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REVIEW

Recent Advances in Controlling the Internal and External Properties of Self-Assembling Cyclic Peptide Nanotubes and Dimers

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One of the most powerful strategies for the preparation of nanotubes is based on the stacking of flat-shaped cyclic peptide components. This strategy allows precise control of the nanotube internal diameter, the external properties and, more recently, the structural characterisitics of the internal cavity. The recent advances in these technologies and the applications of the resulting materials are described.

1. Introduction

Nanometre-scale tube-like structures (nanotubes) are amongst the most pursued nanocomponents based on the variety of proposed applications.[1-7] Most of the anticipated functions, which are inspired by the role of tubular structures in Nature, might be implemented in fields such as optics, electronics, catalysis, separation technologies, medicinal chemistry, molecular transport and sensing amongst others. The successful development of the most relevant applications depends on the ability to prepare these materials with a uniform internal diameter and to have precise control over the length. Although numerous advances have been made in the area of covalently assembled nanotubes,[8-12] the complexity of building nanostructures with suitable dimensions through the controlled formation of covalent bonds has led to the emergence of molecular self-assembly methodologies as powerful tools in nanotube technology.[13] These strategies involve the use of simple units that self-organize and assemble, under specific conditions, to form stable supramolecular structures in which all of the components are joined by non-covalent forces.[14,15] Hydrogen bonds are noteworthy because of their directionality, which facilitates the design of basic subunits for the supramolecular processes. Although hydrogen bonds are considerably weaker than covalent bonds, they determine many of the physical properties of polar compounds. To overcome the limitation in bond strength, the combination of several hydrogen bonds and other cooperative noncovalent bonding interactions is often required to construct stable supramolecular aggregates. In this way these weak interactions are used in the preparation of structures with defined composition, shape, physical, chemical and biological properties. In fact, self-assembly processes are currently amongst the most important bottom-up strategies to obtain functional nanostructures. This importance is due to the simplicity of developing highly convergent synthetic strategies, the built-in error correction and simple control through the design of the basic components.

Under the premises of molecular self-assembly, different approaches have been described to construct tubular structures, which include helically folded linear species, bundles of rod-like components, rolled-up molecular sheets, the juxtaposition of wedge-shaped molecules and the stacking of macrocycles (Fig. 1).[16] The latter strategy allows easy control of the internal diameter of the nanotube because it depends

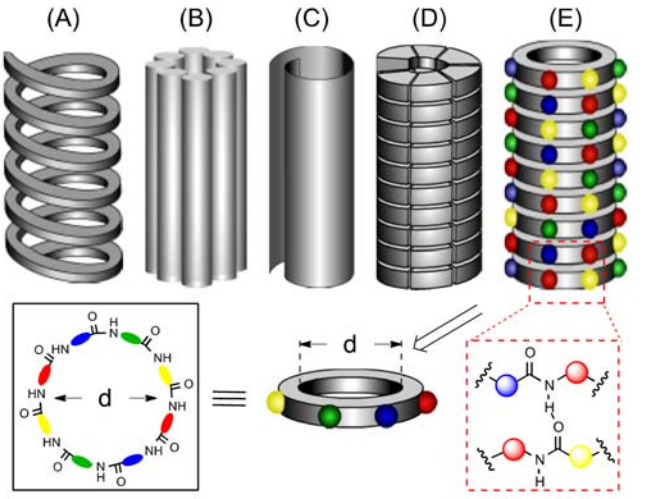

Fig. 1. Schematic representation of self-assembly strategies used in nanotube preparation: (A) helical folding of linear components; (B) assembly of rod-like molecules; (C) rolling-up of sheet-type structures; (D) assembly of sector molecules and (E) stacking of rings, such as cyclic peptides (bottom), whose stacking is favoured by the formation of hydrogen bonds between peptide backbones.

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only on the size of the stacked ring molecules.[17] In addition to the dimensions, the properties of the nanotubes determine both the internal and external topology, and the chemistry of the nanotubes. The type of material used also governs the hardness, biocompatibility, thermal, electrical and optical properties, etc.[3]

Following the previously cited strategies, different biomolecules (DNA, lipids, carbohydrates or peptides) have been used in the preparation of nanotubes.[6] Of these molecules, peptides are the most useful buildings blocks because they offer the possibility of preparing materials with defined properties depending on the selected amino acids and their disposition in the final supramolecular assembly.[18] For this reason, self-assembling cyclic peptide nanotubes (SCPNs) are amongst the most widely pursued nanostructures. This review covers the history and current situation in the design of self-assembling cyclic peptide nanotubes. In particular, the recent strategies that have been employed to control and change the external and internal properties of such nanotubes are reviewed. The control over the internal properties was achieved using γ-amino acids and, for this reason, in this review we place special emphasis on CPs that contain this type of residue.

2. General strategy and characteristics of self-assembly

SCPNs consist of homodetic cyclic peptides (CPs) that stack on top of each other to form a tubular structure through the formation of β-sheet-type hydrogen-bonding interactions.[16,19,20] Each peptide needs to have an even number of atoms in the backbone skeleton and it adopts a flat conformation. To control the flat conformation required for ring stacking, the chirality of each amino acid must be defined.[21] In this conformation all of the amino acid side chains point outwards in a pseudo-equatorial disposition, while the carbonyl and amide proton of the peptide bonds are oriented perpendicular to the plane of the ring. This special arrangement allows the formation of hydrogen bonds with other peptide subunits owing to the complementarity between hydrogen bond donor and acceptor groups on both sides of the disc structure (Fig. 1E). This conformation is the main requirement to consider for the design of nanotubes based on cyclic peptides. In this approach, the number and type of amino acids that constitute the CP determine the internal diameter of the nanotube. The characteristics of the outer surface depend on the properties of the side chains of the amino acids. Therefore, both characteristics can be easily modulated based on the appropriate selection of the CP sequence. For example, the solubility in lipophilic or aqueous media can be promoted by using residues with hydrophobic or hydrophilic side chains, respectively. The appropriate unit design and optimization of conditions for self-assembly allow the nanotube properties to be tailored for specific applications. In consequence, SCPNs can be used, for example, as ion channels or in other transmembrane functions, as antimicrobial and cytotoxic agents or as solid-supported ion sensors. [6,16,22]

According to this design, a variety of peptide nanotubes with wide structural diversity have been developed.[19,22]

2.1 Tubular ensembles of cyclic *D*,*L*-α-peptides (*D*,*L*-α-CPs)

The first peptide nanotubes were prepared by Ghadiri's group, who synthesized a cyclic octapeptide {*c*-[(*D*-Ala-*L*-Glu-*D*-Ala-*L*-Gln)$_2$-]} in which α-amino acids of opposite chirality (*D*, *L*) were alternated (**CP1** in Fig. 2A).[23] The formation of the nanotube was possible due to the pH-dependent ionization of the glutamic acid side chain. Thus, the peptide was soluble in basic aqueous media but slow acidification of the solution led the CP to self-assemble into microcrystalline aggregates formed by ordered hollow tubes with internal diameters of 7.5 Å. Each tube was formed by cyclic units stacked through antiparallel β-sheet-type hydrogen bonding (Fig. 2A). Furthermore, one year later control of the diameter was confirmed by the preparation of nanotubes with an internal diameter of 13 Å with the peptide *c*-[(*D*-Ala-*L*-Glu-*D*-Ala-*L*-Gln)$_3$-] (**CP2** in Fig. 2A).[24] The media-induced structural changes, such as those observed on changing the pH, represent another remarkable feature of peptides, which makes them especially attractive for the development of smart materials.[25-27]

Other SCPNs have been prepared using basic residues combined with hydrophobic residues in their skeleton,[28-32] or even amphiphilic or hydrophobic peptides.[20,33] Each of these nanotubes has different characteristics due to the aforementioned orientation of the side chains towards the outer surface.

A more precise structural understanding of the SCPNs was achieved through the preparation of the simplest tubular structures, namely the toroidal dimers.[34,35] These systems were obtained by using CPs in which all the amino acids of the same chirality were *N*-alkylated. Consequently, the alkyl groups are projected to the same peptide face of the ring, thus blocking the assembly properties through this face. It was concluded from these studies that the best rings to form nanotubes are the octa- and hexa-peptides. Deca- and dodeca-peptides fail to dimerize because of the difficulties in adopting the required flat conformation. Cyclic tetrapeptides did not lead to the corresponding dimers, probably due to conformational restrictions that prevent the appropriate exposure of the hydrogen bonding donors and acceptors of each CP. The main limitation of this procedure is the steric hindrance between the carbonyl and *N*-alkyl group, which destabilises the flat conformation. Consequently, non-methylated CPs might form nanotubes even though the *N*-methylated counterparts did not form the corresponding dimers – for example, decamers and dodecamers have been shown to form SCPNs.[24,30]

2.2 Tubular ensembles of other cyclic peptides

The results described above encouraged various authors to extend the principles of nanotube formation to other cyclic platforms that contain other residues, such as β-,[36-38] δ-[39] or ε-amino acids.[40] The resulting SCPNs have new properties derived from the structural characteristics of the basic units. Several reviews concerning these systems have been

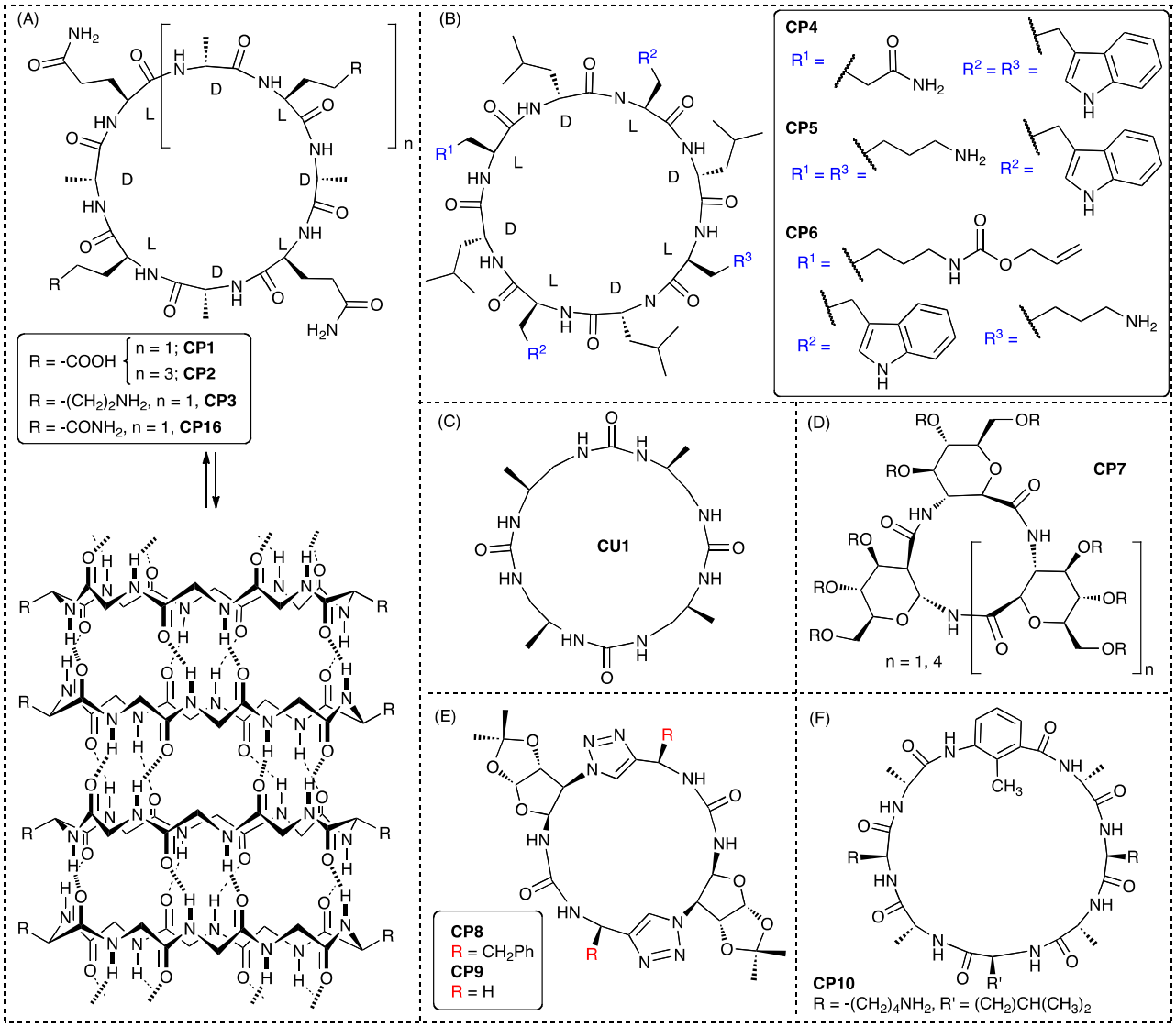

Fig. 2. (A) Schematic representation of the nanotube (bottom) formed by self-assembly of *D,L*-α-CPs (top, **CP1-3** and **CP16**). Structure of (B) different membrane-interacting *D,L*-α-CPs (**CP4-6**) described in this work, (C) a cyclic tetraurea (**CU1**), (D) saccharide-like β-CPs (**CP7**), (E) triazole/urea peptidomimetic macrocycles (**CP8** and **CP9**) and (F) an α-CP containing a γ-amino acid (**CP10**).

published.[6,16,19,20,41] Nanotubes with saccharide-like external surfaces (**CP7**, Fig. 2D), high anisotropy or synthetic ion channel properties have been produced using these types of peptides. γ-Amino acids were also used for the preparation of SCPNs as will be discussed in the next subsection.

Based on the same stacking model, cyclic ureas (Fig. 2C) were also used as precursors of nanotubes.[42-45] The formation of the tubular structure also involves the flat conformation of the macrocycle, with carbonyl groups and amide protons arranged perpendicular to the ring plane, thus facilitating the formation of hydrogen bonds between the neighbouring subunits. In this case, each carbonyl moiety is generally hydrogen bonded to two protons and this provides stronger inter-subunit interactions. The pioneering work of Guichard and co-workers, in which α-Aas were replaced by ureas in a cyclic peptide sequence (**CU1**, Fig. 2C), demonstrated the formation of the tubular structure.[42] Following this strategy, anion transporters,[43] guest gas absorbents[44] and electron donor nanotubes,[45] amongst other systems, have been described. Ureas were also combined with triazole rings to prepare peptidomimetic macrocycles from carbohydrate-derived precursors. Depending on the chirality introduced in the peptide backbone, the macrocycle can self-assemble to form antiparallel dimers (**CP8**, Fig. 2E) or parallel nanotubes (**CP9**, Fig. 2E).[46]

2.3 Tubular ensembles of cyclic α,γ-peptides (α,γ-CPs)

In recent years, numerous examples of peptide structures containing γ-amino acids have been described.[42] The diversity and structural complementarity of these compounds with the α-amino acids make them useful building blocks for the preparation of functional biomaterials.[47] The cyclic γ-residues are particularly interesting because they provide new opportunities to functionalize both the internal and the external surfaces of the nanotube.

Our group was the pioneer in studying this type of CP, specifically using *cis*-3-aminocycloalkanecarboxylic acids (γ-Aca). The cycloalkane moiety confers the rigidity that enables

the peptide to adopt the flat conformation required to stack to form the SCPN (Fig. 3). One of the advantages of the incorporation of γ-residues is the orientation of one methylene group (β-carbon) of the cycloalkylidene moiety of each γ-Aca towards the internal cavity of the ensemble, thus creating a hydrophobic region. The simplest way to design self-assembling CPs of this type is to use nanotube-forming *L,D*-α-CPs and replace any α-Aa residue with a γ-Aca of equivalent chirality (Fig. 3). For this purpose, (1*R*,3*S*)-γ-Aca can be considered as a structural equivalent of the *L*-residues while (1*S*,3*R*)-γ-Aca is equivalent to the *D*-amino acids.[48] Based on this equivalence a variety of CPs can be designed with different numbers and positions of γ-Aca residues. Therefore, CPs formed by alternating α- and γ-residues (α,γ-CPs, **CP11a/b**),[49-51] with these residues combined in a 3:1 ratio (3α,γ-CP, **CP12**),[48] or made exclusively with γ-Acas (γ-CP, **CP13**)[52] were prepared.

Initial studies were carried out with CPs composed of alternating α-amino acids and *cis*-3-aminocyclohexanecarboxylic acid (γ-Ach, Fig. 3, n = 2)[49-51] or *cis*-3-aminocyclopentanecarboxylic acid (γ-Acp, Fig. 3, n = 1).[53] This alternating arrangement of amino acids leads to two non-equivalent faces of the disc-shaped conformation, i.e., the α- and γ-faces, depending which amide groups are projected towards each face. The different distances between the amide proton and the carbonyl group of α-amino acids compared to the γ-residues cause α-faces to interact with α-faces and γ-faces with γ-faces in an antiparallel fashion. Therefore, two different types of interactions (α-α and γ-γ) are generated in an alternating manner along the SCPN structure (Fig. 3). Studies into the thermodynamics of these interactions were carried out using models based on *N*-methylated CPs.[49] In general, the α-α-interactions were stronger than the γ-γ, perhaps due to the steric interactions between the methyl and carbonyl groups in the *N*-methylated α-amino acids.[50,51,54] The ability to control the diameter by this approach, especially for Acp-based cyclic peptides, was confirmed through the synthesis and study of tetra-, hexa-, octa-, deca- and dodecapeptides.[48-53,55-56] The internal diameter of these CPs ranged from 4 to 17 Å and they form, apart from the tetrapeptides, the corresponding dimers in non-polar solvents with large association constants. For Ach-based CPs control of

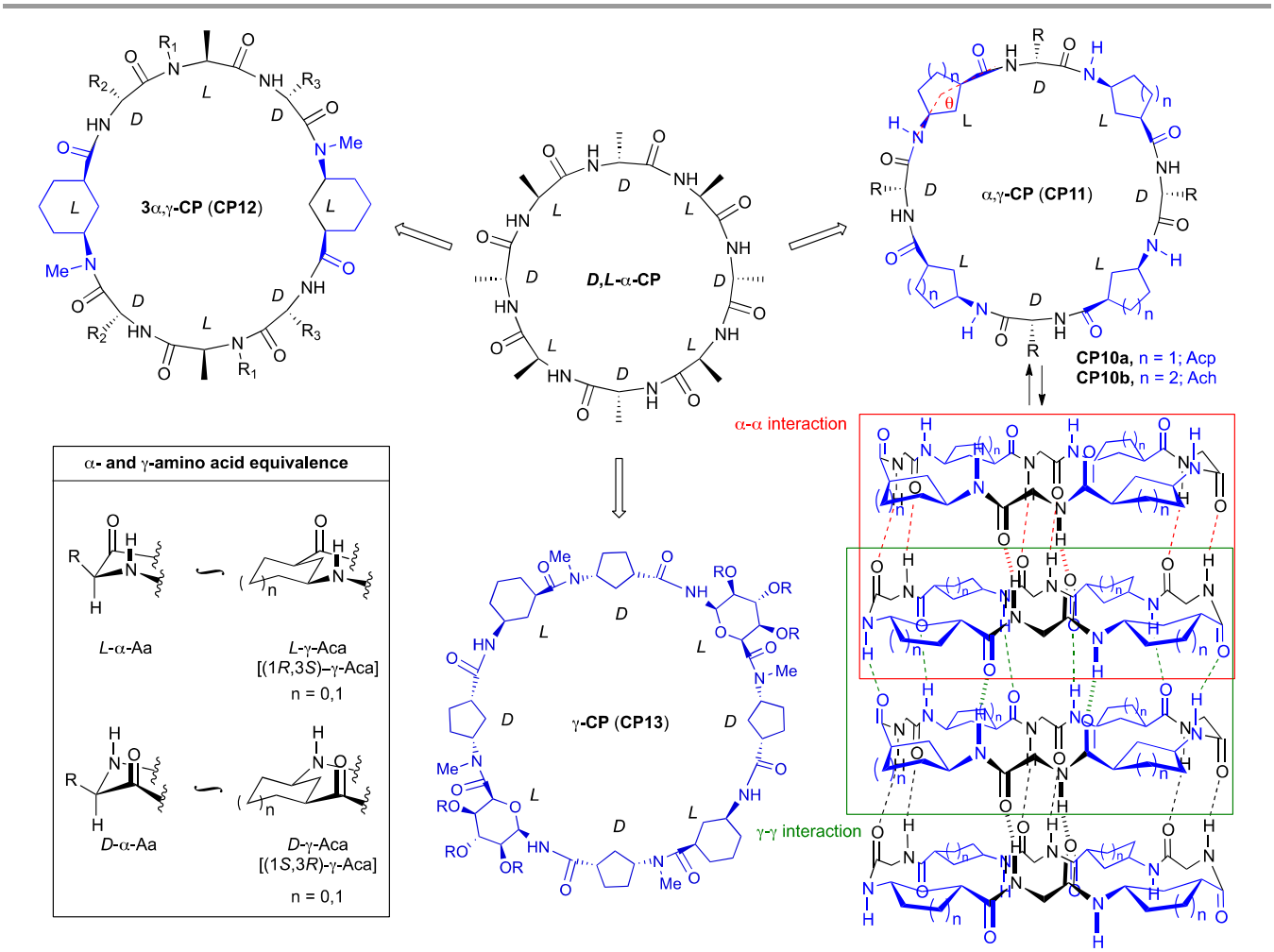

Fig. 3. Equivalence of α- and γ-amino acids (bottom left) and design of novel nanotube-forming CPs by exchanging two (**3α,γ-CP**, **CP12**), four (**α,γ-CP**, **CP11**) or eight (**γ-CP**, **CP13**) α-amino acids (in black) for the equivalent γ-Acas (in blue) of a *D,L*-α-CP. Schematic representation of the nanotube (bottom right) formed by the self-assembly of α,γ-CPs. The two types of hydrogen bond interactions involved in nanotube formation are highlighted (α-α in red, and γ-γ in green).

the diameter is limited to tetra-, hexa- and octa-peptides due to the rigidity and smaller cone angle (θ) of the cyclohexyl moiety.[48-51] Heterodimeric assemblies, in which Ach-based CPs interact with Acp-based CPs, were also prepared and interestingly they are even more stable than the corresponding homodimers (Fig. 4).[55-56] A remarkable aspect of this process is that heterodimer formation is mainly due to the interactions established at the level of the peptide backbone and not by the side-chain/side-chain interactions. This selectivity in heterodimer formation allows the modification of the side-chain properties and the anchoring of diverse groups to generate different functions, such as electron or energy transfer active systems (Fig. 4).[56-58] Additional information about the applications of this type of SCPNs will be provided in the following sections.

As mentioned above, other α- and γ-amino acid combinations are also possible and these allow more precise tuning of the assembly diameter and properties of the pore. Considering the aforementioned correspondence between α- and γ-amino acids, another class of SCPN-forming CP was prepared in which both types of residues were combined in a 3:1 ratio (3α,γ-CP (**CP12**) in Fig. 3).[48] The studies carried out with the latter dimeric models highlighted the structural role played by methyl groups, since the self-assembly process depended on the number and position of these groups. Some monomers can remain in the flat ring conformation and dimerize through antiparallel β-sheet-type hydrogen bonding or fold into double reverse turns. Finally, CPs composed by only γ-amino acids of

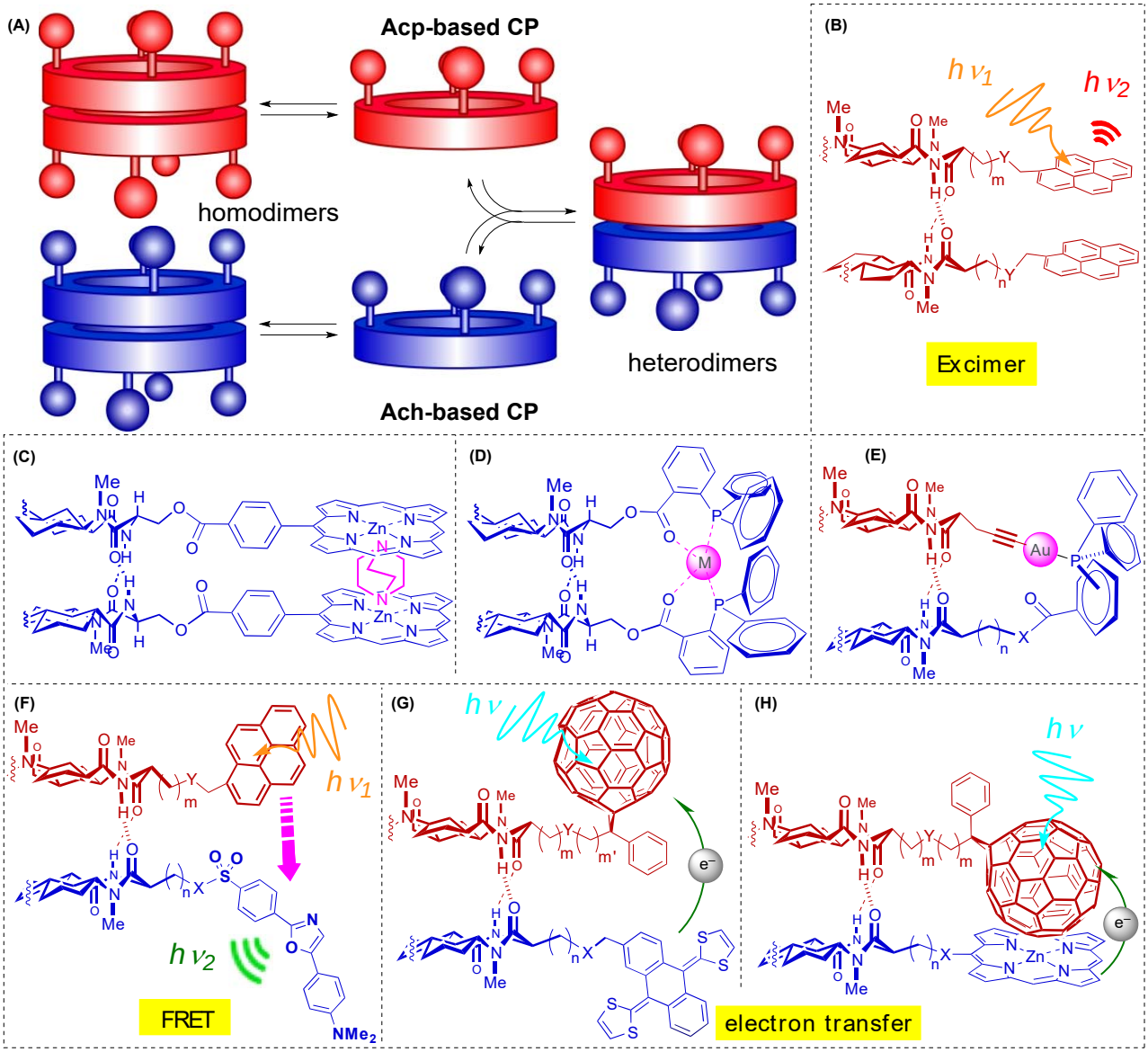

Fig. 4. (A) Selective formation of heterodimers through the combination of Acp- and Ach-based CPs. (B) Model of excimer-emitting homodimer. (C) Model of the selective recognition of diamino ligands (porphyrin-DABCO-porphyrin) with homodimer tweezers. (D and E) Models of the homo/heterodimer formation by means of the coordination with metals. (F) Model of heterodimers of CPs bearing pyrene and dapoxyl groups for efficient energy transfer process (FRET). (G and H) Model of the supramolecular donor-acceptor systems (tetrathiafulvalene-fullerene) and (Zn-porphyrin-fullerene) based on heterodimeric self-assembly in electron transfer processes.

alternating chirality were also prepared (Fig. 3). Interestingly, the self-assembly properties of the γ-CPs were shown to depend on the ring size. While tetrapeptides self-assemble into nanotubes through hydrogen-bond-mediated parallel-type interactions,[59] the γ-octapeptides (**CP13**, Fig 3) dimerize through an antiparallel β-sheet interaction.[52]

3. External functionalization of SCPNs

3.1 Tuning of external properties using peptide sequences

As mentioned above, the appropriate selection of amino acid side chains allows the modulation of the properties of the SCPN outer surface. In this way, the CPs can be designed to interact with phospholipid bilayers to induce nanotube formation by using hydrophobic residues. Thus, Ghadiri's group created the first transmembrane nanotube able to self-assemble in a lipid bilayer, by selecting hydrophobic side chains on the cyclic octapeptide *c*-[*L*-Gln-(*D*-Leu-*L*-Trp)$_3$-*D*-Leu-] (**CP4**, Fig. 2B). The resulting channels displayed transport activities for potassium and sodium ions greater than 10^7 ions s^{-1}.[29] The effect of the amino acid composition of octapeptides formed by four *D*-Leu and four different *L*-amino acids was recently explored in vesicles.[60] The authors found that CPs bearing charged side chains formed large pores while neutral CPs generated monomeric pores. Nanotube bundles derived from amphipathic CPs that have side chains with an amine group (**CP5** and **CP6**, Fig. 2B) gave larger pores. These pores were characterized by their calcein transport activity. The alignment of SCPNs in nanostructured ionic liquids has been reported for similar cationic amphipathic CPs, such as the cyclic octapeptide *c*-[(*L*-Gln-*D*-Ala-*L*-Lys-*D*-Ala)$_2$-] (**CP3**, Fig. 2B).[61] The process is based on the use of the structured domains of ionic liquid crystalline gels to align the CP by itself. The use of this strategy allows the preparation of supported liquid membranes with ordered domains that contain channels. An alternative strategy used to obtain the vertical orientation of the SCPNs involves using the layer-by-layer deposition technique.[62] The resulting molecular tube showed efficient charge transfer properties with a low current attenuation constant. This strategy might provide the opportunity to control precisely the electronic properties at the molecular level through the selection of a suitable sequence of CPs to form the nanotube stack.

These artificial transmembrane single channels are size selective to polar molecules.[30] It was shown that molecules such as glucose or glutamic acid can migrate from one side of the bilayer to the other through the internal cavity of decapeptides, while the octapeptides were not active. The decapeptide was also tested for the transport of antitumour drugs.[63] The authors employed liposome models and found that small (<1.0 nm) polar anticancer drugs can be transported through the phospholipid bilayers in a size-selective and dose-dependent manner. The fastest diffusion rate was observed for 5-fluorouracil (5-FU), the smaller drug studied. The combinatory use of 5-FU and a *D*,*L*-α-CP enhanced the antitumour potency both in cell lines and *in vivo*. The authors proposed, based on computational methods, that the drug was transported by a hopping mechanism through the internal pore of the SCPNs.

The ion transport properties can be modulated by incorporating charged cyclic peptides at the end of the nanotube (molecular cap), as exemplified by the generation of one of the first heteromeric noncovalent transmembrane channels with altered transport characteristics.[64] In this way, rectification, i.e., non-ohmic current-voltage relationship, was observed when an ionic peptide was added to only one side of the membrane. The observed values were consistent with the addition of the polar CP at the channel entrance. For example, the addition of anionic caps led to an increase in conductance. Cyclic β-peptides were also studied as ion channels and these showed similar conductivity values to the equivalent *D*,*L*-α-CPs.[37] More recently, our group has demonstrated that α,γ-CPs can also be inserted in lipid membranes to form channels, which were able to transport alkali metal ions despite having a partially hydrophobic cavity.[65]

All these studies demonstrate that when the amino acid sequence is dominated by hydrophobic amino acids, the resulting nanotube is oriented nearly parallel to the alkyl chains of phospholipids, as required for a transmembrane channel. However, SCPNs formed with amphipathic cyclic peptides lie parallel to the lipid membrane and these provide antimicrobial properties.[22,33] FTIR studies confirmed the mechanistic prediction of the peptides in the membrane.[66] It was also observed that amphipathic and cationic *D*,*L*-α-CPs, which have structural similarities to amyloid plaques, can act as inhibitors of Alzheimer's disease by preventing the aggregation of Aβ proteins into toxic forms.[67] More recently, similar results were described for the inhibition of the aggregation of α-Synuclein, a process associated with Parkinson's disease.[68]

SCPNs made of CPs that contain saccharide-derived β-amino acids were prepared (**CP6**, Fig. 2D), thus modifying the external properties of the nanotubes to favour interaction with lectins.[38] A cyclic β-CP composed of a β-naphthylalanine, two β-alanines, and an ethylenediamine-succinic acid linker was also used in the peptide nanotube formation. The resulting molecular assemblies showed exciton coupling of the Cotton effect and predominant monomer emission, which suggests that the side chains of the SCPNs are aligned in a helical way.[69]

3.2 Small molecule covalent functionalization

The side chains can be used as anchor points for the attachment of other functional molecules without interfering with the self-assembly process. Therefore, the conjugation of different molecules to the periphery of the nanotube-forming CPs expands the properties of SCPNs. In this sense, Ghadiri's group prepared CPs that are analogous to the natural antimicrobial agents mannopeptimycins by attaching saccharides onto the side chain. In the resulting CPs the specific glycosylation of membrane-active *D*,*L*-α-CPs reduced mammalian cell toxicity while maintaining the bactericidal potency.[70] The same group prepared CPs containing 1,4,5,8-naphthalenetetracarboxylic diimide (NDI) attached to the side

chain of a lysine residue.[71] Fluorescence studies carried out with these dimer-forming CPs allowed the assembly process to be studied and showed that steric interactions between the side chains play an important role in the dimerization process. A nanotube-forming CP bearing four NDI units self-assembled into electronically delocalized nanotubes that were hundreds of nanometres in length due to the alignment of the NDI moieties promoted by peptide-peptide interactions.[72] Thus, intermolecular peptide self-assembly is an effective template for the fabrication of one-dimensional materials with potential applications in optical and electronic devices.

Fullerenes were also employed to modify the external surface of peptide nanotubes and potentially to generate 1D molecular wires. In this case α,γ-CPs coupled to one C_{60} moiety were able to self-assemble into nanotubes in which the fullerenes were pointing outwards on both sides of the tube (180° orientation) to form 1-D arrangements.[73] The presence of C_{60} helped in the characterization of the tubular ensembles by STM, which showed two parallel wires of 1 nm height. More recently, cyclic β-peptides bearing a tetrathiafulvalene (TTF) in the side chain were also prepared.[74] These peptides self-assembled to form nanotubes in which all the TTF groups are aligned. The resulting crystals showed typical features of p-type semiconductors, with two conductive states observed depending on the applied potential.

Based on the homodimer-heterodimer equilibria discussed above, light-harvesting/light-converting ensembles have been developed with a characteristic organization of donor and acceptor units that were able to act as efficient artificial photosystems. For this purpose CPs bearing a fullerene moiety were used (Fig. 4F-G).[57] The heterodimer was composed of an α,γ-cyclic hexapeptide bearing an electron-donor unit (π-extended tetrathiafulvalene derivative) coupled by a β-sheet-like hydrogen-bond system to the second CP, which bears the photoactive electron-acceptor unit (fullerene moiety) (Fig. 4F). More recently, a similar system was prepared but a Zn-porphyrin moiety was used as the electron-donor group (Fig. 4G).[75] The major difference observed in these studies was the existence of an attractive interaction between the porphyrin and the fullerene component, which stabilized the heterodimer ensemble with both groups remaining in close proximity in space. The formation of this ensemble increases the efficiency of the through-space electron transfer process. The CP-porphyrin hybrids were also used with the aim of controlling the homodimer shape (β-sheet register) by external chemical signals.[76] The ability of Zn-porphyrins to coordinate amines, such as pyridines or tertiary amines, was used to develop molecular tweezers that interact with diamines such as DABCO or bipyridines (Fig. 4D). This interaction induces the formation of the dimer in which the porphyrin moieties stack on top of each other. Alternatively, the addition of metals that coordinate the ligand moieties attached to the CPs was used to control the formation of the homo- or hetero-dimer species that facilitates the interactions (Fig. 4D and E).[77]

A multicomponent network of pyrene and dapoxyl-derivatized CPs was prepared by controlling the homo-/heterodimer equilibria (Fig. 4F).[58] The system takes advantage of excimer or fluorescence resonance energy transfer (FRET) effects to follow the self-assembly process to generate different supramolecular combinations with specific signal output (Fig. 4B and 4F). Similar results were obtained when the pair pyrene/perylene pair was used with octapeptides.[56]

Furthermore, β-CPs containing saccharide residues were modified to incorporate a DNA base in the side chain of a homoaspartic acid (**CP14**, Fig. 6A).[78] The tetra-β-CP was composed by three β-glucosamino acids and the aforementioned β-homoaspartic acid with the guanine moiety attached at its side chain. This nucleobase forms a hydrogen bonded G-quartet in the presence of metal ions, mainly potassium.[79] The authors used this property to induce self-assembly of the CP in water upon the addition of potassium chloride to generate bundles of four nanotubes through the formation of G-quadruplexes.

3.3 Macromolecule covalent functionalization

Polymers have been conjugated to *D*,*L*-α-CPs in order to change the nanotube properties.[80-95] These macromolecules not only offer the possibility of introducing a wide variety of functionalities on the outer surface of the nanotube but also provide some control over the length by preventing the unlimited stacking of peptides. Different strategies have been developed for the preparation of hybrids of CPs and polymers and these approaches can be performed either before or after SCPN formation.

In 2005, Biesalski's group published the first example of a nanometre-sized peptide-polymer hybrid structure.[80] The strategy was based on the preparation of peptide nanotubes from a *D*,*L*-α-CP conjugated to a polymer initiator group. Upon SCPN formation the subsequent surface-initiated polymerization around the nanotube coated it with a covalently bound polymer shell (Fig. 5A). The same authors later investigated the effect of modifying the polymer molecular weight and they proved that while the external diameter increases with molecular weight, the length decreases, probably due to the increased bulkiness of the polymer.[81] Furthermore, it was found that this effect was independent of the polymer type [poly(*n*-butyl acrylate), poly(N-isopropylacrylamide) or polystyrene]. More recently, another grafting from approach was used to prepare cross-linked polymeric nanotubes by growing ethylene glycol dimethacrylate from SCPNs.[82] The combination of solvents plays an important role in the assembling properties of the CP. For example, the use in the polymerization process of DMSO instead of mixtures of acetonitrile/toluene or isopropanol/toluene led to the divergent growth of polymers from the peptide prior to self-assembly of the aggregate (Fig. 5A).[80,83] Light-induced polymerization was achieved using a β-CP bearing a diacetylene unit at the side chain to form SCPNs with a polydiacetylene chain along the tube, with a length of ca. 100 nm, that might have electronic applications.[84] Similar structures were obtained when the polymer was grafted from the CP before or after nanotube formation.

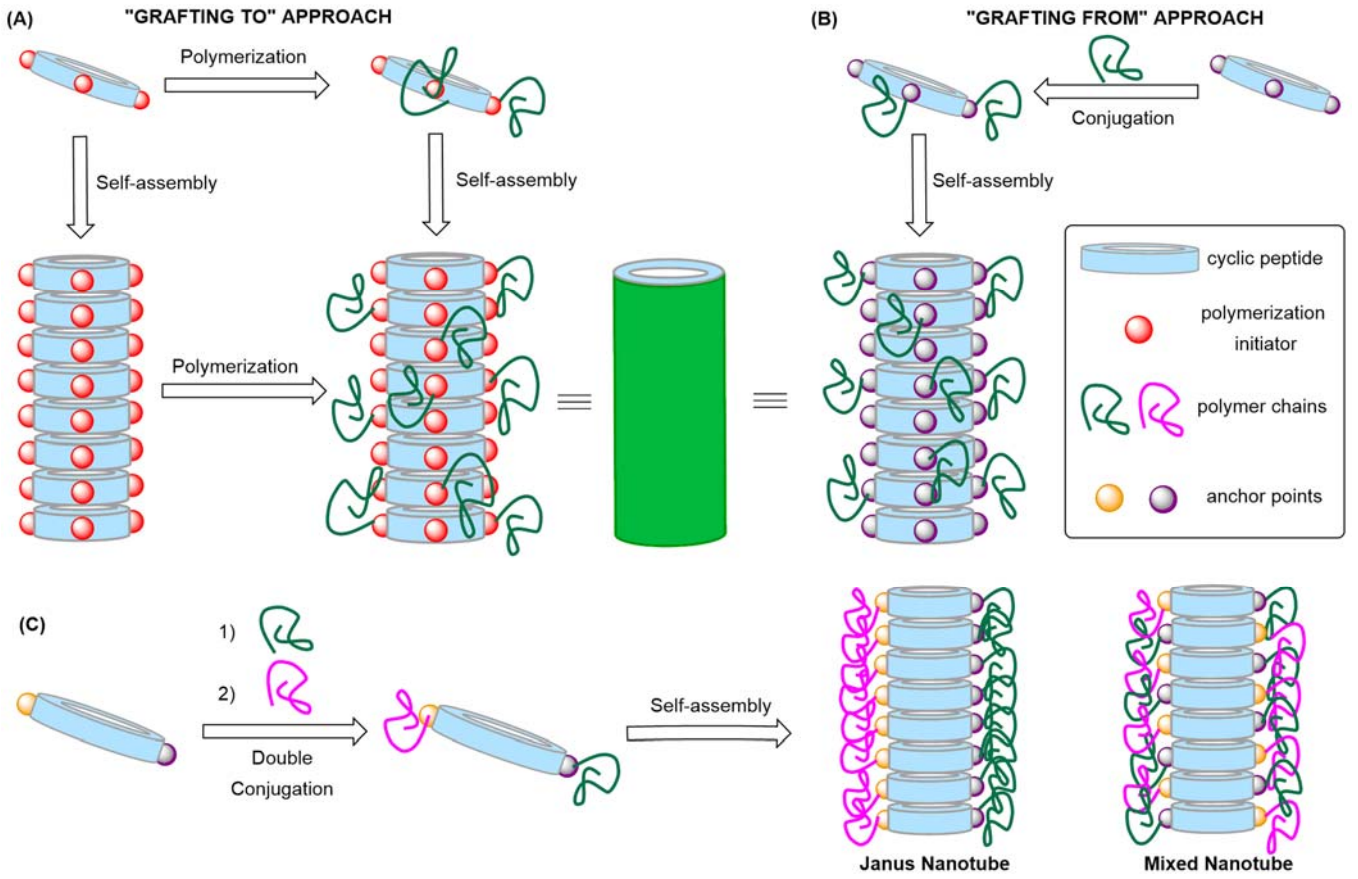

Fig. 5. Scheme for the synthesis of peptide-polymer hybrid structures, following "grafting to" (A) or "grafting from" (B) strategies. (C) Model for the formation of unmixed nanotubes with two different faces (Janus) or mixed nanotubes that depend on the polymer properties.

Grafting to approaches were also employed in the preparation of other peptide-polymer conjugates (Fig. 5B). As Perrier´s group reported recently, both strategies (grafting to and from) generally lead to similar peptide polymer conjugates.[85] For this purpose two poly(*n*-butyl acrylate) chains bearing a free carboxylic acid group were attached through an amide linkage to two Lys located on opposite sides of an eight-residue *D*,*L*-α-CP.[86] More recently, the same group studied the molecular self-assembly of *D*,*L*-α-CPs conjugated to polymers of precise length by a copper-catalysed azide-alkyne cycloaddition reaction.[87] The direct characterization by small angle neutron scattering of the self-assembly process in solution had already been reported.[88] The direct observation of the nanotube formation already showed that control of the solvent mixture, the polymer length and the temperature have a direct impact on the SCPN length. In this context, the self-assembly process of these conjugates in water has also been studied and it was possible to control the aggregation of these supramolecular assemblies by introducing pH-responsive polymeric components.[89] These tailor-made responsive peptide nanotubes might have pharmaceutical applications in gene delivery vectors or as antimicrobial agents.

This strategy allows the introduction of different numbers and types of polymers and also enables the post-modification of the conjugated polymers even after SCPN formation. For instance, the authors reported the use of these materials as carriers for a ruthenium-based anticancer drug.[90] The conjugation of the ruthenium complex with the polymers that form the shell of the SCPNs led to a system with increased cytotoxicity in comparison with the free drug. These results evidence an efficient uptake of the drug and prove that these hybrids are suitable drug carriers. Peptide-polymer conjugates that are capable of self-assembling in lipid bilayers to form artificial channels were also reported. [91,92] A system based on poly(N-isopropylacrylamide) conjugates allowed on-demand control over bilayer channel formation owing to the polymer properties.[91] Another attractive hybrid material has been described for the preparation of nanotubes with dual functionality in the form of either a Janus or mixed polymeric corona (Fig. 5C).[92] The authors developed an orthogonal double functionalization strategy to attach two different polymers in a stepwise manner. The supramolecular ensembles have quite unique structures that combine the nanopore properties of the CP with those derived from the two polymers. The use of immiscible polymers attached to the CP generates Janus nanotubes that form artificial pores made of bundles of these tubes via phase segregation.

Xu and co-workers prepared microporous materials by combining *D*,*L*-α-CPs with block copolymers. [93,94] They also employed a grafting to approach to prepare peptide-polymer

conjugates that were dissolved in a pre-templated block copolymer thin film. A heating-cooling cycle of the material led to the self-assembly of CP-polymer hybrids within the block copolymer matrix to form nanochannels through the polymeric film. These channels were tested for gas transport and it was confirmed that diffusion rates increased with decreasing molecular size of the gas, with carbon dioxide diffusing more rapidly than neopentane. Robust polymer pores with carboxylate groups inside were developed using SCPN templates (Fig. 6B).[95] For this purpose a tetranorbornene-functionalized CP (**CP15**) was polymerized to provide nanotube bundles of around 100 nm in diameter. Finally, hydrolysis of the ester linkage between the CP and the norbornene polymer provided films with pores that were rich in carboxylic acid groups. Granja's group prepared porous liquid crystals by employing dimer-forming α,γ-CPs with mesogenic dendrons attached to the side chains (Fig. 6C).[96] The strategy combines the hydrogen bond interactions between amide groups to form dimers with the mesogenic driving forces promoted by appended dendrons. The packing properties of these materials depend on the number of alkyl chains that contain the attached dendrons. The less ramified dendrons formed two parallel-paired channels while the more highly ramified component provided single pore mesophases. These hybrid materials could be used for the preparation of nanostructured porous materials with controlled pore size (CP diameter) and distribution (mesogenic dendrons).

3.4 Non-covalent functionalization

In addition to covalent modifications, supramolecular external nanotube modification was also studied. This strategy allows the transformation of the properties of SCPNs in a reversible and media-dependent manner. One of the most remarkable achievements was the preparation of hybrids of SCPNs and single-walled carbon nanotubes (SWCNTs).[97] In this work, our group used an α,γ-CP with a pyrene moiety attached to a Lys side chain. This arene is known to interact with carbon nanotubes through π-π stacking interactions.[98] Therefore, these CPs were able to interact with carbon nanotubes and solubilize them in aqueous media. The SWCNTs template the assembly of CPs to form hybrid structures that displayed symbiotic properties, such as enhanced solubility in aqueous media and the electrical conductivity or hardness characteristic of SWCNTs. The nanotube couples were deposited onto mica surfaces and their topography,

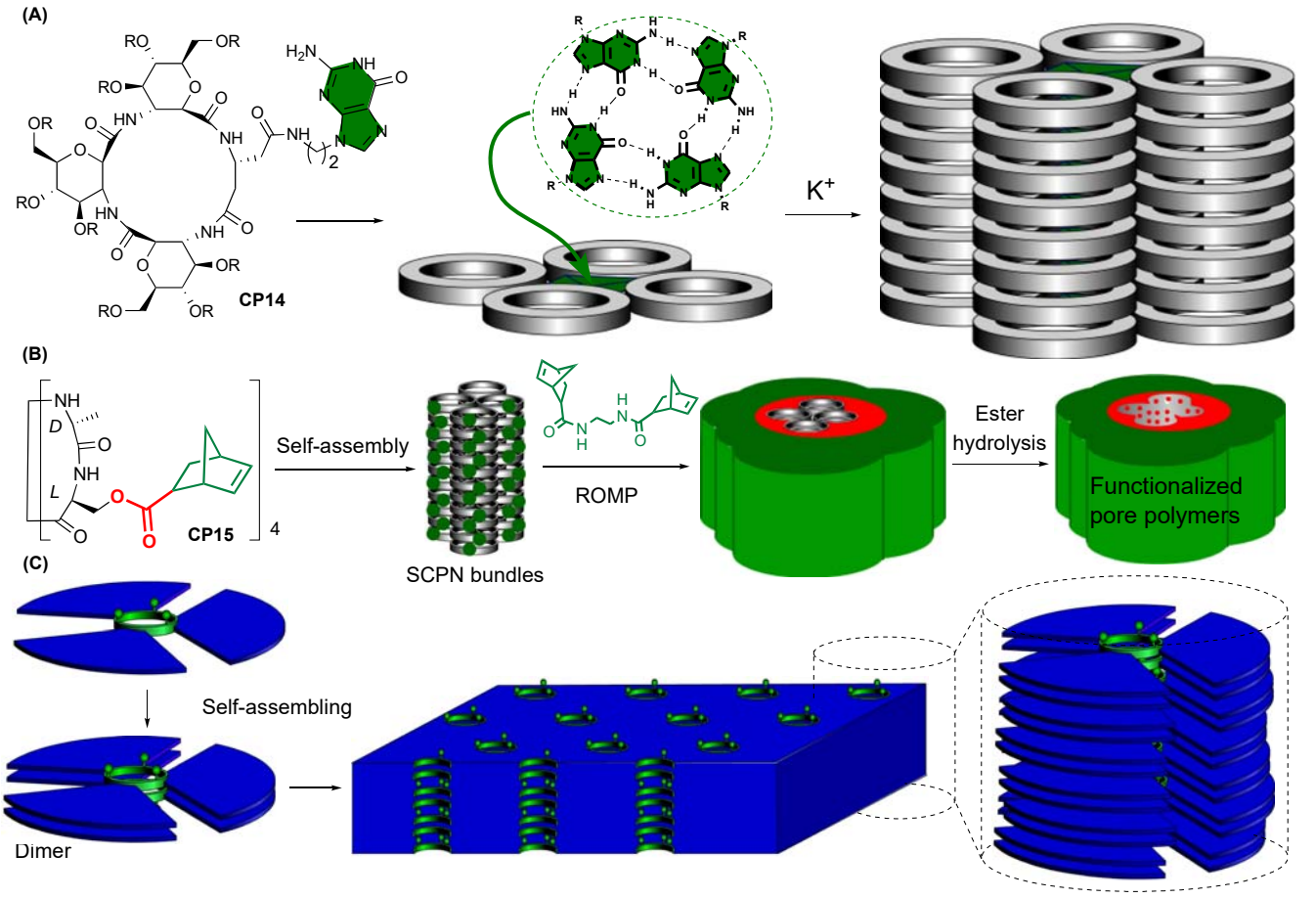

Fig. 6. (A) Model for the self-assembly process of β-CPs (**CP14**) that incorporate a guanine base in one of the peptide chains. The formation of guanine quadruplexes led to the assembly of bundles comprised of four nanotubes aligned in a parallel manner. (B) Model for the preparation of porous polymers through the self-assembly of **CP15** in a block copolymer matrix. (C) Model for the formation of porous liquid crystals using dimer-forming α,γ-CPs conjugated to mesogenic dendrons.

conductivity and elasticity were determined. In addition, nanotube alignment was also observed due to the formation of ordered domains of nanotubes upon evaporation of the couple. Topological defects characteristic of nematic materials, similar to those reported for liquid crystals,[61] were found for concentrated solutions in water/acetonitrile at 5 °C. We also prepared other hybrid materials using novel non-covalent interactions between subnanometric metal(0) silver clusters (SNMCs) and the aromatic (pyrene) moieties.[99] Atomic Force Microscopy (AFM) experiments showed that SNMCs preferentially align on top of the peptide nanotubes. The cluster deposition depends on the concentration and exposure time of the pyrene-modified SCPNs to the aqueous solution of the cluster mixture.

In supramolecular approaches SCPNs have been used in drug delivery. For example, SCPNs loaded with doxorubicin were used for the treatment of breast cancer.[100] In this work, bundles of nanotubes were prepared from hydrophilic CPs containing a cysteine residue. Once loaded with the antitumoural drug, the outer surface of the bundles was modified with a PEG to reduce the toxicity. These supramolecular aggregates led to improved antitumour properties of the drug (cytotoxicity, intracellular absorption and distribution). Alternatively, positively charged SCPNs have been used in gene transfection.[101] For this purpose an octapeptide containing four Lys was modified by the incorporation of a guanidiniocarbonyl pyrrole (GCP) moiety. This weakly basic Arg analogue modified the assembly properties of the CP and caused them to assemble into long cationic aggregates. DNA was attached to the nanotube bundles and they act as vectors in gene delivery to transfect HeLa cells without the addition of any other additive. Studies on the mechanism of gene transfection suggest that the DNA molecules could enter into cells, mediated by the tubular assemblies, without the involvement of endocytosis. Alternatively, tube-like fibres made of cyclic tetrapeptides of alternating chirality [*c*-(*D*-Trp-Tyr)$_2$-] were also studied in DNA delivery.[102] These fibres could interact with plasmid DNA to enhance the duodenal permeability. *In vivo* studies of plasmid/CP formulations also showed a significant increase in oral administration ability.

Recently, mechanical reinforcement of polymeric systems has been proposed through the incorporation of SCPN microcrystals derived from *D*,*L*-α-CPs (*c*-[(Gln-*D*-Leu)$_4$-], **CP16**, Fig. 2) as a structural filler.[103] For example, electrospun fibres of poly(*D*-,*L*-lactic acid), which is one of the commonly used polymers in the fabrication of implantable biomedical materials, were reinforced by this approach. The incorporation of only 8 wt % of peptide nanotubes provided fibres that are at least five times stiffer than the polymer alone. In this sense, the structural and mechanical characteristics of nanotube bundles made from the same CP by itself were also analyzed in a different study.[104] The analysis carried out with the large assemblies suggests a hierarchical organization that provides one of the most robust known nanofibres derived from proteinaceous materials. In fact, the properties exceed those of amyloid fibrils and are on a par with those of silks or suckerins. In addition, although these bundles of SCPNs have half the density of bone, the stiffness and strength are similar. Importantly, these properties are maintained even at the micrometer length scale.

4. Internal functionalization of SCPNs

As mentioned earlier, one of the major advantages of the introduction of γ-amino acids into the cyclic peptide skeleton is the projection of the β-methylene of each γ-amino acid toward the lumen of the nanotube, a situation that provides hydrophobic character to the inner cavity. X-ray diffraction studies on different dimeric structures confirmed that these cavities have amphipathic properties, since chloroform or water molecules were found inside the dimers.[49,105] Computational studies showed that in aqueous media the SCPN cavity is filled with partially ordered water molecules.[106] In addition, DFT calculations also showed that the chloroform molecule in the dimer cavity stabilizes the assembly structure derived from hexapeptides through van der Waals interactions with the methylene moiety of the γ-amino acid.[107] In fact, the authors suggest the possibility, in addition to SCPN stabilization, of controlling its length through the use of polychlorinated oligomers. In contrast, the nanotubes derived from α-amino acids had a hydrophilic pore due to the presence of the carbonyl and amide proton, which are involved in the hydrogen bonds that support the tubular structure. Furthermore, the inner cavity cannot be modified since any alteration of the acyclic amino acids (α, β…) would affect the stacking of the cyclic peptides. This situation has limited the development of some of the applications proposed for this type of structure, such as the preparation of molecular receptors or selective ionic channels, due to the limitation in the preparation of these systems with some functional groups on the inner face. Unfortunately, this type of internal functionalization is far from being achieved. As a consequence, alternative strategies to obtain pores with appropriate functional groups in their internal cavity must be developed.[108] In fact, one of the first approaches for this purpose was the use of *L,L,L,D*-*α*-CP motifs,[109] in which the required flat conformation for SCPN formation is achieved when one of the four amino acids, the second *L*-residue, has its side chain pointing into the channel. In the channel-forming design, the side chains of Ser and Dap (2,3-diaminopropionic acid) are projected into the cavity to provide a polar environment in which the transport activities changed with the pH of the medium.

In the case of the α,γ-CPs, the appropriate functionalization of the β-methylene, either by replacing it with a heteroatom or by introducing a functional group in that position, would allow modification of the internal properties of the ensemble without interfering with the self-assembly properties of the CP. On the basis of the above idea, our group prepared a CP that incorporates in its skeleton the sugar-derived 1-glucuronic acid (γ-Aga) (*D*,*L*-γ-CP in Fig. 3), the hemiaminalic oxygen of which is placed at the lumen, thus modifying the cavity properties of the resulting dimer.[52] Furthermore, the

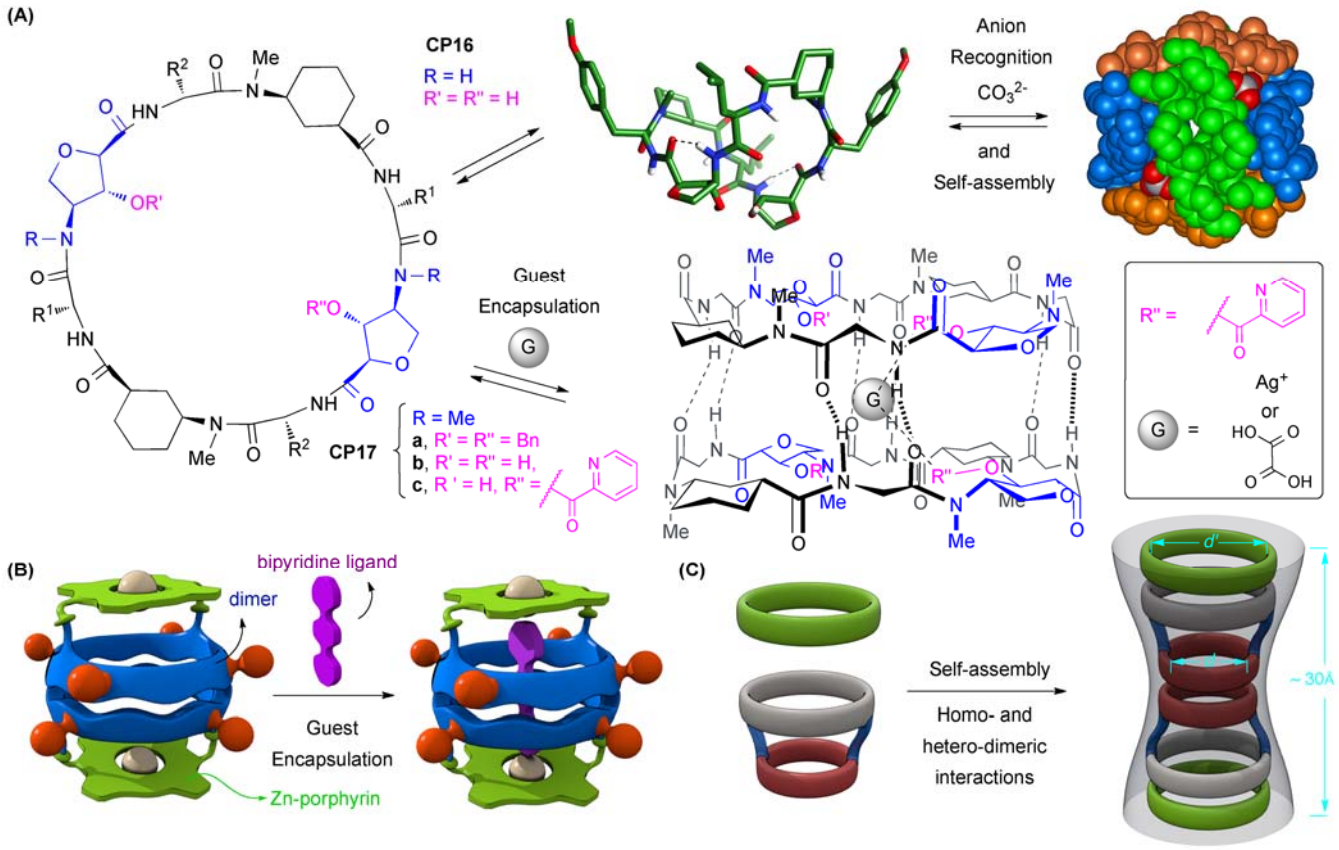

Fig. 7. (A) Structures of cyclic octapeptides that incorporate the γ-Ahf in their skeleton. Depending on the *N*-methylation of this amino acid the CP (**CP17**) can adopt a 'V-shaped' conformation, which self-assembles to form spherical clusters in the presence of anions, or self-assembles to form dimers, which can encapsulate different guests (**CP18a-c**). (B) Model of the cyclic peptide capsule that can recognize and selectively encapsulate large bipyridine ligands. (C) Model for the formation of Venturi-like self-assembling cyclic peptide nanotubes, with precisely controlled dimensions.

remaining hydroxyl groups are exposed on the external face of the ensemble to provide a saccharide-like outer surface. Of the two possible dimeric structures, the alternating dimer, in which the γ-Agas are stacked on top of the γ-Ach residues, was the only observed form. The preferential formation of this ensemble may be due to the repulsive interaction between the lone pairs of the oxygen atom in the eclipsed dimer while a weak O–H–C 'hydrogen bond' can be generated in the alternating form.

Alternatively, functionalization of the β-carbon of the cycloalkane moiety of the γ-residue can also be used to tailor the internal properties of the tubular assembly. For this purpose, it must be considered that the *cis* disposition of the carbonyl and amino groups of the cycloalkane ring ensures the flat conformation of the CP. In addition, the functional group at the β-carbon must be *trans*-oriented with respect to the other two substituents, the carboxylic and amino groups. In this way, when the CP adopts the flat conformation, this group is projected into the inner cavity in a pseudo-equatorial orientation, thus modifying the internal properties of the resulting nanotube without interfering with the self-assembling properties of the CP. With this idea in mind, we synthetized a γ-amino acid with a hydroxyl group on the β carbon, namely 4-amino-3-hydroxytetrahydrofuran-2-carboxylic acid (γ-Ahf).[110] This compound was used in the preparation of the tetrapeptide *c*-[*D*-Leu-*L*-Ahf-*D*-Tyr(Me)-^{Me}*N*-*L*-Acp-], which was able to self-assemble to form the corresponding dimers.[111] The presence of the hydroxyl groups in the inner cavity directs the self-assembly process, thus increasing the stability of the dimer and restricting the equilibrium towards the ensemble in which the two hydroxyl groups are hydrogen bonded. Larger CPs were also prepared using this amino acid, including the octapeptide **CP17** (Fig. 7A).[112,113] Instead of adopting the flat conformation required for the formation of the corresponding dimers, the CP folds in a 'V-shaped' conformation in which the amide proton and carbonyl group of the Ahf residue are hydrogen bonded.[111] Interestingly, the CP retains the ability to assemble but, instead of forming dimers, spherical aggregates arise upon recognition of specific anions, such as chloride, nitrate, hydroxide or carbonate. The cluster contains six CP units and four anions in a tetrahedral arrangement. Interestingly, this peptide presented novel transport properties. While membrane-active SCPNs are cation selective transporters,[29] the new folded peptide is an anion selective antiporter carrier. Very hydrophilic anions, such as sulfate, were not transported.

To avoid the formation of the folded conformation, *N*-methylated-Ahf residues were used. The resulting cyclic octapeptide assembled **CP18** into the corresponding dimers (Fig. 7A).[113] This hydroxyl-free CP (**CP18b**) can only dimerize in

chloroform in the presence of polar molecules, such as methanol or water. This behaviour suggests that the hydrophilic properties of the cavity induce the dimer formation upon entrapping polar molecules.

Furthermore, the modification of the lumen can be achieved by simple chemical transformation of the hydroxyl group and this allows the tuning of the cavity properties. In this way, the incorporation of a picolinic acid moiety, through an ester linkage in one of the Ahf residues, directed the recognition of other molecules, such as silver ions or oxalic acid, inside the dimer cavity (Fig. 7A). This encapsulation not only modified the internal properties of the resulting dimer and the guest but also defined the geometry of the final ensemble. Recently we also described the preparation of molecular cages that recognize and selectively encapsulate large bipyridine ligands (Fig. 7B).[114] For this purpose a Zn-porphyrin moiety was attached on top of a cyclic octapeptide through substituents attached to the nitrogen of the γ-amino acid. The resulting dimer created a closed internal cavity that can bind molecules that can coordinate simultaneously to two zinc ions. The use of ligands with different lengths and properties allowed the cavity dimensions to be estimated.

Xu and co-workers also prepared peptide nanotubes with functionalized cavities by incorporating an aromatic γ-amino acid, namely 3-amino-2-methylbenzoic acid, in a *D*,*L*-α-CP (**CP10**, Fig. 2F).[32,115] However, the introduction of this residue reduces the aggregation properties but allows the preparation of nanotubes in which the methyl groups are pointing towards the inner cavity. The CP was also conjugated to PEG polymers through the Lys side chains to provide precursors with a higher tendency to aggregate, thus rendering more processable SCPNs. This new design allows the easy manipulation of the nanotube formation and this is compatible with the processing window of polymeric membranes. Therefore, it is envisioned that short nanotubes can be integrated into the polymer matrix to fabricate functional membranes.

Finally, Venturi-like nanotubes with a central constraint were reported by us. In these systems two covalently linked CPs with different characteristics, such as diameter and assembling properties, were used (Fig. 7C).[116] The strategy provides the opportunity to control both dimensions of SCPNs: the diameter and the length. In addition, this methodology is especially suitable for the construction of nanotubes with a reduced number of stacked CPs, with nanotubes consisting of three, four or six subunits made by controlling homo- and heterodimer equilibria and media conditions. The shape of these nanotubes resembles the chamber of the selective filter of some natural ion channels. It is envisioned that they might be implemented in the preparation of simple models of these proteins with improved transport properties.

Conclusions

One-dimensional nanomaterials, such as tubes, fibres or rod-like structures, are essential substances in modern science and nanotechnology. Therefore, highly efficient synthetic procedures are required. In this sense, nanotube preparation by stacking of cyclic components represents one of the most powerful and attractive strategies. The simple synthesis, precise control of internal cavity dimensions and ease of modification of SCPNs make them especially attractive. In addition, the potential biocompatibility and robust mechanical properties are characteristics that warrant further exploration in materials science, chemistry and biology. In recent years chemists have been able to develop different strategies to decorate the internal and external surfaces of these nanotubes. The development of molecular-scale building blocks to provide a viable path toward organic nanotubes with molecularly defined interiors is starting to be addressed. Furthermore, the ability to manipulate the nanotube formation – especially through conjugation to polymers – makes these materials compatible with industrial processing. Covalent capture of SCPNs by cross-linking also offers new opportunities to tune the structural and functional properties of the assemblies. The ability to modify the properties of the nanotube pore and to generate internal constraints, as in the Venturi-like nanotubes, opens new opportunities in separation technologies. We also envisage the construction of more efficient and selective synthetic ion channels. For instance, several membrane applications have been proposed for nanotube membranes with controlled internal properties and diameters. In fact, carbon nanotubes with functionalized inner cavities have been proposed using computing models.[117] Unfortunately, this type of internal functionalization is far from being achieved with carbon nanotubes but it might be available now with SCPNs. One step further in membrane processes would be the preparation of synthetic ion pumps that can move ions against concentration gradients using this technology. In this respect, one can visualize molecular motors that move the ions through conformational changes induced by external signals (light, chemical energy and so on). To achieve this goal, 'out of equilibria' systems based on SCPNs will be required.[118] These advantages offer new horizons in nanomaterials science.

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