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Co-Assembly of Peptide Amphiphiles and Lipids into Supramolecular Nanostructures Driven by Anion- π Interactions

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Abstract

Co-assembly of binary systems driven by specific noncovalent interactions can greatly expand the structural and functional space of supramolecular nanostructures. We report here on the self-assembly of peptide amphiphiles and fatty acids driven primarily by anion- π interactions. The peptide sequences investigated were functionalized with a perfluorinated phenylalanine residue to promote anion- π interactions with carboxylate headgroups in fatty acids. These interactions were verified here by NMR and circular dichroism experiments as well as investigated using atomistic simulations. Positioning of the aromatic units close to the N-terminus of the peptide backbone near the hydrophobic core of cylindrical nanofibers leads to strong anion- π interactions between both components. With a low content of lauric acid in this position, the cylindrical morphology is preserved. However, as the aromatic units are moved along the peptide backbone away from the hydrophobic core, the interactions with lauric acid transform the cylindrical supramolecular morphology into ribbon-like structures. Increasing the ratio of lauric acid to PA leads to either the formation of large vesicles in the binary systems where the anion- π interactions are strong, or a heterogeneous mixture of assemblies when the peptide amphiphiles associate weakly with lauric acid. Our findings reveal how co-assembly involving designed specific interactions can drastically change supramolecular morphology and even cross from nano to micro scales.

Supramolecular assemblies of binary systems have great potential in the search for functional systems. Examples include co-assembly of bioactive and non-bioactive molecules to tune the intensity of a response, or the co-assembly of a drug with its vehicle.¹⁻⁵ One of the design aspects of these systems is the use of specific interactions among the components of the binary structure, or to use thermodynamically non-miscible molecular segments in block copolymers or Janus-dendrimers.⁶⁻¹⁰ The literature contains a wide array of examples of self-assembling building blocks with structural features that favor noncovalent bonding among molecules, most commonly hydrogen bonding, metal-ligand interactions, and π -orbital overlaps.¹¹⁻¹⁵ The literature remains scarce on other specific interactions, and co-assembly involving binary systems.

While traditional π -effects, including π - π stacking¹⁶ and cation- π interactions,¹⁷ are relatively well understood in the context of molecular self-assembly, the interaction between negatively charged groups and electron deficient aromatic moieties, referred to as anion- π interactions,¹⁸⁻²⁰ remains less investigated. Anion- π interactions formed by a positive quadrupole on aromatic rings by electron withdrawing substituents were first reported in 2002,¹⁸ and since have been explored by crystallography²¹ and the molecular recognition of anions²² or zwitterions.²³ However, despite growing recognition of the multifaceted roles that anion- π interactions play in biological systems^{24,25} and their potential as design motifs in synthetic biology,^{26,27} to the best of our knowledge their use in self-assembly of monomers into supramolecular nanostructures have not been reported. We report here on the binary self-assembly of two amphiphiles into supramolecular nanostructures driven largely by anion- π interactions. We investigate the morphology of these assemblies as a function of the strength of anion- π interactions and their molecular composition.

We employed a peptide amphiphile and a fatty acid as the two components that should associate primarily driven by anion- π interactions. Peptide amphiphiles (PAs) containing an amino acid sequence substituted by an alkyl chain can form nanostructures with a variety of morphologies in water, mostly depending on their primary structure.²⁸⁻³¹ Fatty acids are of course well known to assemble into a rich variety of supramolecular architectures driven by electrostatic interactions and hydrophobic effects.^{32,33} We used lauric acid due to its relatively high solubility in water and the possibility of matching its 12 carbon alkyl tail to those used commonly in peptide amphiphiles. From a functional standpoint, lauric acid was of interest because of its known antibacterial activity often

retained in nanoscale assemblies.³⁴ We incorporated one fluorinated phenylalanine residue (Z)³⁵ as the electron-deficient amino acid into the V₃A₃E₃ peptide sequence of a PA. The Z amino acid replaced one V residue in different positions, hence the nomenclature of PAs Z1, Z2, and Z3 (Figure 1A). We hypothesized that the alkyl tail of lauric acid would bury itself in the hydrophobic core of supramolecular structures formed and therefore varying the position of the Z residue would allow us to optimize the relative distance between the fluorinated aromatic groups and carboxyl acid headgroups. This would in turn maximize anion- π interactions between PA molecules and lauric acid.

In an effort to establish how the position of the Z residue affects supramolecular morphology, we first characterized the self-assembly behavior of the PAs alone in water by circular dichroism (CD) spectroscopy, cryogenic transmission electron microscopy (cryo-TEM), and small-angle x-ray scattering (SAXS). Similar to the canonical PA that lacks the Z residue,³⁶ CD spectroscopic measurements showed that PA Z1, Z2, and Z3 all adopted predominantly β -sheet secondary structure in solution (Figure 1B), indicating that β -sheet hydrogen bonds can still be formed among amino acid sequences that contain a fluorinated aromatic unit. In terms of morphology, well-defined cylindrical nanofibers with a 7-10 nm diameter were formed by all three PAs in aqueous solution as observed by cryo-TEM (Figure 1C). The morphology of the PA assemblies was also confirmed by SAXS scattering experiments. The scattering signals showed an approximately -1 slope in the low- q region for all the PAs, supporting the cryo-TEM observation of cylindrical nanofibers as the dominant morphology (see Figure 1D). The diameter of the one-dimensional filaments was estimated to be approximately 8 nm, as determined by fitting the scattering curves to a core-shell cylinder model. Collectively, these results demonstrate that all the designed PAs form well-defined one-dimensional nanostructures in water in spite of the presence of the Z residue in the peptide sequence. The Z residue would be expected to contribute to steric interactions within the β -sheets in the internal structure of the supramolecular filaments but obviously does not disrupt the well-known self-assembly behavior of this group of PA molecules.

Figure 1. (A) Chemical structures of PA Z1, PA Z2, and PA Z3 containing perfluorophenylalanine (Z) at various positions. (B) Circular dichroism spectra of PA Z1, PA Z2, PA Z3, and the parent PA in water (50 μ M) at 25 $^{\circ}$ C. (C) Representative cryo-TEM image of cylindrical fibers formed by PA Z1. (D) SAXS profiles of PA solutions plotting scattered intensity versus the scattering vector q (log–log plot) in water. Scattering intensities are offset vertically for clarity and the fitting curves for the scattering data are shown in black.

We subsequently studied the binary co-assembly of the designed PAs and lauric acid by CD spectroscopy. Solutions of PA Z1 or Z2 were mixed with lauric acid and then aged for 2 hours at room temperature. The resulting samples showed a substantial increase in CD intensity at 200 nm compared to that of PA solutions alone (Figures 2A and 2B), suggesting an enhancement of hydrogen bonding among monomers in β -sheets within the assemblies when a small amount of lauric acid is incorporated.^{37,38} The intensity of CD signals at 200 nm reached a maximum value after the addition of approximately 0.4 molar equivalents of lauric acid, and then gradually decreased with further addition (Figure 2, D and E). These results indicate that the β -sheet hydrogen bonds are disrupted by associating the PAs Z1 and Z2 with a large fraction of lauric acid. In marked contrast, mixing the solutions of PA Z3 with lauric acid and aging for 2 hours led to a blue shift for the positive Cotton peak across the entire titration process (Figure 2C). We therefore conclude that association of PA Z3 assemblies with lauric acid alters the hydrogen bonding of PA monomers in supramolecular PA monomers in supramolecular assemblies. As a control experiment, the addition of lauric acid to the parent PA without the Z residue yielded an initial increase in the CD intensity up to a value of 0.25 molar equivalents of lauric acid (Fig. S5). The invariant CD spectral signature of the parent PA upon addition of greater fractions of lauric acid could be attributed to incomplete encapsulation of additional lauric acid and excess of the lipid likely remains free in solution, potentially due to weak

interactions with the parent PA molecules compared with those in binary systems involving PA Z1 / Z2 / Z3. This result suggested that the incorporation of the Z unit allows the PAs to strongly associate with lauric acid molecules driven by anion- π interactions, thus leading to co-assembly of both components.

Figure 2. (A-C) CD spectra of mixed aqueous solutions of PA Z1 (A), PA Z2 (B), and PA Z3 (C) with lauric acid (LA) aged for 2 hours at 25 °C. (D, E) CD intensity changes at 200 nm as a function of LA/PA molar ratio for PA Z1 (D) and for PA Z2 (E). (F) Ratio of CD intensities at 194 and 204 nm for PA Z3 as a function of LA/PA molar ratio.

To qualitatively verify the anion- π interaction between lauric acid molecules and the PAs as well as monitor the location of lauric acid molecules within the assemblies, we carried out ^{19}F NMR experiments on the PAs alone and also on the binary co-assemblies with lauric acid. The *ortho*-, *meta*-, and *para*-fluorine atoms from the phenyl groups were clearly identified in the ^{19}F NMR spectra. Using the conditions that showed a maximum in the CD intensity, we found that simultaneously dissolving the PAs and lauric acid in a molar ratio of 1:0.4 led to separation of the signals into two distinct resonances for both the *meta*- and *para*-fluorine atoms. In the cases of PA Z1 and PA Z2, the main peaks for the *meta*- and *para*-fluorine atoms exhibited down- and upfield shifts, respectively, while the minor peaks of *meta*- and *para*-fluorine of PA Z3 displayed down- and upfield shifts, respectively. The substantial separation and shift of the NMR signals of fluorine atoms are in principle attributed to the anion- π interactions between the PAs and fatty acids.³⁹ It is important to note that although the PAs also contain three carboxylic acids in glutamic acid residues, the fluorine

NMR signals on the PAs alone do not show any shift or separation, suggesting that anion- π interactions are negligible in PA assemblies. Overall, the ^{19}F NMR results strongly indicate that the PAs and lauric acid molecules are successfully co-assembled and associate via anion- π interactions. In particular, positioning the Z residue closer to the N-terminus of the PAs results in greater separation and shift of the fluorine signals, indicating that the PAs interact with lauric acid molecules via anion- π interactions that increase in strength in the following order, PA Z1 > PA Z2 > PA Z3. The ^{19}F NMR results also imply that lauric acid molecules prefer to reside within the hydrophobic core of the PA assemblies. To confirm changes in the ^{19}F NMR signals induced by anion- π interactions, one trifluoro-ethyl laurate (F-ethyl laurate) without the negatively charged headgroup was used to investigate the effect of its encapsulation on the fluorine signals in the ^{19}F NMR spectra. In the mixture of F-ethyl laurate and PA Z1, the fluorine signals did not show any shift or separation, indicating that encapsulation of small molecules into the PA assemblies does not give rise to the change of fluorine signals. These additional NMR experiments clearly demonstrate the occurrence of anion- π interactions between the investigated PAs and lauric acid.

Table 1. Thermodynamic parameters for the complexes formed between the PA molecules and lauric acid in a 1:0.4 molar ratio in water at 25 °C.

	PA Z1 + LA	PA Z2 + LA	PA Z3 + LA
Binding Constant / M^{-1}	$5.49 \times 10^4 \pm 5357$	$4.56 \times 10^4 \pm 1215$	$9.95 \times 10^3 \pm 252$
ΔH / kcal mol^{-1}	-9.11 ± 0.29	-2.52 ± 0.24	-3.64 ± 1.08
ΔS / cal mol^{-1}	8.86 ± 0.84	-6.94 ± 0.58	-12.87 ± 1.34
ΔG / kcal mol^{-1}	-11.75	-0.45	0.19

We further quantified the interaction between the PAs and lauric acid using isothermal titration calorimetry (ITC)⁴⁰ by recording the thermodynamic parameters obtained upon the addition of lauric acid molecules to pre-formed PA assemblies. The thermodynamic parameters of the formation of the complexes containing PAs and lauric acid in a 1:0.4 molar ratio are shown in Table 1. Addition of lauric acid into all three PA solutions resulted in a negative enthalpy change, suggesting the enhancement of β -sheet hydrogen bonds among PA monomers in their assemblies. Interestingly, only a positive entropy change was observed in the formation of the complex of PA Z1 and lauric acid, possibly indicating the release of structured water molecules when these highly favorable interactions occur between PA Z1 and lauric acid carboxylate groups⁴¹. ITC results confirm that the

affinity between lauric acid and the PAs is enhanced due to anion- π interactions, the strength of which varying depending on the location of the Z residue within the PA structure.

Figure 3. (A-C) Cryo-TEM images of mixtures of PA Z1, PA Z2, and PA Z3 with lauric acid in a molar ratio of 1:0.4, and (E-G) conventional TEM images of mixtures in a 1:1 molar ratio without staining (schematic illustrations of the morphologies are shown in the insets). (D, H) SAXS profiles of the LA-PA aqueous solutions in a molar ratio of 1:0.4 (D) and 1:1 (H) (the profiles plot scattered intensity versus the scattering vector q (log-log plot); scattering intensities are offset vertically for clarity and the fitting curves for the scattering data are shown in black in (D)).

Following our experimental observations of the strong association between the designed PAs and lauric acid molecules, we investigated the morphology of the binary co-assemblies using conventional TEM, cryo-TEM, SAXS. Dissolving in water at the same time PA Z1 or Z2 and lauric acid in a molar ratio of 1:0.4 resulted in the formation of cylindrical nanofibers as revealed by cryo-TEM, similar to the nanostructures formed by the PAs alone. In contrast, mixing PA Z3 with lauric acid in a 1:0.4 molar ratio led to formation of ribbon-like flat nanostructures. This morphological difference between the co-assemblies involving PA Z3 and PA Z1 or Z2 is consistent with the variations of CD spectral changes upon addition of lauric acid, which revealed a blue shift for PA Z3 and an increase in signal intensity for PA Z1 and PA Z2. While intercalation of fatty acids into the hydrophobic interior of the PA cylinders may expand the space between the palmitoyl tails of all three PAs within their corresponding assemblies, the location of the Z residue and fatty acids as well as the strength of noncovalent interactions within the assemblies all contribute to the differences in nanostructures observed with the different PA sequences. In the binary system of PA Z3 and lauric

acid, we hypothesize that the co-assembled lipids partially translocate toward the interior of the β -sheet region driven by anion- π interactions, changing the packing geometry of hydrophilic and hydrophobic segments compared with that associated with supramolecular structures formed by PA Z3 alone. Thus, we propose this change causes a morphological transition from cylindrical fibers formed by PA Z3 alone to ribbon-like structures consistent with the theory proposed by Israelachvili *et al*⁴² formed by the binary system. However, in the systems consisting of PA Z1 or Z2 and lauric acid, lipids prefer to localize in the hydrophobic core, thus maintaining the packing geometry associated with cylindrical fibers for the binary systems. We further increased the content of lauric acid in the co-assemblies to a 1:1 molar ratio in order to study how the molecular composition of the structures influences their morphology. In contrast to the cylindrical fibers formed by PA Z1 and lauric acid molecules in a 1:0.4 molar ratio, conventional TEM revealed that large vesicles were formed by PA Z1 and lauric acid molecules in a 1:1 molar ratio. Dynamic light scattering confirmed the presence of these large structures and was used to estimate a hydrodynamic radius of approximately 700 nm. On the other hand, mixing PA Z2 or Z3 with lauric acid in a 1:1 molar ratio led to the formation of a heterogeneous collection of cylindrical filaments and vesicles or ribbons and vesicles, respectively, indicating formation of a heterogeneous collection of supramolecular co-assemblies. In the case of PA Z1, the morphological transition for the 1:1 binary co-assemblies is likely the result of a homogeneous geometry change in the complex formed by these two strongly interacting molecules. In contrast, the relatively weak interactions between the other PAs and fatty acids induce formation of binary co-assemblies containing varying amounts of fatty acids, and therefore a heterogeneous mixture of nanostructures.

We carried out atomistic molecular dynamics (MD) simulations⁴³ on the co-assemblies of PAs and lauric acid using the Gromacs MD package⁴⁴ under the GROMOS force field.⁴⁵ After 300 ns simulations the secondary structure of peptide segments of PA molecules surrounded by water and sodium counter ions reached a steady state and the cylindrical morphology of the PA assemblies was maintained (Figure 4A). Compared with the parent PA, the simulation results show that incorporation of the Z residue gives rise to a decrease in β -sheet secondary structure within the assemblies (Figure 4B). Furthermore, positioning the Z residue closer to the C-terminus (from PA Z1 to Z2 and Z3) gradually lowers the content of β -sheet structures. The simulation results are consistent with

experimental findings obtained from the CD intensity of the β -sheets formed by the various PAs. The β -sheet composition was estimated by MD simulations as 9.3%, 7.4%, and 3.5% for PAs Z1, Z2, and Z3, respectively. These results indicate that the bulky Z residue constrains formation of β -sheets potentially due to steric hindrance, which interferes with coupling between hydrogen bond donors and acceptors, particularly when the Z residue is located in the interior of the β -sheet region in the case of PA Z3.

Figure 4. (A) Cross-sectional snapshots for PA cylindrical fibers without and with lauric acid in a molar ratio of 1:0.2 after a 300 ns simulation (Cyan: alkyl tails in PA molecules; Blue: LA molecules ; Yellow: β -sheets strands). (B) Percentage of β -sheets in secondary structures of the PAs and their co-assemblies with lauric acid in 1:0.2 molar ratio. (C) Cross-sectional distribution profile of lauric acid molecules in the nanofibers as a function of normalized radius.

MD simulations on the PA and lauric acid binary systems were carried out under the same condition for the PAs alone. In 300 ns simulation we observed unstable nanofibers in binary co-assemblies of PA Z3 and lauric acid at the 1:0.4 molar ratio, a finding which is consistent with the morphological transitions from cylindrical fibers formed by PA Z3 alone to flat ribbons formed by the binary system in a 1:0.4 molar ratio as observed in TEM experiments. Hence, in our simulations we investigated initial structures consisting of each PA and lauric acid in a 0.2 molar ratio. The lauric acid molecules are localized either randomly or regularly within the initial structures to maximize the

reliability of the simulations (Fig S8, B and C). After relaxation for at least 200 ns, the initial cylindrical morphology was maintained and the lauric acid molecules reorganized within the fibers (Figure 4A). This suggests that co-assembly of PAs and LA in a 1:0.2 molar ratio leads to the formation of stable, well-defined cylindrical nanofibers. β -sheet content in the binary complexes increases for all the three PAs compared to neat PA assemblies (Figure 4B). This result is consistent with the experimental observation in CD spectra which suggest that encapsulation of lauric acid molecules enables the optimal formation of β -sheet hydrogen bonds within the assemblies. In a more detailed evaluation, we profiled the cross-sectional radial distribution of lauric acid molecules within the stable cylindrical fibers. Our simulations revealed that all of the added lauric acid molecules were confined within the PA assemblies and the radial distribution of lauric acid strongly depends on the position of the Z residue within the PA structures (Figure 4C). In the case of PA Z1, lauric acid molecules are primarily buried in the hydrophobic core (Figure 4C, black curve), whereas in the cylindrical fibers formed by PA Z2 or Z3, lauric acid molecules translocate outward relative to the periphery of the fibers. The distribution of the co-assembled lauric acid molecules correlates with the location of the pentafluorobenzene moiety within the nanofibers (Figure 4C), indicating that the majority of lauric acid molecules are localized around the Z residue. MD simulations strongly imply that anion- π interactions between the PAs and lauric acid play a critical role in their association, and thus control the location of fatty acids within the co-assemblies.

Figure 5. (A) Strength of π,π -stacking interactions estimated from simulations among aromatic groups in PA molecules in the absence and presence of lauric acid molecules and (B) the anion- π interactions between the PAs and lauric acid molecules (PA:LA=1:0.2). (C) Normalized radial distribution function (RDF) of the distance between the polar carboxylic acid functional group of lauric acid molecules and the center of mass of pentafluorobenzene rings within the nanofibers (PA:LA=1:0.2).

MD simulations also allow us to further estimate the strength of the π,π -stacking and anion- π

interactions within the co-assemblies by evaluating the short-range electrostatic and steric interaction energies per PA monomer. The π,π -stacking interaction energy present in the assemblies formed by PAs alone is nearly identical and determined to be approximately $-25 \text{ kcal mol}^{-1}$ (Figure 5A), suggesting that the relative distance and orientation of the phenyl groups is independent of their location within the PA structures. However, co-assembling lauric acid with the PA molecules results in variations of the strength of π,π -stacking depending on the position of the Z unit within the peptide backbone. The strength of π,π -stacking interactions among PA monomers was calculated to be -15 , -30 , $-35.3 \text{ kcal mol}^{-1}$ for the binary systems containing PA Z1, Z2, and Z3, respectively. These results indicate that the co-assembled lauric acid molecules alter the relative distance and orientation of the aromatic units within the assemblies due to their strong association with the PA molecules based primarily on anion- π interactions. The largest increase in π,π -stacking interactions induced by co-assembly with lauric acid molecules occurs in PA Z3 and we hypothesize that this contributes to the morphological transition from the cylindrical fibers formed by PA Z3 alone to the ribbons formed by the PA-lauric acid complexes, as revealed by TEM and SAXS experiments discussed above. Generally the simulation results support the concept that supramolecular morphology of the co-assemblies is directly affected by the strength of noncovalent interactions among components.

Based on the atomistic simulations, the anion- π interaction energy between the Z residue and the carboxylic acid unit in lauric acid molecules increases from PA Z1 to PA Z2 and PA Z3 (Figure 5B). This result from simulations strongly suggests that PA molecules containing the Z unit closer to the N-terminus of peptide backbones exhibit the strongest anion- π interactions with lauric acid molecules. This is possibly due to the preferential localization of lauric acid molecules in the hydrophobic core of the co-assemblies, thus leading to a short distance between the electro-deficient aromatic units and carboxylic acids that promote strong anion- π interactions. Therefore, we profiled the radial distribution function (RDF) of the distance between the polar carboxylic acid functional group of lauric acid molecules and the mass center of the pentafluorobenzene rings within the assemblies (Figure 5C). In the case of PA Z1, the charged head of lauric acid molecules are predominantly localized within 0.4 nm of the aromatic units, a distance which places them in close proximity and favors the anion- π interaction. However, the primary distance between the carboxylic

acid of lauric acid and the center of mass of the pentafluorobenzene ring within the PA Z2 and Z3 assemblies increases to 0.6 - 0.7 nm, a distance that is typically associated with lone pair- π interactions.⁴⁶ This indicates that the main driving force for the association of PA Z2 or Z3 with lauric acid molecules is linked to lone pair- π interactions between the charged head of lauric acid and the fluorinated phenyl groups and additional anion- π interactions. Overall, our simulation results clearly demonstrate that the anion- π interaction between PA and lauric acid molecules determines the location of the encapsulated lauric acid molecules within the co-assemblies.

We have designed and synthesized three peptide amphiphiles containing one pentafluorophenyl alanine at different positions in the peptide backbones. Incorporation of the fluorinated phenyl groups that serve as electro-deficient units promotes anion- π interactions between peptide amphiphiles and fatty acids. Both experimental and computational results confirm that localizing the fluorinated aromatic unit close to the N-terminus of the peptide backbone allows for strong anion- π interactions in the binary systems, due to the preferential localization of fatty acids in the hydrophobic core of the co-assemblies. With such localized fatty acids, the binary systems co-assemble into cylindrical fibers, consistent with the morphology of the assemblies formed by peptide amphiphiles alone. However, positioning the aromatic unit in the interior of β -sheets promotes translocation of fatty acid molecules toward the hydrophilic region of binary assemblies, leading to a morphological transition from cylindrical fibers formed by the PA molecules alone to ribbon-like structures formed by the binary system. We also discovered that the strong association between PA and fatty acid molecules allows the binary systems with a large content of fatty acids to form uniform structures, while weak interactions give rise to a heterogeneous collection of assemblies.

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