**ABSTRACT:** We report a novel molecular architecture of peptide–phospholipid coassemblies. The amphiphilic peptide Ac-18A-NH$_2$ (18A), which was designed to mimic apolipoprotein α-helices, has been shown to form nanodisc structures with phospholipid bilayers. We show that an 18A peptide cysteine substitution at residue 11, 18A[A11C], forms fibrous assemblies with 1-palmitoyl-2-oleoyl-phosphatidylcholine at a lipid-to-peptide (L/P) molar ratio of 1, a fiber diameter of 10−20 nm, and a length of more than 1 μm. Furthermore, 18A[A11C] can form nanodiscs with these lipid bilayers at L/P ratios of 4−6. The peptide adopts α-helical structures in both the nanodisc and nanofiber assemblies, although the α-helical bundle structures were evident only in the nanofibers, and the phospholipids of the nanofibers were not lamellar. Fluorescence spectroscopic analysis revealed that the peptide and lipid molecules in the nanofibers exhibited different solvent accessibility and hydrophobicity from those of the nanodiscs. Furthermore, the cysteine substitution at residue 11 did not result in disulfide bond formation, although it was responsible for the nanofiber formation, suggesting that this free sulfhydryl group has an important functional role. Alternatively, the disulfide dimer of 18A[A11C] preferentially formed nanodiscs, even at an L/P ratio of 1. Interconversions of these discoidal and fibrous assemblies were induced by the stepwise addition of free 18A[A11C] or liposomes into the solution. Furthermore, these structural transitions could also be induced by the introduction of oxidative and reductive stresses to the assemblies. Our results demonstrate that heteromolecular lipid–peptide complexes represent a novel approach to the construction of controllable and functional nanoscale assemblies.

**INTRODUCTION**

The self-assembly of biomolecules is a key component in the creation of nanomaterials. These nanomaterials have a wide variety of structures and have been shown to have functional applications that can be applied to drug delivery, bioimaging, and biosensors. Biomolecules, including proteins and peptides, nucleic acids, lipids, and carbohydrates, exhibit significant promise as building blocks in the synthesis of functional nanoassemblies, as a result of their plasticity, biocompatibility, and biodegradability. The design and synthesis of these nanoscale assemblies, incorporating these biomolecules, has been extensively studied, resulting in versatile architectures in a variety of shapes, sizes, and surface properties. For instance, phospholipids are known to self-assemble into spherical micelles, hollow vesicles (liposomes), and hexagonal phase and planar lamellar structures, based on their chemical structures and the surrounding conditions. Peptide-based nanosystems have also been examined and shown to achieve well-ordered, tunable structures, such as nanofibers, nanotubes, and nanoparticles that can adopt specific secondary polypeptide chain structures, including β-sheets and α-helices. Furthermore, the conjugation of hydrophobic aliphatic groups to hydrophilic biomolecules, including peptides and DNA, results in amphiphilic building blocks including peptide amphiphiles and DNA amphiphiles. The conjugation of these biomolecules has been shown to be a powerful strategy in the initiation of self-association, via hydrophobic interaction, and in the construction of uniform nanoassemblies with representative functional hydrophilic moieties on their surfaces. Previous studies have also demonstrated the structural transition of these nanoassemblies, as a result of external stimuli, and the structure-dependent release of incorporated pharmaceuticals. Therefore, the regulated structural transition of these assemblies implies that they are functional nanomaterials with important implications for nanomedicine. Whereas the majority of self-assembling nanostructures previously examined are composed of only a single building block, ubiquitous biological complexes, including ribosomes, viruses, and lipoproteins, are mixtures of molecules in different classes: proteins, nucleic acids, carbohydrates, and lipids. Here, we show that cooperative noncovalent interactions between the molecules of different types can result in the generation of a well-organized bioactive complexes that can be applied to drug delivery, bioimaging, and biosensors.

**Supporting Information**

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complex. Furthermore, although it is a challenging task to design, synthesize, and manipulate nanoassemblies, consisting of a combination of different types of building blocks, we show that these constructions can mimic biological structures. However, our knowledge of the design principles for heteromolecular nanobioassemblies remains limited because of a lack of an appropriate model system for investigating the self-assembly processes.

Lipoproteins are nanoscale particles composed of lipids and proteins, including apolipoprotein A-I, that circulate in the blood and transport cholesterol. Recently, a discoidal lipoprotein-like particle, the lipid nanodisc, has become of particular interest in the fields of biochemistry, biophysics, and medicine. This lipid nanodisc consists of an amphiphilic α-helical polypeptide chain containing a membrane scaffold protein, a fragment of apolipoprotein A-I, and has been shown to wrap around phospholipid bilayers. Discoidal nanoparticles have also been shown to form, via self-assembly, between the apolipoprotein mimetic peptide Ac-18A-NH₂ (18A) and phospholipid mixtures, as described by Anantharamiah et al. In this study, we examine peptide-based lipid nanodiscs as a model system for self-assembling heteromolecular complexes based on the following characteristics: First, these nanoparticles can spontaneously form by simple mixing in a buffer. Second, synthesis, amino acid mutation, and chemical modification of peptides are relatively straightforward. Third, the dimensions, structures, and functions of these complexes are tunable. Finally, α-helical peptides and phospholipids are both cylindrical molecules, despite possessing very different amphiphilic structures. Amphiphilic α-helical peptides have opposing polar and nonpolar faces oriented along the long axis of the helix, whereas phospholipids have a polar headgroup connected by long hydrophobic acyl chains. Therefore, we expect that the structure and function of these binary nanoassemblies will be governed by a delicate balance between the structure and the stoichiometry. Previously, we have shown that the native chemical ligation of thioester-modified peptides stabilizes nanodiscs and that the diameter of the disks is dependent on the L/P molar ratio in solution. We also demonstrated the dynamic self-reproduction properties of these lipid–peptide nanodiscs and that these lipid–peptide interactions are highly dependent on the amino acid sequence. For example, replacing amino acid residues of 18A by phenylalanine at various positions or connecting two amphiphilic helices by a short linker affected the solubilization of phosphatidylcholine liposomes. However, the relationship between the amino acid sequence of these amphiphilic peptides and the structure of noncovalent peptide–lipid nanoassemblies remains unclear.

Here, we show that a single amino acid substitution in the apolipoprotein mimetic peptide 18A causes a drastic structural transition in peptide–phospholipid nanoassemblies. The mutant form of 18A, 18A[A11C], in which an alanine at residue 11 is replaced by a cysteine residue, forms not only nanodiscs with 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) but also nanofibers, depending on the L/P ratio or reductive conditions affecting the cysteine side chain. The component molecules adopted different structures and exhibited altered solvent accessibility in these assemblies. Furthermore, we also observed a reversible interconversion between these heteromolecular nanoassemblies.

## RESULTS

### Effect of Amino Acid Substitution on 18A[A11X]–POPC Nanoassembly Formation.

To investigate the role of the primary structure of the amphiphilic 18A peptide on nanodisc formation with the POPC lipid bilayer, we generated several 18A variants via amino acid substitution at residue 11: 18A[A11X], where X = S, T, N, V, M, Y, D, G, or C (Figure 1). The native residue at position 11, alanine, is near the center of the primary structure at the interface between the hydrophobic and hydrophilic faces of the amphiphilic α-helix. Therefore, we hypothesized that a mutation at this location would result in a change to the physicochemical properties of the amphiphilic peptide, affecting the formation of the peptide–lipid nanoassemblies. We examined hydrophilic residues (serine, threonine, asparagine, and aspartic acid), hydrophobic residues (valine and methionine), and an aromatic residue (tyrosine) for changing the surface property of the peptides. Glycine and proline were used for disrupting the α-helical structure of the peptides. Substitution by the cysteine residue, which can form a disulfide bond, was also investigated.

Figures 2 and S1 show the resulting changes in the normalized right-angle light scattering intensity of POPC large unilamellar vesicles (LUVs), with a mean diameter of ~100 nm, followed by the addition of the mutated 18A peptides. The scattering intensity gradually decreased with the addition of 18A at L/P molar ratios of 1, 2, and 4, resulting in the formation of nanodiscs with smaller hydrodynamic diameters than those of POPC vesicles, as previously reported (Figure 2a). Dynamic light scattering (DLS) analysis showed that the distribution of the hydrodynamic diameter was less than or equal to 10 nm (Figure S2a), in accordance with the previous reports describing the discoidal particles observed via transmission electron microscopy (TEM) imaging. Similar to 18A, the mutated 18A[A11X] peptides (where X = S, T, N, V, M, Y, and G) also exhibited reduced right-angle light scattering intensities of the POPC vesicles, indicating the formation of small particles, including nanodiscs (Figure S1). The 18A[A11G] mutation at an L/P ratio of 4 resulted in an increase in the scattering intensity for 1 h, while the signal decreased near zero for 1 day (data not shown), indicating a transient aggregation of the peptide and lipid vesicles. However, the 18A[A11P] mutation showed no significant change in the scattering intensity, suggesting no nanodisc formation. This is likely due to the propensity of proline to disrupt α-helical structures. The disruption of charge distribution in the...
indicated that the nano-fluorescein-labeled 18A[A11X] peptides and POPC lipids. The observed fibrillar structures, at optical resolutions, suggested a lateral association of the nanostructures, with widths of >10−20 nm, were observed in the TEM images (Figure 2d). Rouleaux structures, characteristic of 18A[A11C] fibers, were indeed a mixture of lipids and peptides, prior to the preparation of the peptide–lipid assemblies. The subsequently observed colocalization of rhodamine and fluorescein signals in the fibrous aggregates indicated that the nanofibers were indeed a mixture of lipids and peptides. The observed fibrinous structures, at optical resolutions, suggested a lateral association of the nanofibers, forming thick fibril bundles, as seen in the TEM images (Figure S3). Furthermore, we were able to precipitate these aggregates by centrifugation (10 000g, 10 min), and although the fluorescein-labeled 18A[A11C] peptide did not form nanofibers with POPC autonomously, approximately 40% of the peptide was incorporated into the nanofibers formed by the unlabeled 18A[A11C] (data not shown).

Nanofiber and Nanodisc Formation between 18A[A11C] and POPC. In contrast to these mutated 18A peptide variants described, we found that the cysteine substitution at residue 11, 18A[A11C], increased the scattering intensity when the L/P ratio was equal to 1, indicating the formation of coassemblies larger than the initial size of the POPC vesicles (Figure 2b). Our DLS results indicated that the hydrodynamic diameter of these assemblies was approximately 1 μm when an isotropic spherical particle is assumed (Figure S2b). Furthermore, fibrous assemblies, with widths of 10−20 nm and lengths of >1 μm, were observed in the negatively stained TEM images (Figures 2c and S3). However, in the absence of lipid vesicles, 18A[A11C] did not exhibit significant light scattering, indicating that lipid molecules are required for the assembly. Therefore, to confirm the presence of the nanofiber assemblies observed in the TEM images between the 18A[A11C] peptide and the POPC lipids, we employed fluorescence microscopy imaging (Figure 3). POPC liposomes, treated with rhodamine-labeled dioleoylphosphatidylethanolamine (DOPE) and fluorescein-labeled 18A[A11C], were combined with unlabeled peptides prior to the preparation of the peptide–lipid assemblies. The subsequent scattering intensity at an L/P ratio of 4 and the formation of nanoscale particles similar to the other 18A-derived peptides (Figures 2b and S2b). Discoidal particles of 18A[A11C]−POPC, with diameters of 10−20 nm, were observed in the TEM images (Figure 2d). Rouleaux structures, characteristic of nanodiscs, were also evident (Figure 2d, arrowheads). These results indicate that 18A[A11C]−POPC nanodiscs are formed when the L/P ratio is equal to 4. The right-angle light scattering intensities, as a function of the POPC–18A[A11C] molar ratio, are shown in Figure S4. The scattering intensities of the lipid vesicles increased when the L/P ratio was low (<2); however, they decreased at the higher L/P ratios of 3 to 6. At L/P ratios of 8 and 16, no changes were observed in the signal, suggesting that at lower concentrations the peptide did not induce deformation of the vesicle structures. We have also found that the nanofiber could not be formed at a low concentration of 18A[A11C] (<10−6 M), even at an L/P ratio of 1, probably because of the reduced amount of the peptide bound to the lipids (data not shown).

To further investigate the molecular basis of the observed nanofiber formation of 18A[A11C] and POPC, we substituted the cysteine residue at 11 with S-methyl-l-cysteine and found that this mutant did not result in nanofiber formation but did allow for nanodisc formation at an L/P ratio of 1, 2, and 4, as determined by a decrease in the light scattering intensity.
We also found that the position of the cysteine residue was critical for the nanofiber formation (Figure S1). We synthesized three mutants: 18A[L3C], 18A[ASC], and 18A[F6C], in which the cysteine residues were incorporated at the hydrophobic face, the hydrophilic face, and the hydrophobic/hydrophilic interface of the α-helical structure, respectively. These peptide variants did not result in an increase in the light scattering intensity when added to the POPC vesicles, indicating that nanofibers were not formed. However, it is notable that 18A[A11C] did not form intermolecular disulfide bonds in these nanofibers and nanodiscs, which we confirmed by high-performance liquid chromatography (HPLC) analysis (Figure S6). Although metal ions accelerate the formation of disulfide bonds, the addition of EDTA-2Na suppressed the formation of disulfide bonds by preventing interaction between the metal ions and the thiol groups. By contrast, the synthesized 18A[A11C] disulfide dimers result in a decrease in the scattering intensity in combination with the POPC vesicles, resulting in the formation of small particles at L/P ratios of 1, 2, and 4 (Figure 4), as shown in the TEM images of the formation of nanodiscs. Furthermore, the circular dichroism (CD) results indicate that the free sulfhydryl group of cysteine at the hydrophilic interface of the peptide nanoassembly formation by 18A[A11C] dimers. (a) Changes in the right-angle light scattering intensity of the POPC LUVs, followed by the addition of 18A[A11C] dimers at 650 nm. L/P ratios equal to 1 (blue), 2 (red), and 4 (green) are shown at a POPC concentration of 40 μM. The peptide concentrations were calculated as monomer units. The scattering intensity was normalized to the intensity of the POPC LUVs before the addition of the peptide. All measurements were recorded in 20 mM MOPS/1 mM EDTA-2Na buffer (pH 7) at 25 °C. (b) Negatively stained TEM images of 18A[A11C] dimer–POPC assemblies at an L/P ratio equal to 1.

Molecular Structure of the 18A[A11C] Peptide and POPC Lipid Nanoparticles. The CD spectrum of 18A[A11C] in buffer revealed an α-helical structure, as shown by the local minima at approximately 208 and 222 nm, similar to the monomer nanodiscs at an L/P ratio of 4 (Figures S7 and S9, see below). These results indicate that the monomer and dimer peptides experienced similar nanodisc environments. These results indicate that the free sulfhydryl group of cysteine at residue 11 plays a pivotal role in the nanofiber formation.

The CD spectrum at an L/P ratio equal to 4 also indicated the presence of α-helical structures formed by the peptide in the nanodiscs similar to the 18A peptide as shown in previous reports. The peptide and lipid structures in the nanofibers were further investigated using a solid-state NMR (SSNMR) spectroscope (Figure Sb,c). The natural abundance of 13C in the nanofibers, with a magic angle spinning (MAS) at 14.6 kHz, exhibited narrow peak widths (e.g., ~1.2 ppm at 18 ppm for Ala5/17Cβ), indicating uniform molecular structures in the assembly. The carbonyl 13C peak at approximately 178 ppm, indicative of the peptide backbone, showed a downfield secondary shift, suggesting that 18A[A11C] adopted an α-helical-rich structure, in accordance with the CD spectrum results. The static 31P spectrum of POPC in the nanoparticles exhibited a powder pattern, rather than the uniaxial symmetrical pattern typically observed in the lamellar phase, suggesting a deformation of the lipid bilayer. The narrower bandwidths observed (~130 ppm), in contrast to those of solid phospholipid powders (~200 ppm), indicate the residual dynamics of the lipid molecules in the assemblies. Limited sensitivity of the spectrum makes the detailed analysis difficult. Although the dynamics of the lipid headgroup may deform the spectrum obtained by cross-polarization (CP), the experimental spectrum indicates that the headgroup is in a static state. This experimental
spectrum is quite different from that for lipid bilayers in which lipids rotate uniaxially.31

**Characterization of the 18A[A11C]−POPC Assemblies by Fluorescence Spectroscopy.** To characterize the structure of the 18A[A11C]−POPC coassemblies, we performed fluorescence spectroscopic analysis. The tryptophan fluorescence spectrum of 18A[A11C] showed an increase in the signal intensity and a blue shift in the maximal wavelength, coincident with the formation of nanofibers and nanodiscs followed by the addition of POPC vesicles, suggesting that the tryptophan side chain was localized to the hydrophobic regions of these assemblies (Figure 6).32 To determine the accessibility of the tryptophan residue to the solvent, we performed a fluorescence quenching assay. Tryptophan fluorescence was measured in the presence of acrylamide at an excitation wavelength of 280 nm in 20 mM MOPS/1 mM EDTA-2Na buffer (pH 7) at 25 °C. L/P ratios equal to 0 (black), 1 (blue), and 4 (green) are shown at an 18A[A11C] concentration of 10 μM. (b) Quenching of tryptophan fluorescence of the 18A[A11C]−POPC assemblies by acrylamide at an excitation wavelength of 295 nm. Emission wavelengths equal 330 nm (L/P = 1), 335 nm (L/P = 4), and 350 nm (L/P = 0). The ratio of the fluorescence intensity before and after the addition of acrylamide (F0/F) was plotted as a function of quencher concentration. The error bars represent the mean ± SD (n = 2).

![Figure 6](image.png)

**Figure 6.** Tryptophan fluorescence of 18A[A11C]−POPC nanoassemblies. (a) Fluorescence spectra at an excitation wavelength of 280 nm in 20 mM MOPS/1 mM EDTA-2Na buffer (pH 7) at 25 °C. L/P ratios equal to 0 (black), 1 (blue), and 4 (green) are shown at an 18A[A11C] concentration of 10 μM. (b) Quenching of tryptophan fluorescence of the 18A[A11C]−POPC assemblies by acrylamide at an excitation wavelength of 295 nm. Emission wavelengths equal 330 nm (L/P = 1), 335 nm (L/P = 4), and 350 nm (L/P = 0). The ratio of the fluorescence intensity before and after the addition of acrylamide (F0/F) was plotted as a function of quencher concentration. The error bars represent the mean ± SD (n = 2).

The solvent accessibility of the lipid headgroups of these assemblies was also examined. Dipalmitoylphosphatidylethanolamine (DPPE) (0.5 mol %) labeled by nitrobenzoazide (NBD) fluorophore was added to the assemblies. The measured fluorescence intensity of NBD increased from nanofiber to nanodisc to LUV, indicating differential hydrophobic environments (Figure 7a).34 The NBD fluorescence was irreversibly quenched by the addition of the water-soluble quencher sodium dithionite. Approximately half of the fluorescence of the POPC LUVs was quenched followed by the addition of the quencher, indicating that the NBD-DPPE molecules in the outer leaflet of the lipid bilayer vesicles were accessible to the solvent as predicted (Figure 7b).35 Nearly all fluorophores were quenched in the nanodisc and nanofiber assemblies, suggesting that all of the lipid molecules were accessible to the solvent. The kinetics of fluorescence quenching for the NBD fluorophores by dithionite also varied among the different assemblies, with the nanofibers displaying the highest rate of quenching (Figure 7c). These results clearly indicate that the microenvironment surrounding the peptides and lipids is dependent on the morphology of these assemblies. Furthermore, our results show that the headgroups of the lipids that were integrated into the nanofibers had the highest solvent accessibility, reflecting a deformation of the bilayer structures, as shown in our 31P NMR measurements.

To further determine the structure of these nanoassemblies, we examined the composition of the entrapped aqueous compartment of the POPC vesicles, nanodisks, and nanofibers, prepared in a buffer containing calcein dye (Figure 7d).36 A membrane-impermeable ion, Co2+, was added to the solution to quench the fluorescence via the formation of a chelate complex with calcein. The entrapped volumes of these assemblies were determined via quenched fluorescence signals. POPC vesicles with a diameter of approximately 100 nm displayed an entrapped volume of 2.5 L/mol total lipid, in accordance
with the theoretical volume of 3.0 L/mol for vesicles with a diameter of 100 nm. Entrapped aqueous phases were nearly absent in the nanodiscs and nanofibers, indicating no sequestered aqueous compartments in the nanofiber and nanodisc assemblies.

**Induced Transitions between Fibrous and Discoidal 18A[A11C]–POPC Assembly.** Determining the precise timing of the transition to supramolecular assemblies is challenging. Therefore, we evaluated these structural transitions via the use of external stimuli. We alternately added peptide and lipid vesicles to the POPC vesicle solution, thereby altering the L/P ratio from 4 to 1. The right-angle light scattering intensity both decreased and increased, in a stepwise manner, as shown in Figure 8a, suggesting the alternate formation of nanodiscs and nanofibers in this range. These transitions, from nanodisc to nanofiber, appeared to be reversible, which is supported by the observation that the final scattering signals were not influenced by previous lipid–peptide mixing (Figure 8b). These results demonstrate that reversible morphological transitions can be triggered by a change in the molar ratio of lipid to peptide.

As shown in Figure 4, the disulfide-linked dimers of 18A[A11C] formed nanodiscs even at an L/P ratio equal to 1, whereas monomeric 18A[A11C] formed nanofibers. The addition of tris(2-carboxyethyl)phosphine (TCEP), which reduces disulfide bonds to free sulphydryl groups, resulted in a large increase in the scattering intensity, for up to 1 h, of the 18A[A11C] dimer–POPC nanodiscs at an L/P ratio equal to 1 (Figure 8c). This indicates the formation of nanofibers resulting from the interaction with 18A[A11C] monomers. Nearly all of the dimeric 18A[A11C] reverted to monomers 1 h after the addition of TCEP, as confirmed by HPLC. Furthermore, upon the addition of the oxidizing agent K3Fe(CN)6 to the 18A[A11C] monomer–POPC nanofibers, scattering was decreased after 9 h, indicating a transition from nanofiber to nanodisc via dimer formation (Figure 8d).

Therefore, our results indicate that the morphologies of these nanoassemblies can be controlled by the reduction of the cysteine residue in the 18A[A11C] peptide.

**DISCUSSION**

Cooperative self-assembly of biomolecules of different classes represents a novel strategy for constructing biomaterials with a variety of structures and functions. Here, we demonstrate that the amphiphilic α-helical peptide 18A[A11C] and POPC can cooperatively assemble into not only nanodiscs at an L/P ratio equal to 4 but also nanofibers at an L/P ratio of 1, as shown by our TEM and fluorescence imaging results. Previous studies have shown that the diameter of the lipid bilayer is regulated by the L/P ratio in 18A nanodiscs. By contrast, the nanofiber morphology in the 18A[A11C]–POPC mixture at an L/P ratio of 1 suggests a significantly different arrangement of the constituent molecules in the assembly from those of the nanodiscs. Furthermore, these 18A[A11C]–POPC nanofibers have an apparent novel molecular architecture. These 18A[A11C]–POPC nanofibers formed α-helical bundles, as shown in our CD and 31P NMR spectroscopy results. This configuration is distinct from the amyloid fibrils formed by pathogenic peptides such as amyloid-β, in which the β-strands run perpendicular to the fiber axis, although the fibrillation of amyloid peptides has also been reported to be facilitated by lipid membranes. Similar β-sheet structures have been reported in nanofibers formed by peptide amphiphiles. Our 18A[A11C]–POPC nanofibers resemble the peptide nanofibers described by Woolfson et al., exhibiting similar CD spectra and TEM images, where the leucine zipper motifs formed helical bundles, resulting in the assembly of nanofibers. However, our results clearly demonstrate that phospholipid molecules were also integrated into the nanofibers. The 31P NMR spectrum, showing a nonuniaxial symmetry pattern, suggests a deformation of the lamellar structure of the lipid bilayer in these nanofibers. Although the amphiphilic α-helix of 18A[A11C] does not have a defined, complementary interface for bundle formation similar to the leucine zipper motif, the POPC molecules appear to bind to the hydrophobic patches between the associated α-helices and stabilize the fibrous aggregates. The disruption of the lipid bilayers observed in these nanofibers also differs from the protein-mediated tube formation of lipid membranes induced by BAR domains. Domanov and Kinnunen reported that lipid nanotubes, with diameters of less than 250 nm, formed upon the addition of an antimicrobial peptide to supported phospholipid bilayers at an L/P ratio of approximately 5. Lee et al. demonstrated that a de novo designed amphiphilic peptide formed Golgi-like nanotubules with Golgi-specific lipids. The morphology of the tubules is similar to that of our 18A[A11C]–POPC nanofibers in terms of the length and diameters. However, the tubules were formed at L/P ratios of 2.5–10, suggesting that the stoichiometry and the structures of constituent molecules in these assemblies are different.
Our results indicate that nanofiber formation by these 18A-derived peptides is specific to the A11C mutation. Furthermore, a free sulphydryl group is required. Other amino acid substitutions, including the structurally conservative amino acids such as alanine, serine, and threonine, did not result in nanofiber formation. However, previous studies have shown that the conservative amino acid substitution of a cysteine residue does not always maintain the structural and molecular recognition properties of the proteins. Cysteine residues have been shown to play several pivotal roles in molecular interactions. Intra- and intermolecular van der Waals interactions, weak hydrogen bonding, and slight hydrophobicity appear to be critical for the nanofiber formation. Nonhydrogen bonding interactions with carbonyl, amide, and aromatic groups in proteins have also been reported to be important for these molecular interactions. Interestingly, Cys11 disulfide bond formation induced nanodisc formation even at an L/P ratio of 1, suggesting that the intermolecular cross-linking of the cysteine residues constrained peptide conformations to unfavorable positions for nanofiber formation. Thus, connecting two adjacent peptides at their hydrophobic/hydrophilic interfaces may be more favorable for nanodisc formation when taking the double belt model into account, in which two amphiphilic \( \alpha \)-helical chains wrap around the lipid bilayers. A cysteine mutation at the other side of the hydrophobic/hydrophilic interface (F6C mutant) did not result in the fiber formation, suggesting that the position of residue 11 was also important for the interhelical interactions in the nanofibers. Further structural analysis of these nanofibers using imaging and spectroscopic techniques, including cryo-electron microscopy and high-resolution SSNMR analysis combined with isotope-labeled peptides and lipids, will provide an increased understanding of the mechanisms underlying these molecular interactions at an atomic resolution.

The 18A[A11C]–POPC complexes exhibited a reversible transition between the nanodisc and the nanofiber depending on the L/P ratio and redox state of the cysteine residue. The structural transitions of nanobioassemblies, resulting from external stimuli, have been reported in multiple systems, including liposomes, peptide nanofibers, and peptide amphiphiles. Structural changes can be critical for bioactivity, including the release of incorporated drug molecules, which is necessary for the development of nanomedicines. We found no internal aequorin phase sequestration from the external solution in the 18A[A11C]–POPC nanofibers, unlike in our observations of the POPC liposomes (Figure 7d), indicating that they are unsuitable for carriers of hydrophobic drugs and imaging agents. However, this 18A[A11C]–POPC nanodisc/nanofiber system has potentially attractive applications for nanomaterials. We observed significant changes in the surface accessibility in the integrated molecules during the disk-to-fiber transition (Figures 6 and 7). This indicates that the molecular recognition of these surface-presented molecules may be regulated by structural transitions. The wide-ranging changes in the size (nanometers to micrometers) between the nanodiscs and nanofibers allow them to be easily separated using basic laboratory techniques such as centrifugation. Furthermore, emerging evidence suggests that nanodiscs are suitable substrates for the structural and functional analysis of membrane proteins via the incorporation of the protein in the lipid bilayer of the disks and dispersal in solution. Indeed, the structures of membrane proteins, including outer membrane protein X and transient receptor potential vanilloid 1 ion channel, into nanodiscs have been shown by solution NMR spectroscopy and cryo-electron microscopy, at atomic resolutions, respectively. Nanodiscs also show promise as potential drug carriers. Alternatively, peptide-based nanofibers have been examined for use in diverse applications including tissue engineering. Therefore, the stimuli-induced transitions seen in our nanodisc-to-nanofiber assemblies may enable the further manipulation of multifunctional nanomolecular components.

### CONCLUSIONS

In this study, we show that the amphiphilic peptide 18A[A11C] forms both nanodiscs and nanofibers cooperatively with the phospholipid POPC. We also demonstrate that the morphologies of these heteromolecular complexes are influenced by the amino acid sequence of this peptide, the L/P ratio, and the redox state of the cysteine residue. Furthermore, the reversible structural transition between the nanofiber and the nanodisc could be induced via external stimuli. These lipid–peptide nanofibers have a unique supramolecular architecture, in which the \( \alpha \)-helical peptide bundles are associated with nonlamellar phospholipid molecules. Our findings provide insights into the mechanisms underlying the formation of supramolecular structures, utilizing a mixture of biomolecules from different classes, and demonstrate a novel, rational design for nanomaterials that may be utilized in future applications involving peptide–lipid nanoassemblies.

### EXPERIMENTAL PROCEDURES

**Peptide Synthesis.** Peptides were synthesized manually using Fmoc-based solid-phase synthesis. To generate fluorescein-labeled 18A[A11C], a fluorescein moiety was introduced into the N-terminus of the peptide using 5-carboxyfluorescein in the presence of \( N,N' \)-disopropylcarbodiimide and 1-hydroxybenzotriazole in dimethylformamide on resin. The dye-labeled proteins were protected from light to avoid photodegradation. The peptides were cleaved from the resin using trifluoroacetic acid/water/1,2-ethanediol/triisopropanol (92.5/2.5/2.5/2.5) for 3 h and purified via reverse-phase HPLC (RP-HPLC) on a C18 HPLC column over a gradient of water to acetonitrile containing 0.1% trifluoroacetic acid. The peptides were characterized by HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Autoflex-T1, Bruker Daltonics, Billerica, MA). The 18A[A11C] disulfide-linked dimers were prepared using 18A[A11C] monomers (1 mM) incubated in a buffer (20 mM MOPS, pH 7) containing 50% dimethyl sulfoxide for 1 week and purified using RP-HPLC. Lyophilized peptide powder was dissolved in water, and the concentration was measured at an absorbance of 280 nm. Peptide stock solutions were stored at −25 °C until use.

**Lipid Vesicle Preparation.** POPC, with a purity greater than 99%, was purchased from the NOF Corporation (Tokyo, Japan). NBD-DPPE and rhodamine-DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). The LUVs were prepared via the extrusion method as previously described. Briefly, aliquots of lipids in chloroform/methanol (2/1) were transferred to round-bottom flasks, and the solvent was removed using a rotary evaporator. The residual lipid film was dried under vacuum overnight. A buffer solution (20 mM MOPS/1 mM EDTA-2Na, pH 7) was added to the lipid film to form multilamellar vesicles (MLVs) and subjected to five cycles...
of freezing and thawing. The MLV suspension was filtered through a 100 nm pore polycarbonate filter 31 times to form the LUVs. The size of the LUVs was determined using a DelsaMax Core dynamic light scattering instrument (Beckman Coulter, Brea, CA).

**Light Scattering.** Formation of the supramolecular assemblies by peptides and lipids was monitored by right-angle light scattering using an F-4500 fluorescence spectrometer (Hitachi, Tokyo, Japan) with a quartz cell of 4 mm path length set in a cell holder at 25 °C. The peptides (0, 10, and 40 μM) and the LUVs (40 μM) were mixed into the buffer (20 mM MOPS/1 mM EDTA-2Na, pH 7). Excitation and emission wavelengths were measured at 650 nm with slit widths of 5 nm. The DLS of the nanodiscs and nanofibers was measured using a DelsaMax Core instrument (Beckman Coulter, Brea, CA).

**CD Spectroscopy.** The CD spectra of the assemblies in the buffer (20 mM MOPS/1 mM EDTA-2Na, pH 7) were measured at 25 °C using a J-805 spectrometer (JASCO, Tokyo, Japan), with a 1 mm path length quartz cell. Eight scans were averaged for each sample. The spectrum of the lipids in the LUVs was subtracted as a blank.

**Tryptophan Fluorescence and Quenching Assays.** The fluorescence spectra were measured using an F-4500 fluorescence spectrometer (Hitachi, Tokyo, Japan). Before acquisition of the tryptophan fluorescence spectrum, 18A-[A11C] (10 μM) and POPC LUVs (0, 10, and 40 μM) were mixed in the buffer (20 mM MOPS/1 mM EDTA-2Na, pH 7) in a quartz cell with 4 mm path length at 25 °C and incubated for at least 1 h. The excitation wavelength was set to 295 nm. Acrylamide was added to the sample solutions to quench the fluorescence signals of the tryptophan residues. The fluorescence intensity, as a function of the acrylamide concentration, [Q], was analyzed using the Stern–Volmer equation, \[ F_0/F = 1 + K_{SV}[Q] \], where \( F_0 \) and \( F \) are the intensities in the presence and absence of the quencher, respectively. The Stern–Volmer constant \( K_{SV} \) was measured using least linear fitting of the plot.

**NBD Fluorescence and Quenching Assays.** POPC/NBD-DPPE (99.5/0.5) LUVs (40 μM lipid) and 18A[A11C] (0, 10, and 40 μM) were mixed into the buffer (20 mM MOPS/1 mM EDTA-2Na, pH 7) in a quartz cell with 4 mm path length at 25 °C and incubated for 2 h. Fluorescence intensities were recorded at an excitation wavelength of 460 nm and an emission wavelength of 534 nm. During signal recording, sodium dithionite in 2 M tris(hydroxymethyl)-aminomethane solution was added into the cell after 120 s to a final concentration of 8 mM to quench the NBD moieties accessible to the solvent. Triton X-100 was added to the sample (1%) to disrupt the assemblies after 960 s.

**Evaluation of the Entrapped Aqueous Volume.** POPC LUVs were prepared in a buffer containing calcine dye (20 mM MOPS/0.1 mM calcine, pH 7). LUVs (1 mM) were incubated in polypropylene tubes in the absence and presence of 18A[A11C] (0.25 and 1 mM) for 2 h at 25 °C. The samples were diluted 100-fold in a calcine-free buffer (20 mM MOPS, pH 7) to a final volume of 300 μL. The fluorescence intensity at 530 nm was recorded at an excitation wavelength of 490 nm. After 120 s, a CoCl₂ solution was added to a final concentration of 0.1 mM to quench the calcine fluorophore outside of the assemblies by the chelation of Co²⁺. Then, 30 μL of 10% Triton X-100 (1%) was added to complete the quenching by disrupting the assemblies after 360 s. The fraction of the entrapped calcine in the assemblies, \( E \), was calculated as \( E = (F_2 - 1.1F_0)/(F_1 - 1.1F_0) \), where \( F_1 \) and \( F_2 \) are the fluorescence intensities of the calcine before and after the addition of CoCl₂, respectively, and \( F_0 \) is the intensity after the addition of Triton X-100. The entrapped volume of the aqueous phase in the assemblies for 1 mol of lipid was determined by \( V = E/[\text{Lipid}] \) (L/mol), where \([\text{Lipid}]\) indicates the concentration of POPC before the dilution (1 mM).

**Transmission Electron Microscopy.** A glow-discharged Cu grid coated with a thin carbon film (300 meshes) was placed on a droplet of the sample for 5 min. Then, the excess sample solution on the grid was removed using a filter paper. Next, the grid was placed on a droplet of 2% phosphotungstic acid for 30 s and dried after the removal of the excess solution. TEM images were obtained using a JEM-1400 transmission electron microscope (JEOL Inc., Tokyo, Japan) operated at 80 keV.

**Fluorescence Microscopy.** Nanofibers were prepared by mixing peptides and lipid vesicles as described above. Here, LUVs doped with 1 mol % of rhodamine-DPPE were used. Fluorescein-labeled 18A[A11C] (5 mol %) was added to the nonlabeled peptide. The sample solution was placed on a glass slide and sealed by a coverslip. Fluorescence imaging was conducted using the BX51 microscope equipped with a 100 W mercury lamp and a DP50 digital camera (Olympus, Tokyo, Japan). The U-MWIB2 (excitation filter, 460–490 nm; emission filter, 510 nm; and dichromatic mirror, 505 nm) and U-MWG2 (excitation filter, 520–550 nm; emission filter, 580 nm; and dichromatic mirror, 565 nm) mirror units were employed for the detection of fluorescein and rhodamine fluorescence signals, respectively.

**SSNMR Spectroscopy.** Nanofiber sample (5 mg) was packed into a 3.2 mm MAS ZrO₂ rotor by ultracentrifugation (120 000 g, 20 min) using the Beckman Optima L-90K ultracentrifuge equipped with the SW40Ti rotor. A custom-made rotor adaptor was utilized for the direct packing of the nanofibers into the NMR rotor from the suspension. SS ¹³C and ³¹P NMR experiments of nanofibers were performed using JEOL ECA II spectrometers at ¹H resonance frequency of 700 and 500 MHz, respectively. ¹³C NMR was observed with a repetition time of 3.0 s at an MAS frequency of 14.6 kHz and a sample temperature of 5 °C. ³¹P NMR was observed under a static condition with a repetition time of 7.0 s at 25 °C. Time-domain ¹³C and ³¹P signals were acquired under two-pulse phase modulation ¹H decoupling at an rf amplitude of 78 kHz following CP from ¹H with a contact time of 2.0 ms. The free induction decays for ¹³C and ³¹P NMR with a spectral width of 100 kHz were multiplied by an exponential function with a dumping rate of 50 and 300 Hz, respectively, and were zero-filled to 4096 points using the Delta NMR software (JEOL Inc., Tokyo, Japan). The ¹³C NMR spectrum was obtained by subtracting the background signals recorded for a blank rotor. The ¹³C chemical shift was referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid.

**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00424.

Nanofiber formation between 18A[A11X] peptide and POPC, DLS analysis, TEM images, HPLC analysis, and CD spectra (PDF)
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