, strained-promoted or metal-catalysed (CuAAc) alkyne-azide cycloaddition, thiol-ene reaction, isocyanate-amine addition, and amide bond coupling, see Scheme 6. Previously, amphiphilic conjugates have been synthesised with and without purification of the polymer intermediates depending on their chemistries. The resulting amphiphilic polymer-peptide conjugates have been purified successfully using low-polarity solvents such as methyl tert-butyl ether. The development of these conjugates has led the discovery of hierarchical amphiphilic systems, which not only self-assembly into nanotubes but also further aggregate due to hydrophobic interactions in water, see Figure 3b. More recently, these systems referred to as ‘tubisomes’ have also been shown to deliver the anti-cancer drug Doxorubicin photo-responsively.[84]

A notable limitation of these conjugation reactions is the restrictive solvent range at which they can be carried out in. The insolubility of many of these cyclic peptides in most solvents, due to their lateral aggregation, mean that conjugation reactions are typically only viable in highly hydrogen bond competitive solvents such as DMF, DMSO, or TFA.



Scheme 6. Conjugation chemistries used to attach polymers and small molecules to the periphery of the peptide unimer.

Below is are tables summarising the various chemistries and peptides used to form a range of self-assembling cyclic peptide conjugates. In Table 1, various cyclic peptides which utilise a singular conjugation and attachment chemistry have been collated. In Table 2, cyclic

peptides that use orthogonal chemistries to attach different compounds to the periphery of the peptide have been collated.

Table 1. Table summary of self-assembling cyclic peptide conjugates formed by a singular conjugation chemistry.

CP structure	**Conjugated moieties**	**Attachment chemistries**	**Reference**
CP-NH_2	PEG-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	77
CP-N_3	pBA-CHCH or pS-CHCH	1,3-dipolar cycloaddition -$CHCH^a$ (polymer), -N_3 (CP)	85
CP-CHCH	CHCH-pBA-CHCH or CHCH-pS-CHCH	1,3-dipolar cycloaddition -$CHCH^a$ (polymer), -N_3 (CP)	85
CP-Pyr/Dap	Pre-installation using L-Lys (Pyr/Dap) amino acid	Pyr/Dap funtionalised lysine used in SPPS	86
CP-OH/NH_2	(o,p) -phosphinobenzoic acid (PhosBA)	Amide bond formation -COOH (PhosBA), -NH_2 or -OH (CP)	87
CP-COOH	PVA-OH	Complexation H-bonding -OH (Polymer), -COOH (CP)	*88*
CP-BIB	pEGMA (grafting from)	Pre-installed ATRP initiator -COOH (initator), -NH_2 (CP)	**89**
CP-$(NH_2)_2$	pDMA-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	83
	pDMA-*b*-pBA-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	83
	pEtOx-S-S-CH_2-CH_2-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP) n.b. polymers can be selectively removed by disulfide cleavage	90
	pEtOx-S-CH2-CH2-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	90
	pBA-COOH	Amide bond formation -COOH (polymer), -NH_2 (CP)	67, 91
	pBA-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	92
	pBA-*co*-pFluoresceinA	Amide bond formation -COOH (polymer), -NH_2(CP)	91
	pHPMA-*co*-pPUEMA	Amide bond formation -COOH (polymer), -NH_2(CP)	40

		n.b. Pyridines can be used to complex Iridium drug	
	PEG-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	*77*
	pPEGA-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	*77*
	pDPA-*b*-pHPMA	Amide bond formation -NHS (RAFT agent), -NH_2(CP)	*93*
	pDMAEMA-COOH	Amide bond formation -NHS (RAFT agent), -NH_2(CP)	81
	pNAM-NHS	Amide bond formation -NHS (RAFT agent), -NH_2(CP) or -NHS (polymer), -NH_2(CP)	*76, 94*
	p^{t}BA-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	pHEA-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	p^{n}HexAm-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	pLA-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	pHEA-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	pNIPAM-NHS	Amide bond formation -NHS (polymer), -NH_2 (CP)	94
	PEG-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	*95, 96*
	pS-COOH	Amide bond formation -COOH (polymer), -NH_2(CP)	*95*
CP-$(N_3)_2$	pBA-CHCH	1,3-dipolar cycloaddition -CHCH^a (polymer), -N_3 (CP)	*97-99*
	pHEA-CHCH	1,3-dipolar cycloaddition -CHCH^a (polymer), -N_3 (CP)	98, 100
	pDMAEA-CHCH	1,3-dipolar cycloaddition -CHCH^a (polymer), -N_3 (CP)	*98*
	pS-CHCH	1,3-dipolar cycloaddition -CHCH^a (polymer), -N_3 (CP)	*98*
	p^{t}Bu-CHCH	1,3-dipolar cycloaddition	*98, 100, 101*

		-CHCH[a] (polymer), -N_3 (CP) n.b. Removal of ^{t}Bu upon TFA deprotection to reveal AA	
	pHEA-*co*-pCEMA -CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP) n.b. Finkelstein reaction was used to attach the RAPTA-C	102
	pI-*co*-p^{t}Bu-CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP) n.b. Removal of ^{t}Bu upon TFA deprotection to reveal AA	103
	pEtOx-CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP)	104
	pMMA-CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP)	95
CP-$(TEG)_2$	Pre-installation using L-Lys (TEG) amino acid	TEG funtionalised lysine used in SPPS	*105 106*
CP-$(NH_2)_3$	PEG-COOH	Amide bond formation -COOH (polymer), -NH_2 (CP)	77
	pNIPAM, pS, pBA (grafting from)	Pre-installed ATRP initiator -COOH (initator), -NH_2 (CP)	*107*
CP-$(N_3)_4$	pHEA-CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP)	100
	pBA-CHCH	1,3-dipolar cycloaddition -CHCH[a] (polymer), -N_3 (CP)	99
CP-SH	pDMA-S-S-PDS	Disulfide formation -PDS (polymer), -SH (CP)	108
	pPEGA-S-S-PDS	Disulfide formation -PDS (polymer), -SH (CP)	108

Table 2. Table summary of self-assembling cyclic peptide conjugates formed by orthogonal chemistries.

Orthogonal Chemistry			
CP structure	**Conjugated moieties**	**Attachment chemistries**	**Reference**
N_3-CP-NH_2	PEG-COOH pPEGA-COOH	Amide bond formation -COOH (polymer), -NH_2(CP) 1,3-dipolar cycloaddition	84

		-CHCH[b] (polymer), -N_3 (CP)	
CP-$(NH_2)_3$ Protected DdeNH-CP-$(NHBoc)_2$	Cy3/5–NHS pDMA-COOH	Amide bond formation -NHS (Cy dye), -NH_2 (CP) Amide bond formation -COOH (polymer), -NH_2(CP)	83
	Cy3/5–NHS pDMA-*b*-pBA-COOH	Amide bond formation -NHS (Cy dye), -NH_2 (CP) Amide bond formation -COOH (polymer), -NH_2(CP)	*83*
H_2N-CP-SH	PEG-NHS pPEGA-S-S-PDS	Amide bond formation -NHS (polymer), -NH_2 (CP) Disulfide formation -PDS (polymer), -SH (CP)	108
N_3-CP-$(Phe)_2$	PEG-CHCH Phe-COOH	1,3-dipolar cycloaddition -CHCH[b] (polymer), -N_3 (CP) Amide bond formation -COOH (Phe), -NH_2 (CP) n.b. Cucurbiturils were selectively bound to the Phe through host-guest interactions	*109*
N_3-CP-NH_2	pPEGA-NCO pBA-strained alkyne	Disubstitued urea bond -NCO (polymer), -NH_2 (CP) 1,3-dipolar cycloaddition -CHCH[b] (polymer), -N_3 (CP)	110
N_3-CP-NH_2	Cyanine 3/5-NHS CHCH-linker-NHS PEG-NH_2	Amide bond formation -NHS (Cy dye), -NH_2 (CP) 1,3-dipolar cycloaddition -CHCH[c] (linker), -N_3 (CP) Amide bond formation -NHS (CP-Cy3/5), -NH_2 (PEG)	78
N_3-CP-$CHCH_2$	pBA-NHS pS-SH	Amide bond formation -NHS (polymer), -NH_2 (CP) Thiol-ene reaction -SH (polymer), -CH-CH_2 (CP)	75
N_3-CP-$CHCH_2$	pBA-NHS pCHA-SH	Amide bond formation -NHS (polymer), -NH_2 (CP) Thiol-ene reaction -SH (polymer), -CH-CH_2 (CP)	*75*

1,3-dipolar cycloaddition was conducted through three different routes. [a]Microwave-assisted in the presence of CuAAc catalyst. [b]Pre-funtionalised of polymer with strained alkyne group. [c]After first amide coupling is completed, the cyclic peptide is pre-funtionalised with amine group by attaching linker through strained alkyne–azide reaction. Abbreviations: BA= n-butyl acrylate, ^{t}Bu= tert-butyl acrylate, S= Styrene, DMA= *N,N*-Dimethylacrylamide, EtOx= 2-ethyl-2-oxazoline, NHS= *N*-Hydroxysuccinimide, FluoresceinA= fluorescein acrylate, HPMA= 2-hydroxypropyl methacrylamide, PUEMA= (Pyridin-4-ylmethyl)ureido)ethyl)methacrylate, PEGA= poly ethyl glycol acrylate, DPA= 2-(Diisopropylamino)ethyl methacrylate, DMAEMA= 2-(Dimethylamino)ethyl methacrylate, DMAEA= 2-(Dimethylamino)ethyl acrylate, NAM= 4-Acryloylmorpholine, HEA= 2-Hydroxyethyl acrylate, ^{n}HexAm= n-Hexylacrylamid, LA= lauryl acrylate, NIPAM= N-isopropylacrylamide, CEMA= 2-chloroethyl methacrylate, I= isopyrene, TFA= trifluoro acetic acid, AA= acrylic acid, PDS= pyridyl disulfide, Cy= Cyanine, Phe= Phenyl alanine, NCO= isocyanate group, CHA= cyclohexane acrylate, BIB= α-Bromoisobutyryl initiating group, EGMA= Ethylene glycol dimethacrylate, MMA= methyl methacrylate, Pyr= Pyrene, Dap= Dapoxyl, PhosBA= phosphinobenzoic acid.

Dynamic behaviour of cyclic peptide-polymer nanotubes

Investigating the dynamic behaviour of cyclic peptide-polymer nanotubes

Since the conception of self-assembling cyclic peptide nanotubes, many have envisioned their promising use in bio-therapeutics. The advent of water-soluble cyclic peptide systems, made possible by the attachment of hydrophilic polymers, has enabled our group to work on realising their potential as drug delivery vectors. However, to explain the biological results and improve the design of these systems in the future, a better understanding of the self-assembly was needed. The foremost question was whether the cyclic peptide nanotubes were dynamic or non-dynamic assemblies, especially in the context of aqueous environments.[78]

Using the orthogonal chemistries discussed in detail above, self-assembling cyclic peptide with FRET dyes were developed to study their dynamic behaviour. Utilising FRET dyes on the periphery, which are proximity dependent, the mixing of dye conjugates could be used to directly inform us of the exchanging CPs between the supramolecular assemblies. In the context of cyclic peptides, Granja *et al.* previously employed FRET to study the hydrogen bonding interaction of small dimeric cyclic peptides.[86] As both the dye and polymer are essential to the monitoring and composition, a ‘bottom up’ approach was used to synthesise an asymmetric self-assembling peptide, with two different conjugation sites. The orthogonality of the azide and amine on the cyclic peptide enabled the selective attachment of both a dye and polymer to the periphery, a detail discussion of this can be found above.

Using these two model conjugates, PEG-CP-Cy3 and PEG-CP-Cy5 the dynamic behaviour of these supramolecular polymers in a range of different environments could be elucidated. This work exploited the non-radiative Förster resonance energy transfer (FRET) between the two dyes. For this energy transfer to take place, two important parameters must be met. First, the emission of the donor dye and the excitation of the acceptor dye must spectrally overlap. For this reason, the well-established donor and acceptor pair Cyanine 3 and Cyanine 5, which have high extinction coefficients were used in this study. Secondly, FRET will only take place if the donor and acceptor (Cy3 and Cy5 respectively) are close enough in space for the energy transfer to take place, typically 10-100 Å. The average distance between two CPs has previously been reported to be 4.7 Å and the pore diameter of the CP around 7.5 Å. Therefore, an energy transfer for the presented system is expected if both dyes are incorporated into the same nanotube upon mixing. When these conditions are fulfilled, the energy transfer translates into a decrease in donor emission and an increase in the acceptor emission. Importantly, the design and synthesis of these orthogonal conjugates are not limited to cyclic peptide; this method can be used to measure the dynamics of a host of supramolecular polymers.

The change in fluorescence emission was used to not only prove that the self-assemblies were rapidly disassembling and reassembling but also infer their rate of exchange and extent of mixing. Using this system, the solvent dependence on the rate of exchange between the cyclic peptide-polymer nanotubes was shown to be significant. In highly hydrogen bond competitive solvents, such as DMF, where the CP-polymer conjugates have been shown to be mainly unimeric, no net change in the FRET ratio is observed. The extent of FRET taking place can be defined by the change in the emission bands of the donor and acceptor, expressed as the following equation:

$$FRET\ ratio\ = \frac{I_A}{I_D + I_A} (1)$$

Where I_A and I_D are the total acceptor and donor fluorescence intensities, respectively, upon donor excitation.

Most interestingly, in water where the hydrogen bond competitivity of the solvent is less than in DMF, a dramatic increase in the FRET ratio over time was observed, and in turn a fast rate of exchange between the nanotubular aggregates. In further contrast, in toluene where there is no direct solvent competitivity with the hydrogen bonding sites of the peptide, the rate of exchange is significantly slower than in water. Furthermore, when the final FRET ratio, i.e. when the ratio reaches a plateaux, was compared to a premixed sample in the relevant solvent, the extent of mixing was very high, 90 and 88% in water and toluene, respectively.

This suggests near, but not full (100%), quantitative exchange of the unimers between the nanotubes.

Though anecdotally observed in static light scattering (SLS), the disassembly of the nanotubes upon dilution could not previously be confirmed. This is due to the fact that at higher concentration the effect of intermolecular interactions can affect the number of aggregation obtained via this technique. First, known concentration of Cy3 and Cy5 conjugates were made up in DMF and added to one another, upon removal of the DMF solvent and dissolution of conjugates in water, a premixed sample of a known concentration was obtained. Upon systematic dilution, the FRET emission was shown to decrease, confirming the concentration dependence of these cyclic peptide aggregates. This FRET emission was calculated by comparing the emission of the acceptor band upon excitation at the donor and acceptor independently. Notably, this FRET emission reached a plateau at 0.2 and did not reach 0, suggesting that after a certain concentration, further dilution led to no further net change in the size of the aggregates. This indicates that these systems could have a critical aggregation concentration and may follow a cooperative mechanism. The observation of a concentration independent region in the static light scattering of many of our systems also support this hypothesis. Efforts to elucidate this mechanism using isothermal titration calorimetry and UV-Vis studies are still ongoing.

Most importantly for the biological application of these systems, the dynamic nature of these conjugates was shown to take part inside the complex environment of cells. The combination of FRET and confocal microscopy showed that the different nanotubes could be independently transported into the same cell compartments upon sequential addition of the individual FRET-dye conjugates. This ability to recombine even under such conditions as present in living cells makes these materials appealing candidates as therapeutic vectors.

Controlling the dynamic behaviour of cyclic peptide-polymer nanotubes

With the insight gained from the model self-assembling studies, the next chapter of these systems looked at how this knowledge could be used to improve their intended applications. In a biological setting, many of these dynamic systems will be injected or delivered at low concentrations, where they are likely to disassemble. From the study of model PEG conjugated cyclic peptide nanotubes, the exchange behaviour of CP-nanotubes in water and *in vitro* showed the peptide unimers rapidly exchange between the self-assembled nanotubes. The highly dynamic nature of these supramolecular assemblies explains why many of these systems are very short in length, typically around 10 nm. The hydrophilic polymer arms decorating the peptide nanotube provide a steric barrier to prevent lateral aggregation and in turn improve their solubility in water, however dramatically lower their aggregation number.

For this reason, more recently efforts have focused on creating more stable supramolecular assemblies.[83] There are only a few examples of stabilised elongated uniform nanostructures using the concept of 'living' supramolecular polymerization, discussed in detail *vide supra*. Inspired by the stabilising core-forming block in micelles formed via crystallisation-driven self-assembly, a new generation of supramolecular cylindrical nanostructures with a stabilising hydrophobic region around the peptide nanotube were envisioned. Importantly, the hydrophobic interactions and region around the peptide core prevents the solvent/water molecules from competing with the peptide assembly, which in turn stabilises these aggregates. Moreover, the hydrophilic corona ensure that the nanotubes remain stabilised singular nanostructures in water. Unlike previous hydrophilic cyclic peptide conjugates which show fast dynamics and a low aspect ratio, by introducing a secondary hydrophobic driving force to stabilise the peptide assembly, the formation of more stable supramolecular polymers with lengths above 100 nm were observed.

The FRET exchange studies showed significantly slower exchange rates for the conjugates with the additional hydrophobic stabilising block. Notably, a significant plateau in the FRET ratio took almost 7 days to reach. Most interestingly, this final FRET ratio only reached 40% of the maximum FRET ratio, which was calculated from the premixed sample. Further studies conducted using high resolution stochastic optical reconstruction microscopy (STORM) showed the nanotubes were not readily exchanging but formed discrete supramolecular block copolymer-like structures. This process of understanding and designing plays an integral part to realising the potential biological applications of these, and other, supramolecular polymers.

Conclusion

The functionalisation of self-assembling peptide nanotubes, with polymers and other small molecules, have shown promising biological applications – such as antimicrobials, drug delivery vectors, and artificial ion channels. This viewpoint is a user's guide to synthesising and characterising peptide-based supramolecular conjugates, whilst also emphasising how improving our fundamental understanding of these supramolecular systems we can better design and tailor future systems. The article aims to demystify these complex supramolecular systems by explaining in detail their rational design. Here we highlight the versatility built into these peptide-based self-assemblies in the hopes of inspiring others to create new innovative functional materials using this peptide-based unimer.

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