Nanostructured Materials

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Nanofibers and Lyotropic Liquid Crystals from a Class of Self-Assembling β-Peptides**

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Lyotropic liquid-crystalline (LC) phases, in other words, LC phases induced by the presence of a solvent, form through the assembly of large anisometric particles, such as rigid polymers,[1] viruses,[2] or inorganic rods,[3] as a result of excluded volume interactions (Onsager theory).[4] Small molecules can form lyotropic LC phases if they undergo self-assembly to generate anisometric nanostructures. Aqueous LC phases are commonly observed for small molecules that have clearly segregated hydrophilic and lipophilic segments, such as detergents and lipids.[1a] For such "globally amphiphilic" molecules it is believed that hydrophobically induced association of the lipophilic segments drives the assembly that underlies LC formation. Chromonic LCs are formed from a distinct class of small molecules that self-assemble in water but do not display a simple segregation of hydrophilic and lipophilic segments[5] (they are non-globally amphiphilic). It is difficult to understand how the self-assembly of chromonic molecules (i.e., molecules that form chromonic LCs) is related to the nature and internal organization of lipophilic surfaces because these molecular properties cannot readily be altered in an incremental fashion. Here we describe a class of oligomers that, like the chromonic molecules, are not globally amphiphilic but nevertheless form LC phases in water. The intrinsic modularity of these oligomers allows rational and systematic modification of lipophilic or hydrophilic components, which enables us to define key features that are required for LC formation.

Short oligomers of β-amino acids (β-peptides) are attractive for systematic study of assembly processes leading to LCs because β-peptides can display a diverse range of functionalized side chains, and these oligomers fold into compact and stable conformations that orient the side chains in predictable ways.[6] The most widely studied β-peptide secondary structure is the 14-helix, which is defined by $i,i-2$ C=O···H–N hydrogen bonds (14-membered ring) between backbone amides and contains approximately three residues per helical turn. The high stability of the 14-helix, in combination with ready manipulation of β-peptide sequence, permits considerable control over the nanopatterning of chemical functionality in three dimensions. Here we exploit these features by designing β-peptide oligomers that fold into helical nanostructures and are decorated with functional groups in patterns that either do or do not confer global amphiphilicity on the 14-helix. As described below, this sequence-based patterning strategy has led to the discovery of β-peptide oligomers that are non-globally amphiphilic and exhibit liquid crystallinity in aqueous solution.

Our initial experiments focused on sequence isomers **A** and *iso*-**A** (Figure 1). The latter has a repeating triad motif containing *trans*-2-aminocyclohexanecarboxylic acid (ACHC), β^{3}homophenylalanine (β^{3}hPhe), and β^{3}hLys residues; the lipophilic–lipophilic–hydrophilic ACHC-β^{3}hPhe-β^{3}hLys sequence repeat pattern in *iso*-**A** leads to a globally amphiphilic nanostructure. In contrast, the sequence of **A** does not lead to global segregation of lipophilic and hydrophilic side chains in the 14-helix, but rather to a distribution of lipophilic and hydrophilic side chains around the entire periphery of the helix (Figure 1). The helix from **A** is defined as non-globally amphiphilic. Since global amphiphilicity of β-peptides has previously been associated with liquid crystallinity,[7] we expected *iso*-**A** but not **A** to form a LC phase in water. Surprisingly, however, strong birefringence was observed for aqueous solutions containing **A** $\geq$ 6.5 wt%, (36 mM, Figure 2), but no birefringence was detected for solutions of *iso*-**A** up to the solubility limit $>$ 10 wt% (57 mM, Figure 2).

Inspection of the birefringent domains at high magnification shows an absence of optical textures characteristic of higher ordered mesophases such as smectics and cholesterics;[8] these observations lead us to conclude that the mesophase is likely nematic. The unexpected ability of the non-globally amphiphilic helix to form an LC phase is

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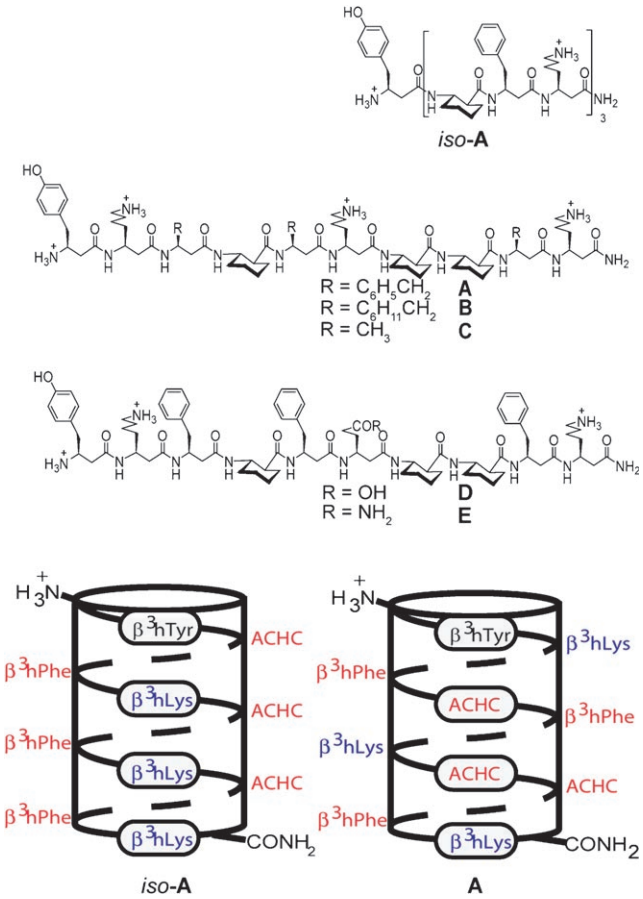

Figure 1. β-Peptide sequences (top) and cylinder representations (bottom) of globally amphiphilic *iso*-**A** and non-globally amphiphilic **A**.

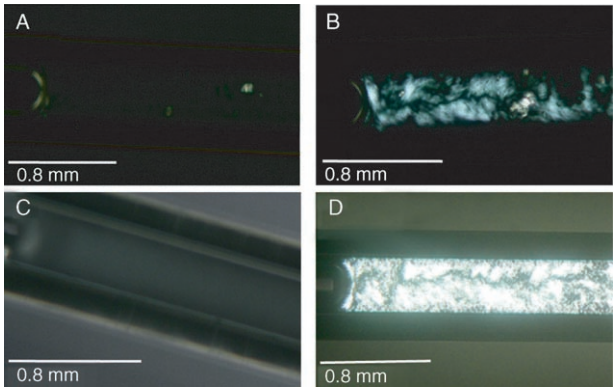

Figure 2. Optical micrographs of solutions of β-peptides between crossed polarizing filters. A) *iso*-**A** (10 wt%, 57 mM), B) **A**, (10 wt%, 57 mM), C) **B** (9 wt%, 51 mM), D) **D** (4 wt%, 23 mM).

intriguing, as is the fact that the globally amphiphilic isomer does not display LC order.

β-Peptide oligomers are inherently modular, and we took advantage of this feature to shed light on the interactions that underlie LC phase formation by **A** in aqueous solution. First, we made systematic changes in the β-peptide sequence to try to elucidate the contributions of the side chains of **A** to the intermolecular interactions that underlie liquid crystallinity. In order to determine whether self-assembly into a LC is driven by hydrophobicity, we replaced the aromatic rings of the three β^3hPhe residues in **A** with cyclohexyl rings (**B**). This substitution abolished the LC phase at all concentrations investigated. Since cyclohexane is more hydrophobic than benzene,[9] the behavior of β-peptide **B** indicates that hydrophobicity alone does not explain LC phase formation by **A**. A second analogue (**C**),[10] in which each aromatic ring is replaced by a hydrogen atom (three β^3hPhe→β^3hAla mutations), also failed to form a LC phase; this allows us to conclude that the aromatic side chains of the β^3hPhe residues in **A** are necessary for assembly into a LC phase. We note that interactions mediated by aromatic side chains of the amino acids phenylalanine, tyrosine, and tryptophan are often found to stabilize tertiary structure in folded proteins.[11]

We next sought to understand the role of electrostatic interactions in the self-assembly process that leads to formation of the LC phase. The three β^3hLys residues and the N terminus of **A** should confer a net charge of +4 on the oligomer at or below neutral pH. Substitution of β^3hLys-6 by β^3hGlu, to form **D**, led to LC phase formation in more dilute solutions as low as 2 wt% (11 mM, Figure 2).[10] This mutation decreases the positive charge of the β-peptide mesogen by at least one unit; the resulting enhancement of LC phase formation suggests that electrostatic forces between β-peptide molecules have a large effect on LC phase formation. To test whether the reduction in minimum LC concentration observed for **D** relative to **A** results from a simple reduction in net charge or from the ability of the β^3hGlu side chain to engage in intermolecular salt-bridge interactions, we examined β-peptide **E**, which contains β^3hGln in place of β^3hGlu. The amide side chain of β^3hGln is similar to the carboxylic acid side chain of β^3hGlu in size and shape but is unable to form an ion pair. The minimum LC concentration for **E** was found to be comparable to that of **D**,[10] which suggests that both mutations influence LC formation through a simple decrease in net charge. Overall, the results of the mutation studies suggest that self-assembly of these non-globally amphiphilic β-peptide oligomers into liquid crystals is promoted by aromatic interactions and regulated by electrostatic repulsions.

Onsager theory predicts that assemblies of molecules with a high aspect ratio will spontaneously form LC phases above a critical concentration through an entropy-driven process.[4] This theory requires large aggregates (>500 monomers)[10] if LC behavior is observed at concentrations as low as 2 wt%. Cryogenic transmission electron microscopy (cryo-TEM) was therefore undertaken in an effort to obtain evidence for formation of high-aspect-ratio assemblies in lyotropic LC phases formed by non-globally amphiphilic β-peptides. At 2 wt% **A**, below the concentration required for LC phase formation, cryo-TEM revealed micron-long fibers intermixed with smaller globular aggregates (Figure 3A). At 8 wt% **A**, which forms a lyotropic LC phase, a densely packed network of fibers was observed (Figure 3B). Nanoscale assemblies of this type could correspond to the high-aspect-ratio mesogens predicted by Onsager theory; in other words, these nanofibers generated by β-peptide self-assembly presumably represent the mesogenic species for LC phase formation. In contrast,

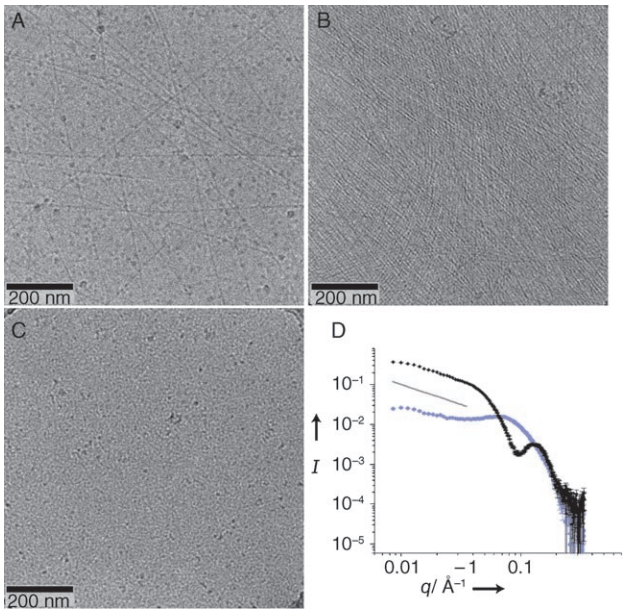

Figure 3. SAXS and cryo-TEM micrographs of A) **A**, (2 wt%, 11 mM), B) **A**, (8 wt%, 44 mM), and C) *iso*-**A** (8 wt%, 44 mM). D) SAXS scattering curves for *iso*-**A** (black diamonds) and **A** (blue-gray circles); a q^{-1} slope is shown as a reference.

only globular aggregates were observed in aqueous solutions of *iso*-**A** over the concentration range studied. This observation is consistent with the inability of β-peptide *iso*-**A** to support lyotropic LC phase formation (Figure 3C).

We performed small-angle X-ray scattering (SAXS) experiments to characterize further the microstructure of the assemblies in the absence of the sample freezing necessary for cryo-TEM. SAXS measurements were conducted with 8 wt% aqueous solutions of sequence isomers *iso*-**A** and **A**. Inspection of Figure 3D reveals that scattering from **A** at low q follows a q^{-1} dependence, consistent with the presence of rodlike aggregates as seen in the cryo-TEM. In contrast, SAXS measurements of *iso*-**A** are consistent with the presence of small globular aggregates. These results confirm that both **A** and *iso*-**A** aggregate in solution, but that only **A** assembles into high-aspect-ratio structures, as inferred from cryo-TEM.[12]

14-Helix formation by β-peptides has been extensively studied in the crystalline state and in dilute solution.[6] We undertook circular dichroism (CD) measurements to determine whether β-peptides of the type discussed here remain 14-helical under conditions approaching those required for the nanofiber formation.[10] Dobson et al. and others[13] have demonstrated that α-helical α-peptides can denature to form β-sheets that aggregate at high concentration, and Aggeli et al.[14] have shown that β-sheet assemblies can form LC phases. β-Peptides **A** and *iso*-**A** displayed strong 14-helical signatures up to 2 wt%,[10] the concentration at which nanofibers begin to form for **A.** A CD spectrum was recorded also for β-peptide **D** at a concentration of 0.8 wt%, which is just below the minimum LC concentration (because our samples are not aligned in the cuvette, the modulation of polarized light from birefringence affects the circularly polarized light necessary for CD experiments[15]). β-Peptide **D** at this high concentration displays a broad minimum near 214 nm, which is characteristic of the 14-helix.[10] Overall, the CD data indicate that both **A** and **D** display 14-helicity even at relatively high concentrations, which is consistent with the strong stabilization of 14-helical structures by ACHC residues.[16] These results suggest that nanofiber formation results from self-assembly of non-globally amphiphilic β-peptides in the 14-helical conformation and argue against a change in β-peptide folding upon self-assembly.

The experiments described above have allowed us to identify a new class of non-globally amphiphilic molecules that can form lyotropic LC phases. A number of analogies can be drawn between our β-peptides and the mesogens that form chromonic LCs, although typical chromonic molecules have structures quite different from those of β-peptides.

1) Our results show that aromatic side chains are crucial for lyotropic LC formation in water by non-globally amphiphilic β-peptides. Similarly, a defining feature of chromonic molecules is the presence of large aromatic surfaces.[5]
2) The dramatic difference between sequence isomers **A** and *iso*-**A** shows that distribution of hydrophilic groups around the molecular periphery is important for LC phase formation within this family of β-peptides. Similarly, hydrophilic groups are generally distributed around the periphery of a rigid aromatic core in chromonic LC mesogens.
3) Variations in the behavior of **A**, **D**, and **E** reveal that electrostatics play a vital role in regulating LC phase formation by β-peptides. Similarly, the effects of added salt on the stability of chromonic LC phases suggest that electrostatic interactions are important in these systems.[17]
4) Our cryo-TEM and SAXS data indicate that LC-forming β-peptides assemble into nanofibers, which are the mesogenic species. Similarly, columnlike nanostructures are detected in chromonic LCs.[17] A significant distinction between these two classes of LC-forming molecules is that β-peptides can be incrementally modified with ease, while chromonic molecules cannot. Therefore, β-peptides offer a versatile system for exploring structure–property relationships among lyotropic LC-forming molecules.

The non-globally amphiphilic β-peptide oligomers described here could provide a foundation for technological advances comparable to those achieved with non-globally amphiphilic small molecules that form chromonic LCs. Chromonic LCs can be compatible with viruses,[18] cells,[18,19] and proteins,[20] and these LCs have been used as sensors for recognition of biomolecules.[20,21] The modular nature of the LC-forming β-peptides is advantageous because additional functionality can be readily introduced, for example, α-peptide sequences with specific recognition properties. Continued efforts to elucidate the relationship between β-peptide sequence, the three-dimensional display of side-chain functionality that results from folding, and self-assembly into nanofibers should allow us to take advantage of β-peptide modularity for tailoring LC properties in this broad context.

Experimental Section

β-Peptide synthesis and purification: See the Supporting Information for full experimental details. Briefly, all β-peptide sequences were synthesized in a CEM MARS microwave reactor using standard solid-phase peptide-coupling reagents. β-Peptides were subsequently cleaved from the solid support and purified by RP-HPLC. Polarized optical microscopy was carried out by dissolving lyophilized β-peptide powders in a known volume of water followed by incubation overnight to ensure dissolution. Aliquots of β-peptide solutions were extracted into a microcapillary, sealed with vacuum grease, and immediately analyzed between crossed polarizing filters. Preparation of samples for cryo-TEM and SAXS is detailed in the Supporting Information. Circular dichrosim measurements were carried out using a thin film of β-peptide solution placed between two quartz slides with a pathlength of either 10 μm or 100 μm.

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