Communication

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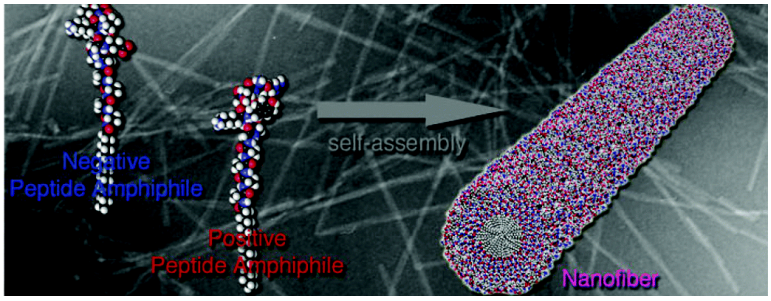

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Self-Assembly Combining Two Bioactive Peptide-Amphiphile Molecules into Nanofibers by Electrostatic Attraction

Krista L. Niece, Jeffrey D. Hartgerink,† Jack J. J. M. Donners, and Samuel I. Stupp*

Departments of Materials Science & Engineering and Chemistry, and the Feinberg School of Medicine, Northwestern University, 2220 Campus Drive, Evanston, Illinois 60208

Received August 20, 2002; E-mail: s-stupp@northwestern.edu

There are instances in the preparation of cell substrates in which combining two biological signals in intimate fashion yields a synergistic effect on cell response.[1−3] Therefore, the development of materials that present two or more peptide signals in a three-dimensional environment could be important to advanced medicine.[4] Here we describe a new approach to self-assembly that combines these features. In our strategy, we combine two oppositely charged peptide-amphiphiles (PAs) in aqueous solution at physiological pH, each bearing a different biological signal. Nanofiber formation in these solutions transforms the liquid into a three-dimensional gel. This method can bring together two amino acid sequences into a single self-assembled nanofiber. This is in contrast to our previously reported PA systems, which required low pH for self-assembly[5,6] and involved only a single molecule. In principle, this system could be delivered to living tissues through a simple injection.

The peptide epitopes on molecules **1**−**4** were chosen as a demonstration of the biomedical potential of the self-assembling systems described here. RGD is the well-known cell adhesion ligand found in fibronectin,[7] while IKVAV[8] and YIGSR[9] are laminin sequences known to interact with mammalian neurons. IKVAV promotes neurite outgrowth in mammalian neurons, while YIGSR appears to play a related role in neuronal cell−substrate adhesion. PAs **1** and **3** have a net negative charge at neutral pH, whereas **2** and **4** have a net positive charge. Electrostatically driven co-assembly between **1** and **2** as well as **3** and **4** thus creates mixed nanofibers that simultaneously present two biological signals to cells.

We demonstrated previously that peptide-amphiphiles containing acidic amino acids could be self-assembled upon neutralization of their charge at acidic pH (below 4)[5] or with the use of divalent cations.[6,10] In this work, we prepared mixed systems, in which oppositely charged PAs self-assemble by forming salt-bridged pairs at neutral pH, and PAs with basic amino acids that can self-assemble at alkaline pH. For this purpose, molecules **1**−**4** were prepared by standard solid-phase peptide synthesis followed by alkylation with the C16 fatty acid, palmitic acid (Figure 1). The peptides thus prepared were characterized by electrospray ionization (ESI) mass spectrometry and found to correspond to their respective expected masses. All four of the PAs were found to dissolve in water at neutral pH at concentrations of 1 mg/mL or less. Molecules **3** and **4** must be fully reduced to eliminate disulfide bond cross-linking and then used under anaerobic conditions or left in an excess of dithiothreitol. Molecules **1** and **2** did not require this treatment.

Solutions of each molecule were prepared at a concentration of 0.1 mg/mL at neutral pH. Molecule **1** was slowly acidified (HCl) and found to precipitate below a pH of 4.5, while increasing the solution's pH (KOH) left the molecule completely dissolved. Conversely, base was slowly added to a solution of molecule **2**, and it was found to be soluble until a pH above 9.5 was reached at which point a precipitate formed. At neutral pH, both molecules were completely dissolved; however, upon mixing these clear solutions, precipitation occurred within seconds with no detectable change of pH. At higher concentrations (5 mg/mL), mixing the oppositely charged amphiphiles caused the immediate formation of a birefringent gel. Molecules **3** and **4** behaved in an analogous fashion.

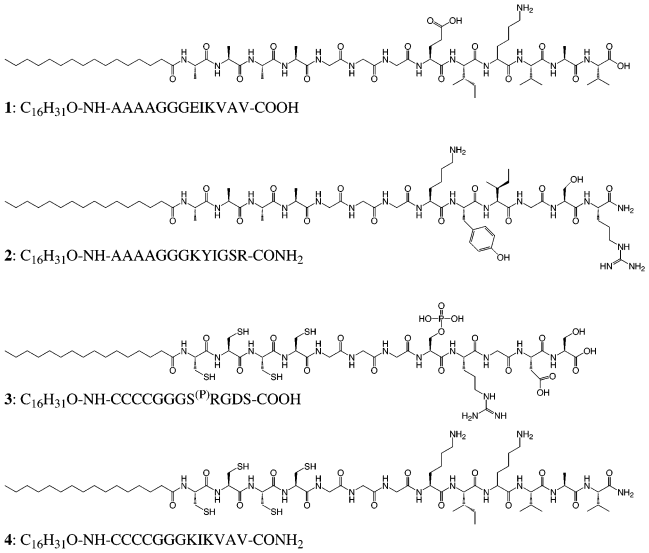

Figure 1. Chemical structures of the four peptide-amphiphiles used for self-assembly. Molecule **1** and **3** self-assemble at acidic pH, and molecule **2** and **4** self-assemble at basic pH, while molecule pairs **1**/**2** and **3**/**4** co-assemble at neutral pH.

The self-assembly of the nanofibers was characterized by negative stain transmission electron microscopy (TEM) and infrared spectroscopy (FT-IR) as well as 2-D NMR spectroscopy. Samples of the precipitated material in each of six cases, one for each individual PA and mixed samples of PAs **1** and **2** (PA **1**/**2**) and PAs **3** and **4** (PA **3**/**4**), when examined by TEM, revealed that in all cases the PA had self-assembled into nanofibers with nearly uniform diameters of 7 ± 1 nm and often many micrometers long. FT-IR showed strong hydrogen bonding based on N−H stretching frequencies between 3276 and 3289 cm^{-1}, and all spectra showed significant parallel β-sheet character based on the position of the amide I band at 1630 cm^{-1}. Additional contributions in this region between 1650 and 1680 cm^{-1} indicate that the peptide region also adopts significant α-helix and random coil characteristics. In the case of PA **1**/**2**, the NOESY spectrum indicates intermolecular close contact (<3 Å) between the γ-protons of valine on PA **1** and the β-proton of serine on PA **2**. Other contacts between residues are also likely to be intermolecular (see Supporting Information). This

† Current address: Department of Chemistry, Rice University. Email: jdh@rice.edu.

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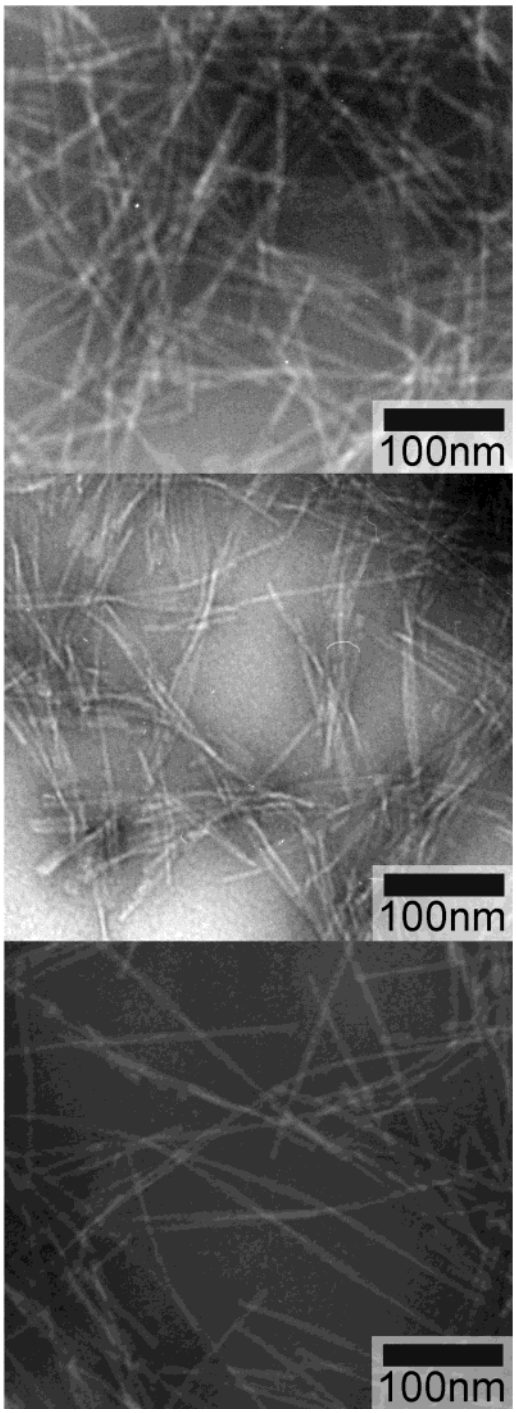

Figure 2. TEM images of three different self-assembled peptide-amphiphile nanofibers. Top: Negatively charged peptide amphipile **1** assembled with acid. Middle: Positively charged **2** assembled with base. Bottom: Nanofibers formed at neutral pH with a mixture of **1** and **2**.

provides spectroscopic evidence for mixing of the two molecules within the nanofibers.

All of these data lead us to suggest the following model of self-assembly. At neutral pH, molecule **1** has a net negative charge of −1. This charge helps to keep the molecule solubilized through electrostatic repulsion from other negatively charged species despite the large hydrophobic bulk of its fatty acid tail. Similarly, molecule **2** has a net charge of +2 at neutral pH and is soluble. When oppositely charged amphiphiles are mixed, self-assembly is initiated as the charges are attractive instead of repulsive.

In our working model of the molecular organization of the PA nanofibers, the hydrophobic alkyl tails are hidden in the center of the micelles with the more hydrophilic peptide segments of the molecules in contact with the aqueous environment. The cylindrical structure of this micelle might be partly explained by the tapered shape of individual molecules,[11] but a second driving force might be β-sheet hydrogen bonding among peptide segments down the long axis of the fibers. This is based on the parallel β-sheet hydrogen bonding conformation observed by FT-IR and on the unusual dominance of the cylindrical self-assembly motif across such a broad concentration range (<0.001% to >10 wt %).[5]

The fact that the mixed self-assembly occurs at neutral pH where the individual molecules are soluble strongly suggests that the self-assembly is driven by an electrostatic attraction involving both positively and negatively charged molecules and not simply hydrophobic collapse involving one or the other PA. We believe that the two oppositely charged molecules are thoroughly mixed within any given nanofiber as opposed to molecules segregating into mixtures of homomeric fibers. If homomeric fibers formed despite the highly unfavorable charge concentrations associated with these structures, we would expect to see the fibers pack into bundles of oppositely charged fibers to reduce electrostatic repulsion. In fact, we see the same amount or less bundling in the mixed PA fibers as compared to the fibers formed from a single PA molecule (Figure 2).

In this Communication, we have reported on a method to form nanofibers from two oppositely charged PAs carrying different biological signals at neutral pH. We have also demonstrated nanofiber formation by these peptide-amphiphiles over a large pH range. Together these new mechanisms of self-assembly may become important in the biomedical application of these materials for either in vitro or in vivo cell therapies.

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Supporting Information Available: TEM micrographs of PAs **3**, **4**, and **3**/**4**, FT-IR spectra, ESI mass spectra, and NOESY, COSY, and 1-D proton NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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