Coassembly of Aromatic Dipeptides into Biomolecular Necklaces

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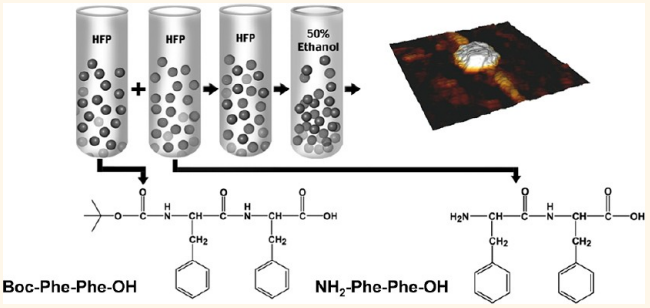
ABSTRACT This paper describes the formation of complex peptide-based structures by the coassembly of two simple peptides, the diphenylalanine peptide and its tert-butyl dicarbonate (Boc) protected analogue. Each of these peptides can self-assemble into a distinct architecture: the diphenylalanine peptide into tubular structures and its analogue into spheres. Integrated together, these peptides coassemble into a construction of beaded strings, where spherical assemblies are connected by elongated elements. Electron and scanning force microscopy demonstrated the morphology of these structures, which we termed "biomolecular necklaces". Additional experiments indicated the reversibility of the coassembly process and the stability of the structures. Furthermore, we suggest a possible mechanism of formation for the biomolecular necklaces. Our suggestion is based on the necklace model for polyelectrolyte chains, which proposes that a necklace structure appears as a result of counterion condensation on the backbone of a polyelectrolyte. Overall, the approach of coassembly, demonstrated using aromatic peptides, can be adapted to any peptides and may lead to the development and discovery of new self-assembled architectures formed by peptides and other biomolecules.



KEYWORDS: self-assembly · peptides · aromatic interactions · molecular recognition · diphenylalanine

Nature utilizes simple building blocks such as nucleic acids, phospholipids, and amino acids to create complex functional structures by the process of molecular self-assembly. In an effort to mimic this process *in vitro*, numerous studies demonstrated the ability of DNA molecules, peptides, and lipids to assemble into ordered structures.[1−7] The potential of these assemblies in a wide range of nanotechnological and biotechnological applications is immense.[6] It includes the integration of the assemblies into sensors to generate devices with higher sensitivity and specificity, the formation of three-dimensional scaffold for tissue engineering, and the combination with inorganic materials for the fabrication of new composite materials.[6]

Peptides, specifically, hold a great promise as biomolecular building blocks since they present substantial diversity—their building blocks include the combination of the 20 natural amino acids and other nonstandard residues, their synthesis in large scale is straightforward, and they can be easily modified with biological and chemical entities.[8,9]

Several classes of designed peptides such as cyclic peptides, amphiphiles, peptide-conjugates and homoaromatic dipeptides can self-assemble into ordered structures.[10−28] This includes tubes, spheres, fibrils, tapes, and hydrogels. The structural diversity of the assemblies formed by these classes of peptides is, however, still limited and there is a great interest in developing new strategies for the construction of intricate morphologies.

This paper presents a new approach for the spontaneous formation of complex architectures by the coassembly of peptides. The premise in the base of this approach is that structural complexity can emerge *in vitro* when the experimental settings match to the ones found in nature. In other words, if the experiment includes a combination of monomers, rather than one type of building blocks, the potential toward complexity is higher. We demonstrate this approach using the coassembly of homoaromatic peptide monomers; however, the same methodology can be adapted to other classes of peptides. This approach can potentially lead to the discovery of new peptide-based structures and to a better control on their assembly.

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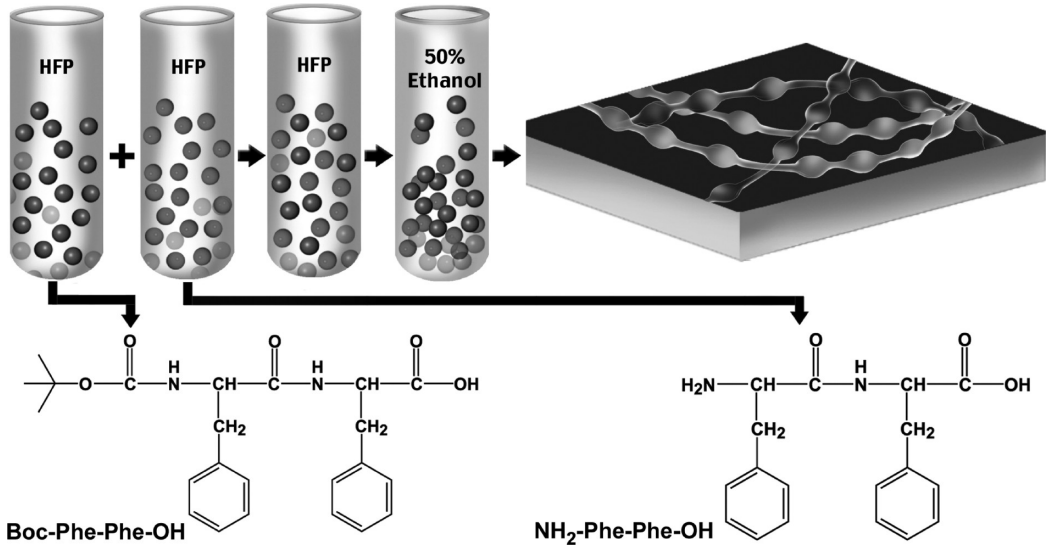

Scheme 1. The coassembly process.

RESULTS AND DISCUSSION

We chose to coassemble two simple peptides that can individually form distinct structures (Scheme 1).[20] One of the peptides is the diphenylalanine peptide (NH_2—Phe—Phe—OH), the core recognition element of the β-amyloid polypeptide which is involved in Alzheimer's disease.[20] This peptide can self-assemble in aqueous solution into hollow tubular structures with nanometric dimensions. The other peptide is a Boc protected analogue of the diphenylalanine peptide (Boc—Phe—Phe—OH). This peptide can form fibrillar structures in water and spherical assemblies with metallic-like stiffness in ethanol.[29] We assumed that these peptides would coassemble into a network that combines tubular and spherical structures.

To coassemble the two peptides, we dissolved each peptide in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). Then, we blended the peptides in several different proportions as indicated in Table 1 and diluted them in 50% ethanol (Scheme 1). The polarized solvent allowed the self-assembly of the peptides.

Based on previous findings, we determined the concentration of the peptides: The diphenylalanine peptide forms a tubular structure at a concentration of 2 mg/mL,[20] while its Boc protected analogue assembles into a spherical structure at a concentration of 5 mg/mL,[29] we therefore chose to examine the coassembly of the peptides under this condition (Table 1, condition 1). Scanning electron microscopy (SEM) analysis revealed the formation of spherical structures presenting thin fibrils on their outer surface (Figure 1a). The diameter of the spherical structures ranged between 800 nm and 2 μm while their elongated extensions had a diameter of 100—400 nm. When the concentration of each peptide was 1 mg/mL or 2 mg/mL (Table 1, conditions 2 and 3, respectively) spherical structures were formed (Figure 1b,c); however, when

TABLE 1. The Final Concentration of the Peptides at Each Experimental Condition

condition no.	Boc—Phe—Phe—OH (mg/mL)	NH_2—Phe—Phe—OH (mg/mL)	assemblies
1	5	2	spherical structures with branching fibers
2	1	1	spherical structures
3	2	2	spherical structures
4	3	3	necklaces
5	4	4	necklaces
6	3	4	necklaces
7	3	5	necklaces
8	5	4	necklaces

the concentration of the peptides was higher (Table 1, conditions 4 and 5) and the final concentration of each peptide was 3 mg/mL or 4 mg/mL, new distinctive structures self-assembled. These peptide-based architecture comprised spherical assemblies with a diameter of several micrometers (1—4 μm) connected by elongated structures with a diameter of few hundred nanometers (~300 to 800 nm) (Figure 2). The assemblies resembled in their morphology to beaded strings as the spherical structures seem to be threaded on the elongated assemblies. We, therefore, termed these assemblies "biomolecular necklaces".

Using SEM, we noticed that the biomolecular necklaces self-assemble within 10 h of incubation at room temperature (RT). In addition, we observed the formation of these structures with other concentrations of peptides (Table 1, conditions 6—8). By sampling different regions of the substrate using SEM, we determined that the assembly process occurred with high yield where the majority of the sample (more than 70%) contained these structures (Supporting Information, Figure S1). Extra-high-resolution SEM (extra-HR-SEM) analysis revealed that the spheres embedded in the

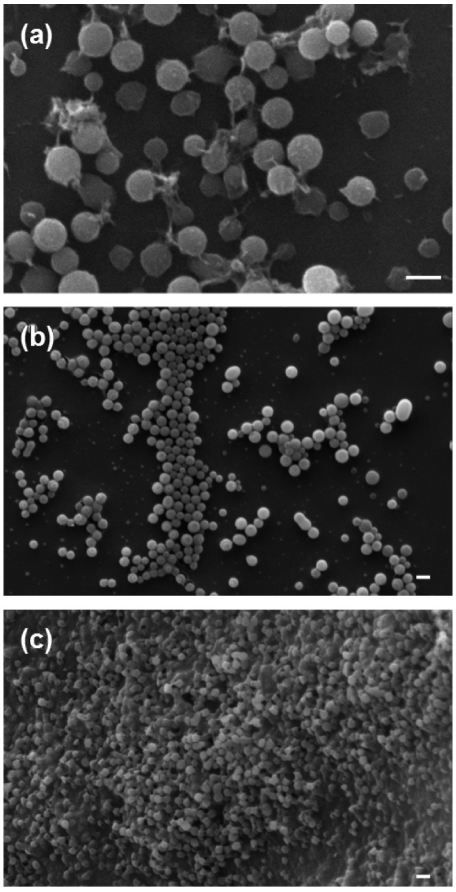

Figure 1. SEM images of the assemblies formed at the different concentrations. (a) Boc–Phe–Phe–OH at 5 mg/mL, and NH_2–Phe–Phe–OH at 2 mg/mL. (b) Boc–Phe–Phe–OH and NH_2–Phe–Phe–OH at 1 mg/mL. (c) Boc–Phe–Phe–OH and NH_2–Phe–Phe–OH at 2 mg/mL. Scale bars represent 2 μm.

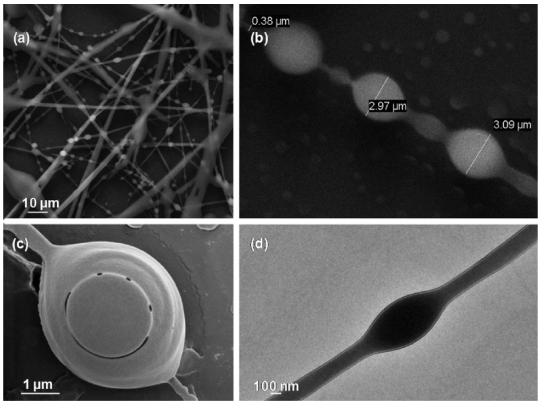

Figure 2. Electron microscopy analysis of the assemblies formed when the concentration of Boc–Phe–Phe–OH and NH_2–Phe–Phe–OH is 4 mg/mL; (a) SEM micrograph of the molecular necklaces; (b) SEM analysis of the diameter of the spherical and fibrillar assemblies; (c) extra-HR-SEM image of the spherical structures; (d) TEM micrograph of an individual molecular necklace.

elongated structures have a multilayered arrangement (Figure 2c). Figure 2 shows a transmission electron microscope (TEM) micrograph of the assemblies. In

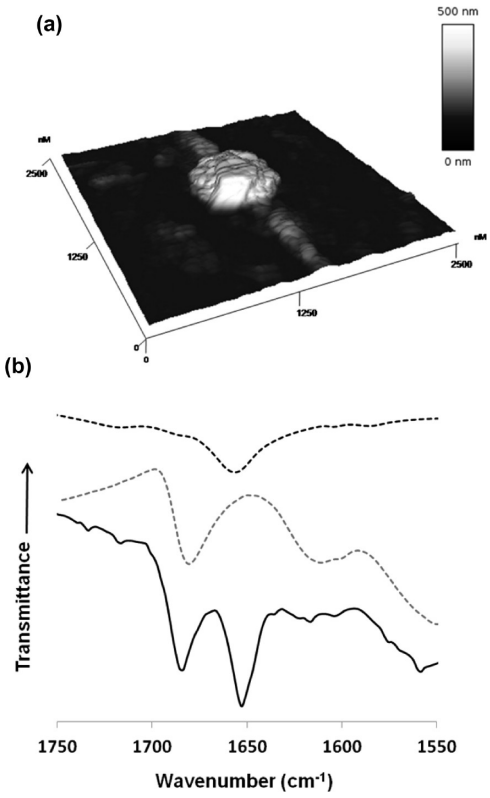

Figure 3. Structural analysis of the self-assembled biomolecular necklaces: (a) three-dimensional AFM topography image of the assemblies; (b) FT-IR analysis of the structures formed by Boc–Phe–Phe–OH (black dotted line), NH_2–Phe–Phe–OH (gray dotted line) and the two peptides together (solid black line).

addition, the three-dimensional arrangement of the structure could be clearly demonstrated using atomic force microscopy (AFM) (Figure 3).

To examine the reversibility of the coassembly process we diluted a solution containing the molecular necklaces and then concentrated the diluted solution. A 10-fold dilution of the solution resulted in the formation of large (~several micrometers in diameter) flat spherical structures (Figure 4a). The structure disassembled completely when the solution was diluted 50-fold and no ordered structures could be detected (Figure 4b). Upon concentrating the 10-fold diluted solution to a concentration of 0.9 mg/mL (for each peptide), spherical structures with a diameter of 1–4 μm formed (Figure 4c). When the concentration was 5.3 mg/mL, beaded strings formed (Figure 4d). These structures had less order than the ones formed according to Scheme 1. These results indicate that the self-assembly process is, to some extent, reversible.

The tubular assemblies formed by the diphenylalanine peptide and the spherical structures assembled by other homoaromatic peptides show good thermal and chemical stability.[21,30] For example, the diphenylalanine-based tubular structures are stable to boiling and even to autoclave treatment. The information on the stability of the assemblies is very important to their

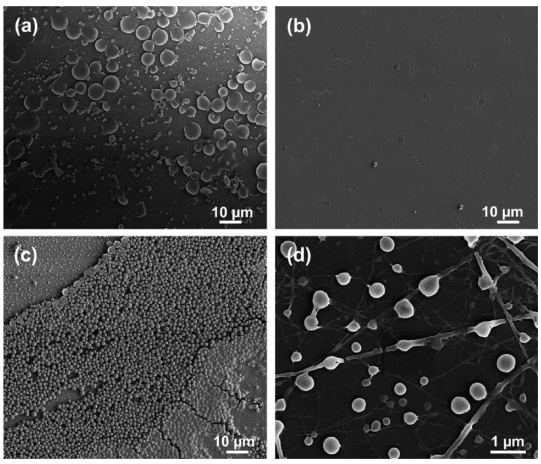

Figure 4. SEM analysis shows the reversibility of the self-assembly process. SEM micrograph of the coassembled structures after dilution of the solution by (a) 10-fold, (b) 50-fold. Evaporation of the 10-fold diluted solution to a concentration of (c) 0.9 mg/mL and (d) 5.3 mg/mL.

integration in hybrid devices. We, therefore, exploited the stability of the biomolecular necklaces under similar conditions. SEM analysis of an aged solution of the two peptides heated to 70 °C for 5 min, showed the formation of spherical structures, rather than necklaces. However, when the solution was cooled to room temperature the biomolecular necklaces assembled in high yield (Figure 5). The stability of the biomolecular necklaces to alkaline and acidic conditions was lower compared to that of the spherical structures formed by the diphenylglycine peptide as the structures disassembled upon exposure to strong acid and base (Supporting Information, Figure S2).[21]

To obtain information on the secondary structure of the self-assembled structures we performed Fourier transform infrared spectroscopy (FT-IR) analysis. The spherical assemblies formed by the peptide Boc—Phe—Phe—OH (5 mg/mL, 50% ethanol) showed a single amide I peak at 1657 cm^{-1} indicating an α helix conformation. The tubular structures formed by the NH_2—Phe—Phe—OH peptide (2 mg/mL, 50% ethanol) showed two distinctive peaks, one at 1613 cm^{-1} and the other at 1682 cm^{-1}. These peaks indicate the presence of a β-sheet secondary structure as was demonstrated previously.[20] The FT-IR spectrum of the biomolecular necklaces formed by the coassembly of the two peptides (condition 5) comprised two peaks: one peak at 1653 cm^{-1} which corresponds with an α helix structure and another peak at 1684 cm^{-1} which relates to a β-turn conformation. (Figure 3b).[31,32] This spectrum is different from the assignment for each individual peptide indicating a unique structure for these assemblies.

In the course of our experiments, we found that in 5% of our experiments a prolonged incubation of the peptide Boc—Phe—Phe—OH, for a period of 2—4 days, resulted in the formation of a mixture comprising

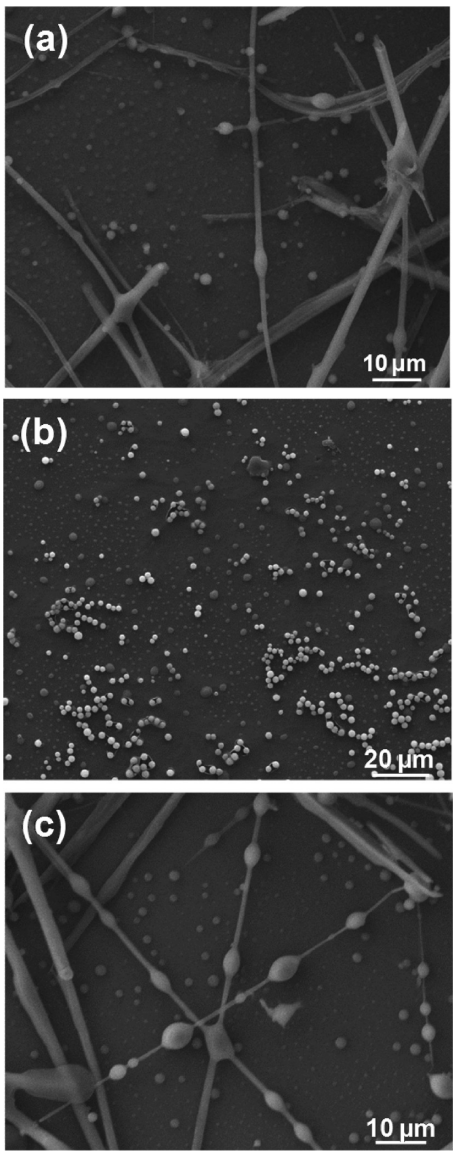

Figure 5. SEM analysis of the structures formed (a) after 1 day of incubation at room temperature, (b) following heating the solution containing the two peptides to 70 °C, (c) upon cooling to room temperature.

tubular and spherical assemblies along with few structures that resembled in their morphology the biomolecular necklaces (Figure 6). These structures assembled only when the concentration of the peptide was relatively high and equaled the concentration of the two coassembled peptides (above 20 mM).

This process of structural conversion is similar to the transition between spherical and tubular structures that occur with a cationic analogue of the diphenylalanine peptide (H—Phe—Phe—NH_2·HCl).[28] For this analogue, peptide nanotubes formed when a solution containing vesicle-like structures was concentrated. In addition, the cationic analogue self-assembled into a metastable state which was termed "necklace-like structure".[28] The structures which we termed biomolecular necklaces are different from these structures in

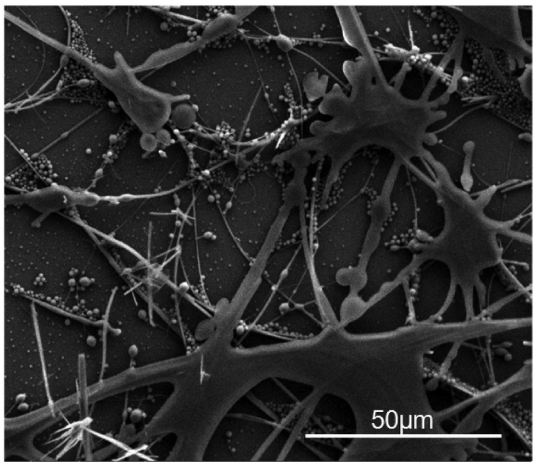

Figure 6. Representative SEM micrograph of the assemblies formed by the Boc−Phe−Phe−OH peptide at a concentration of 20 mM and five days of incubation.

their morphology: The biomolecular necklaces comprise both linear elements and spherical elements, in which the spherical elements are connected by the linear structures, whereas the "necklace-like" structures are sequential joint vesicles.

We, therefore, propose a different mechanism of formation for the biomolecular necklaces. Our suggestion is based on the necklace model for polyelectrolyte chains.[33] Polyelectrolytes are charged polymers with ionizable groups. According to theory and simulations, polyelectrolyte chains with a charge smaller than a critical charge collapse and form dense globules.[34] The globule undergoes a transition into a necklace-like structure, with compact beads joined by narrow strings, as a result of counterion condensation on the backbone of the polyelectrolyte, in order to minimize its free energy.[34] Such condensation can occur by increasing the salt or polyelectrolyte concentration.[33] A similar effect can be achieved by adding polyampholyte chains to the solution.[35] Under the experimental conditions we examined (pH∼3), the peptide Boc−Phe−Phe−OH bear a net negative charge. We assume that the aromatic interactions among the peptide monomers results in the formation of a sequence of negatively charged units which consequently behaves as a polyelectrolyte chain.[33] This polyelectrolyte further collapses into a condensed spherical globule. On the basis of the necklace model, we suggest that an increase in peptide concentration (effectively polyelectrolyte concentration) results in a reduced net charge of the peptide due to counterion condensation on its backbone, and therefore the formation of necklace-like structures at relatively high peptide concentrations. In the coassembly experiments, the overall concentration of the peptides is maintained with a slight increase in the pH of the solution pH, ∼4.5. Under these conditions, the zwitterionic peptide (NH_2−Phe−Phe−OH) acts as a polyampholyte, and its addition leads to the formation of a necklace structure. The electrostatic interactions among the monomers in the coassembly experiments might facilitate the thermodynamic stability of the necklaces formed by the combined peptides. This may be the cause of necklaces formation with a higher yield when compared to the formation of necklaces by the Boc−Phe−Phe−OH peptide. It is important to note that the reversibility of the self-assembly process probably results from the noncovalent interactions that effectively generate the polyelectrolyte chain.

To further test the proposed model, we examined the self-assembly process in the presence of salt. We expected that the addition of salt will promote the formation of necklaces in the same manner as an increase in peptide concentration or the presence of polyampholyte in the solution. We studied the self-assembly of the Boc−Phe−Phe−OH in two different concentrations: a low concentration (5 mg/mL) and a high concentration (9.6 mg/mL) which equals the concentration of the two peptides in the coassembly process. We determined the NaCl concentration to 16 mM since this concentration is identical to the molar concentration of the diphenylalanine peptide in the coassembly experiment. Our findings indicated that the presence of NaCl accelerates the formation of necklaces by the Boc−Phe−Phe−OH peptide when the peptide is at a high concentration. In this case few necklaces self-assembled after 1 day of incubation instead of a more prolonged incubation needed without salt. When the peptide concentration was low (5 mg/mL), spherical structures connected by short linear elements formed. These structures resemble in their morphology a transition state between a globule sphere and necklaces reported for the necklace model of polyelectrolyte chains[36] (Supporting Information, Figure S3).

To examine how general the proposed mechanism is and what are the molecular requirements of the process, we further studied other combinations of peptides. We examined the coassembly of other homoaromatic peptides since we consider that the process is limited by the length of the peptide and its aromatic nature. When the peptide is short and aromatic, it presents structural rigidity and therefore each peptide monomer can effectively act as a monomer in the polyelectrolyte chain. When the length increases the peptide is more flexible and also solubility issues may rise. The Fmoc−Phe−Phe−OH peptide with 9-fluorenylmethyl carbamate protecting group on its N termini, is another negatively charged analogue of the diphenylalanine peptide. Under the same experimental conditions we used for the other individual peptides, this peptide self-assembled into a hydrogel in 50% ethanol as demonstrated before with water.[22] When the Fmoc−Phe−Phe−OH peptide coassembled with the NH_2−Phe−Phe−OH peptide, only tubular structures were formed. These tubular structures divided into small nanometric fibers at their ends (Supporting Information, Figure S4a). The Ac−Phe−Phe−OH

peptide, an additional negatively charged analogue of the diphenylalanine peptide formed large aggregates when combined with the NH_2–Phe–Phe–OH peptide. This peptide, as an individual monomer self-assembles into elongated structures. However, when the peptides Boc–Phe–Phe–OH and Fmoc–Phe–Phe–OH coassembled, structures that resembled the biomolecular necklaces were observed (Supporting Information, Figure S4). The necklaces in this case were shorter and less continuous than those created by the combination of the peptide Boc–Phe–Phe–OH and the NH_2–Phe–Phe–OH. On the basis of these observations we conclude that the formation of biomolecular necklaces is directed not only by the aromatic nature of the peptide and its structural rigidity, but also by the ability of the individual peptide to form a dense globule in solution.

CONCLUSIONS

We have shown the coassembly of two aromatic dipeptides into the structure of beaded strings. These findings suggest a new strategy for the formation of complex assemblies by simple dipeptides. We demonstrated this approach utilizing two peptides; however, the diversity concealed in this approach is immense and derived from the large variety of natural and unnatural amino acids. In addition, peptide-based structures can be easily decorated with chemical and biological entities such as antibodies and enzymes, and therefore present endless opportunities as scaffolds for biosensors, tissue engineering, and drug delivery. The biomolecular necklaces present a structural arrangement of spherical elements with a diameter at the micrometer range and elongated assemblies with a diameter at the nanometer. This combination of dimensions at different scales makes the necklaces appealing as a mold for casting components of metal or semiconductors for electronic applications. In addition, the necklaces might serve as a new biomaterial as they are soluble in water, biocompatible, and present self-healing properties. Moreover, the necklaces show high structural similarity to beaded filaments formed by the high molecular weight glycoprotein fibronectin.[37] Fibronectin plays a critical role in many fundamental biological processes such as embryogenesis, angiogenesis, wound healing, and tissue repair, where it regulates basic cell adhesion, growth, proliferation, migration, differentiation and generates an insoluble matrix which provides a scaffold important for cell attachment, migration, and further matrix deposition.[37] The resemblance in morphology between the biomolecular necklaces formed by the peptides and the beaded filaments formed by fibronectin suggests that the newly discovered necklaces might be useful as a new biomaterial with the advantage of spontaneous formation by simple and short peptides.

EXPERIMENTAL SECTION

Materials. The diphenylalanine and its analogues Boc–Phe–Phe–OH and Fmoc–Phe–Phe–OH were purchased from Bachmen (Bubendorf, Switzerland). The peptide Ac–Phe–Phe–OH was purchased from Shanghai Hanhong Chemical Co., Ltd. (Yancheng City, Jiangsu Province, China). The peptides were synthesized by solution synthesis and do not contain TFA salt. To avoid any preaggregation, new fresh stock solutions of the peptides were prepared for each experiment. The fresh stock solutions of the diphenylalanine peptide and Boc–Phe–Phe–OH were prepared by dissolving the lyophilized forms of the peptides in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) to a concentration of 100 mg/mL. Owing to its low solubility in HFP, the Fmoc–Phe–Phe–OH was dissolved to a concentration of 50 mg/mL. For the assembly of the individual peptides, the diphenylalanine peptide was diluted in 50% ethanol to several different concentrations (2–10 mg/mL), the Boc–Phe–Phe–OH peptide to a concentration of 5 mg/mL (low concentration) or 9.6 mg/mL (high concentration), and the Fmoc–Phe–Phe–OH to a concentration of 4 mg/mL, using the same solvent. In coassembly experiments, the peptides stock solutions in HFP were blended together and then diluted to the desired final concentration with 50% ethanol. For example, in the case of condition 5, each peptide was diluted to a final concentration of 4 mg/mL in 50% ethanol. In the case of coassembly of the Boc–Phe–Phe–OH with the Fmoc–Phe–Phe–OH different concentrations were used: 5:2, 3:3, and 4:4 mg/mL, respectively. In the case of NH_2–Phe–Phe–OH and Fmoc–Phe–Phe–OH the concentrations were 2:2, 3:3, and 4:4 mg/mL. The Ac–Phe–Phe–OH could not be dissolved in HFP, and therefore the coassembly with the diphenylalanine peptide was done by blending the peptides in their lyophilized form, dissolving them to a concentration of 100 mg/mL in HFP, and then diluting them to a final concentration of 4:4 mg/mL. It should be noted that due to the highly volatile nature of the solvent the experiments are sensitive to small changes in the concentration of the peptides.

Self-Assembly in the Presence of Salt. A stock solution of the Boc–Phe–Phe–OH in HFP was diluted to a final concentration of 5 mg/mL or 9.6 mg/mL in 50% ethanol with 16 mM NaCl. The concentration of NaCl is identical to the final concentration of NH_2–Phe–Phe–OH in the coassembly experiment.

Assembly and Reassembly. To examine the reversibility of the self-assembly process, a solution containing the biomolecular necklaces was diluted 10-fold and 50-fold. To concentrate the sample the 10-fold diluted solution was placed in a desiccator and the solvent was allowed to evaporate. Samples were taken out at different time points, and the concentration was calculated by measuring the volume of the solution.

Thermal and Chemical Stability. To examine the thermal stability of the necklaces, a solution containing the self-assembled peptides was heated in a water bath to 70 °C for 5 min. Then, a drop of 5 μL of the hot solution was placed on a glass coverslip, dried at RT and prepared for SEM analysis. After being cooled to RT, a 5 μL drop of this solution was placed on a glass cover, dried, and analyzed using SEM. In the case of stability to alkaline conditions, NaOH was added into the peptide solution to a final concentration of 1 M. In the case of stability in acidic conditions, TFA was added to the nanostructure solution to a final concentration of 10% TFA. After 8 h peptide solutions were placed on glass coverslips and analyzed by SEM.

Scanning Electron Microscopy (SEM). A drop containing 10 μL of the peptides solution was placed on a glass coverslip and allowed to dry at RT. The peptides on the glass were coated with gold using a Polaron SC7640 Sputter Coater. SEM images were taken using a Quanta 200 ESEM operating at 20 kV.

High Resolution Scanning Electron Microscopy (HR-SEM). Samples were prepared in the same manner as for the SEM and viewed using an extra-high-resolution scanning electron microscope, MagellanTM400L operating at 1 kV.

Atomic Force Microscopy (AFM). Samples were prepared in the same manner as for the SEM analysis and studied using a JPK NanoWizard3 (JPK instruments, Germany) working in an AC mode.

Transmission Electron Microscopy (TEM). A drop containing 10 μL of the peptides solution, incubated at RT for 10 h was placed on 200-mesh copper grid, covered by carbon-stabilized Formvar film (Electron Microscopy Science, PA, USA). After 1 min, excess fluid was removed and then negatively stained with 10 μL of 2% uranyl acetate in water. After 0.5 min, excess fluid was removed from the grid. The samples were viewed using a Tecnai T12 G2 Spirit (Cryo-TEM) operating at 120 kV.

Fourier Transform Infrared Spectroscopy (FT-IR). Infrared spectra were recorded using a Nicolet 6700 FT-IR spectrometer with a deuterated triglycine sulfate (DTGS) detector (Thermo Fisher Scientific, MA, USA). Peptide solution were deposited on a CaF_2 plate and dried by vacuum. The peptide deposits were resuspended with D_2O and subsequently dried to form thin films. The resuspension procedure was repeated twice to ensure maximal hydrogen-to-deuterium exchange. The measurements were taken using a 4 cm^{-1} resolution and averaging 2000 scans. The transmittance minimal values were determined by the OMNIC analysis program (Nicolet).

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: SEM analysis demonstrating the yield of the coassembly process, the chemical stability of the biomolecular necklaces, the self-assembly in the presence of salt, and the coassembly of other homoaromatic dipeptides. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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