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Photo-Crosslinking Induced Geometric Restriction Controls the Self-Assembly of Diphenylalanine Based Peptides *

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The diphenylalanine (FF) motif has been widely used in the design of peptides that are capable of forming various ordered structures, such as nanotubes, nanospheres and hydrogels. In these assemblies, FF based peptides adopt an antiparallel structure and are stabilized by $\pi-\pi$ stacking among the phenyl groups. Here we show that assembly of FF-based peptides can be controlled by their geometric restrictions. Using tripeptide FFY (L-Phe-L-Phe-L-Tyr) as an example, we demonstrate that photo-crosslinking of C-terminal tyrosine can impose a geometric restriction to the formation of an antiparallel structure, leading to a structural change of the assemblies from nanosphere to amorphous. This finding is confirmed using far-UV circular dichroism, Fourier transform infrared spectroscopy and atomic force microscopy. Based on such a mechanism, we are able to control the gel-sol transition of Fmoc-FFY using the geometric restriction induced by photo-crosslinking of C-terminal tyrosine groups. We believe that geometric restriction should be considered as an important factor in the design of peptide-based materials. It can also be implemented as a useful strategy for the construction of environment-responsive "smart" materials.

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Self-assembly is a powerful strategy to create a great variety of materials with ordered structures.[1−6] The assembly processes are typically controlled by either thermodynamics or kinetics and driven by a variety of non-covalent interactions, such as hydrophobic interaction, hydrogen bonding, electrostatic interaction, $\pi-\pi$ stacking, Van der Waals force and chiral interaction. The self-assembly of oligopeptides is one of the representative self-assembly strategies that have been extensively studied to form many nanoscale structures, e.g. nanotubes, nanoribbons and nanospheres and macroscopic materials (e.g. hydrogels).[7−13] These peptide-based assemblies have shown great potential as functional biomaterials and nanodevices.[14,15]

Diphenylalanine peptide (L-Phe-L-Phe, FF) is probably the most studied motif in the field of peptide self-assembly.[14,16−25] It was initially derived from the core region of Alzheimer's amyloidic β structures.[12,18,25,26] Owing to the $\pi-\pi$ stacking among the phenyl groups, it can be assembled into nanotubes,[25] nanoplates,[18] nanospheres,[27] hydrogels[22,28] and organogels,[19] etc. These assemblies have found many applications, such as nanofabrication, drug/DNA delivery, tissue engineering and biosensors.[8,11,12,29]

The structural arrangement of the two phenyl groups in various FF assemblies has been revealed by spectroscopic methods and x-ray studies.[16,18,22,30,31] It was found that the FF peptides form antiparallel β-sheets with two phenyl groups interlocked to each other (Fig. 1(a)). However, despite great advances in the study of the structure of FF motif, the effects of neighboring chemical environment, such as terminal charge and adjacent amino acids on the assemblies, are far from clearly understood. It was found that changing the terminal groups can tune the assembly of FF from nanotube to nanosphere,[17] closed-cage,[27] or even macroscopic hydrogels.[16,28] Such diverse structures may be due to the effect of terminal charges or neighboring amino acids on the structural arrangement of the two phenyl groups.

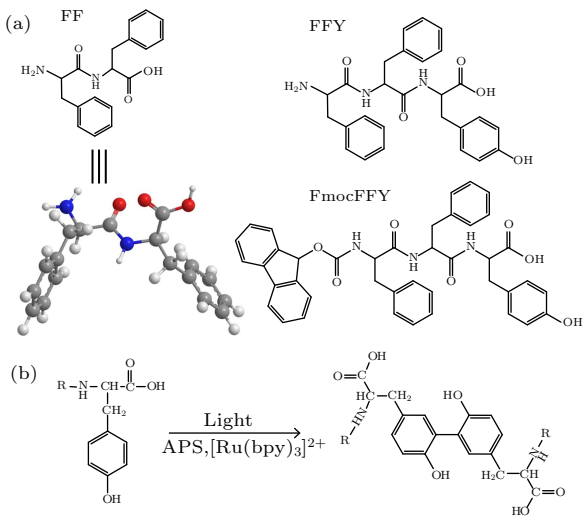

Fig. 1. (a) schematic of the diphenylalanine- (FF) and FF-based peptides used in this study. (b) Photo crosslinking of tyrosine induces centrosymmetry to the peptide, leading to geometric restriction of the assembly process.

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In this study, we show that the assembly of FF peptides can also be affected by their geometric restriction. Although geometric restriction has been recognized as an important factor that affects protein folding,[32] its effect on peptide self-assembly is somewhat overlooked. This might be because the effect of geometric restriction in short peptides is typically not significant and difficult to implement. Here, we use tripeptide FFY (L-Phe-L-Phe-L-Tyrosine) as an example (Fig. 1(a)) to study the effect of geometric restriction on their self-assembly. We introduce a Tyrosine to the FF peptide because it can be crossed-linked to dityrosine via a Ruthenium ($[Ru(bpy)_3]^{2+}$) catalyzed photochemical process (Fig. 1(b)). This process allows the crosslinking of two tyrosine residues that are in close proximity into dityrosine adduct and leads to rapid and quantitative formation of dityrosine crosslinks between FFY peptides. Once tyrosines are crosslinked, the two aromatic rings tend to point to opposite directions with a centrosymmetry (Fig. 1(b)). Such an arrangement makes the formation of an antiparallel β-sheet not structurally feasible due to steric hindrance, and geometrically restricts the self-assembly process.

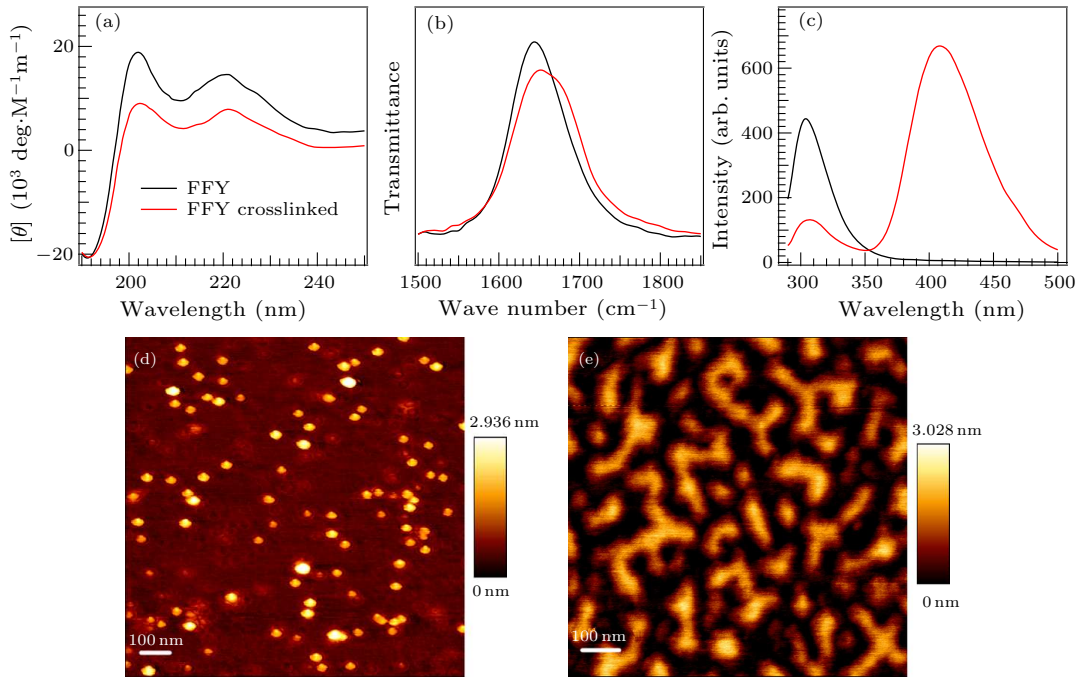

Fig. 2. Crosslinking of tyrosine introduces geometric restriction to FFY and modulates its assembly. (a) CD spectra of FFY before (black) and after crosslinking (red) at a concentration of 1 mg/mL. (b) FTIR spectra of FFY before (black) and after (red) crosslinking (taken from lyophilized samples). (c) Fluorescence spectra of FFY before (black) and after (red) crosslinking. (d) AFM image of FFY assemblies. (e) AFM image of crosslinked FFY assemblies.

For comparison, we first study the process of FFY self-assembly in a phosphate buffer saline (PBS, 20 mM) solution. We synthesized the peptides using Fmoc based solid-phase chemistry on a Symphony Quartet peptide synthesizer (Protein Technologies Inc., USA) and purified them via reverse-phase chromatography on a AKTABasic pH/C 10 ml System (Amersham Biosciences).[33,34] FFY was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL. Then this stock solution was diluted into PBS at a final peptide concentration of 1 mg/mL under rigorous stirring. It is worth noting that insufficient stirring may lead to inhomogeneous assemblies. The self-assembly process was allowed to proceed for three hours before the characterization of the physical properties of the assemblies. We used far-UV circular dichroism (CD, J-810 CD spectropolarimeter, Jasco, Japan) and Fourier transform infrared spectroscopy (FTIR, VECTOR22 FTIR, Bruker, Germany) to characterize the secondary structure of FFY in the assemblies. As shown in Fig. 2(a), the CD spectrum of FFY shows two positive peaks at wavelengths of 200 nm and 217 nm, respectively. This infers the anti-parallel β-sheet structure of FFY. The CD spectrum of FFY resembles that of FF, indicating that attaching tyrosine residue at the C-terminus does not affect the assembly process. The FTIR spectrum of FFY also confirmed the anti-parallel structure of FFY. As shown in Fig. 2(b), the IR absorption shows a peak at 1640 cm^{-1}, corresponding to the amide I peak for C=O stretching. The IR spectrum is very similar to that of FFF (L-Phe-L-Phe- L-Phe) and is slightly shifted to higher wave number compared with that of FF.[18] We then used an atomic force microscope (AFM, Nanowizzard Ⅱ, JPK) to characterize the morphology of assembled FFY. As shown in Fig. 2(d), FFY assembles into small nanospheres in PBS, with a diameter of about 50 nm. It is worth noting that the

nanospheres formed by FFY are very similar to those of CFF (L-Cys-L-Phe-L-Phe), but distinct from the nanotubes formed by FF.[27]

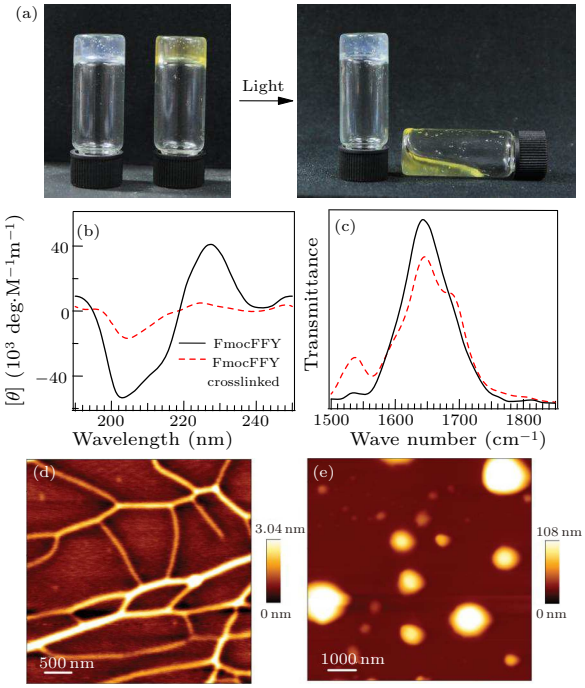

Fig. 3. Crosslinking of tyrosine introduces geometric restriction to FmocFFY and modulates its gel-sol transition. (a) Gel-sol transition of FmocFFY can be controlled by light-induced crosslinking of tyrosine. The left bottle contains 5 wt% FmocFFY in PBS and the right bottle contains 5 wt% FmocFFY, 10 mM APS and 200 μM $[Ru(bpy)_3]^{2+}$. (b) CD spectra of FmocFFY before (black) and after (red) tyrosine crosslinking. The samples were diluted to 0.25 mg/mL for CD measurement. (c) FTIR spectra of FmocFFY before and after tyrosine crosslinking. (d) AFM image of FmocFFY assemblies. (e) AFM image of FmocFFY assemblies after tyrosine crosslinking.

We then test how tyrosine crosslinking affects the structure of the assemblies. We added 10 mM ammonium persulfate (APS) and 200 μM $[Ru(bpy)_3]^{2+}$ to the FFY solution and mixed it by vortex for 1 min. It is noted that adding APS and $[Ru(bpy)_3]^{2+}$ does not affect the structure of the assemblies. The solution was then subjected to white light illumination for 2 min. The light-illuminated sample (the light intensity is 300 mW/cm^2) was vortexed for one more minute and equilibrated for three hours before the characterization. Since dityrosine shows its characteristic fluorescence peak at about 410 nm, we used fluorescence spectra to verify the crosslinking process. As shown in Fig. 2(c), the uncrosslinked FFY shows a fluorescence peak at about 305 nm, corresponding to the emission of tyrosine (black line). However, after illumination, this peak decreased significantly (red line) and a new peak centered at about 410 nm with much higher amplitude rose up, indicating the formation of dityrosine. Using a calibration curve, we estimated that around 80% of tyrosine in FFY was crosslinked. We again used CD and FTIR to characterize the secondary structure of FFY in the assemblies. As shown in Fig. 2(a), the crosslinked FFY shows much less ellipticity at wavelengths of 200 nm and 217 nm, indicating that crosslinking affected the antiparallel conformation. Similarly, in FTIR (Fig. 2(b)), the crosslinked FFY showed additional peaks at 1690 cm^{-1} which were not found in uncrosslinked FFY. However, due to the difficulties in the peak assignment, we were not able to draw an affirmed conclusion on the resulted secondary structures of crosslinked FFY. We then used AFM to characterize the morphology of the crosslinked FFY (Fig. 2(e)). Instead of seeing well-separated uniformed nanospheres, the crosslinked FFY is largely amorphous. However, there are still some isolated nanospheres, which may be due to incomplete photo-crosslinking. Taking these observations together, it is clear that geometric restriction induced by tyrosine crosslinking significantly affects the morphology of the self-assembled FFY. Crosslinking of tyrosine introduces centrosymmetry to neighboring FF motifs, which imposes a geometric restriction on the formation of an FF antiparallel β sheet structure and disrupts the secondary structure of FFY in the assemblies.

In order to further illustrate the effect of geometric restriction on the self-assembly process of diphenylalanine-based peptides, we used Fmoc-FFY (Fig. 1(a)), a peptide with a fluorenylmethoxycarbonyl group (Fmoc) at the N terminus, as an example to study the effect of tyrosine crosslinking on its gel-sol transition. Fmoc-FFY was first dissolved in DMSO at a concentration of 50 mg/mL. Then, this solution was injected to PBS with rigorous stirring. Fmoc-FFY is able to form solid gel in PBS at a concentration of 10 mg/mL (Fig. 3(a)). It hung over the bottom of the bottle, showing a characteristic feature of a hydrogel. The gelation process is very fast and takes place in less than 5 min. We used a far-UV CD spectrum to characterize the secondary structure of Fmoc-FFY in the hydrogel. As shown in Fig. 3(b), Fmoc-FFY shows a β-sheet structure with a negative peak at about 215 nm. This spectrum is somehow "atypical", as there are still some contributions from $\pi - \pi$ stacking to the ellipticity at the far-UV region. This is consistent with the CD spectrum of Fmoc-FF observed by Ulijn and coworkers.[16] However, upon photo-induced crosslinking for 2 min (300 mW/cm^2, white light), the Fmoc-FFY gel became a viscous floating solution (Fig. 3(c)). Such a transition of the structures of Fmoc-FFY is clearly due to geometric restriction induced by tyrosine crosslinking. Indeed, in the CD spectrum of crosslinked Fmoc-FFY, the negative peak was almost disappeared. FTIR studies also showed a new peak at around 1690 cm^{-1}, indicating the structural change of Fmoc-FFY upon photo-crosslinking. To further understand the macroscopic transition of Fmoc-FFY triggered by geometric restriction, we used AFM to study the detailed structural changes of the micro-

scopic structures of Fmoc-FFY upon crosslinking. As shown in Fig. 3(d), Fmoc-FFY self-assembles into entangled nanotubes with diameters of about 100 nm, a very character of hydrogels. However, after crosslinking, it is turned to nanospheres of diverse sizes ranging from 50 nm to 1 μm (Fig. 3(e)).

Recently, Fmoc-FF and Nap-FF (a naphthyl group at the N-terminus of FF) have been proposed as efficient gelation motifs in the design of peptide-based hydrogels (so called "samogen").[22,28] Using these motifs as a backbone, many functional peptides such as RGD (Arg-Gly-Asp) have been introduced to incorporate functional units to hydrogels. However, in some systematic studies, it was found that linking proline to the C-terminus of these motifs dramatically affects the gelation process.[35] Since proline has unique phi and psi angles, we may also introduce geometric restrictions on the peptide assembly, similar to that induced by tyrosine crosslinking. Moreover, introducing cysteines or other crosslinkable motifs adjacent to the FF motif has also been found to affect the self-assembly process upon crosslinking.[27] Therefore, geometric restriction should be an important factor to be considered in the design of peptide assemblies.

Since introducing geometric restriction can effectively alter the self-assembly process of FF motif, we may use it as a novel way to engineer environment-responsive material, such as the one shown in this study. We anticipate that any modifications to peptides, such as ligand binding and chemical reactions, which can introduce geometric restriction to the peptide, can cause similar transition as the tyrosine crosslinking. Moreover, the peptides with implemented geometric restrictions can also serve as a novel class of smart materials. and the self-assembly is controlled by the reactions that release such geometric restriction. The studies along this direction are well under way.

In summary, we have shown that the self-assembly process of diphenylalanine-based peptides can be controlled by geometric restriction induced by neighboring groups. This effect is achieved through the alternation of the secondary structure of FF in the peptides and can be triggered by a chemical reaction: tyrosine crosslinking. This finding is of fundamental importance in the design of peptide-based materials and may be adopted as a useful strategy in the design of environment-responsive smart materials.

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