Nanocapsules Hot Paper

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Covalent Self-Assembly and One-Step Photocrosslinking of Tyrosine-Rich Oligopeptides to Form Diverse Nanostructures

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Abstract: *We present covalently self-assembled peptide hollow nanocapsule and peptide lamella. These biomimetic dityrosine peptide nanostructures are synthesized by one-step photopolymerization of a tyrosine-rich short peptide without the aid of a template. This simple approach offers direct synthesis of fluorescent peptide nanocages and free-standing thin films. The simple crosslinked peptide lamella films provide robust mechanical properties with an elastic modulus of approximately 30 GPa and a hardness of 740 MPa. These nanostructures also allow for the design of peptidosomes. The approach taken here represents a rare example of covalent self-assembly of short peptides into nano-objects, which may be useful as microcompartments and separation membranes.*

Self-assembly of biomaterials inspired by natural creatures has received tremendous attention owing to their unique physical, chemical, and biological properties, which make them promising candidates for applications in various fields.[1–4] In particular, a variety of peptide-based self-assembled nanostructures have been synthesized through non-covalent interactions, such as hydrophobic interaction, π–π stacking, hydrogen bonding, and electrostatic interactions. However, these non-covalent interactions are intrinsically weaker than the covalent bond, and can be disassembled by changes in the surrounding environment, such as pH and temperature.[3] In addition, most previous studies have been directed toward specific molecular designs (for example, amphiphilic peptides) in which multistep processes are needed to facilitate self-assembly.[4] Thus, simple and short peptides that can induce crosslinks between side-chains are very attractive building blocks for the versatile design of nano-objects with tunable properties.[2,3]

Intriguingly, tyrosine–tyrosine crosslink networks were found in resilin, an elastic protein of the cuticle of most insects and arthropods, and in the outer layer of yeast ascospore walls, which all showed outstanding mechanical properties such as elasticity.[5,6] Biological crosslinking systems with dityrosine bonds inspired attempts to mimic their rubber-like functions with high resilience. The elasticity and Young's modulus were increased by controlling the number of dityrosine crosslinks in recombinant resilins,[5a,6c] silk protein,[5b] and suckerin hydrogels.[5d] However, low tyrosine content and diverse sequences in the complex structures of the natural proteins hindered the development of rationally-designed micro- and nano-structured materials with satisfactory Young's moduli. Furthermore, control of the morphology of self-assembled nano-objects required tedious post-treatment steps and use of templates (surfactants, spheres). Therefore, a single-step covalent self-assembly of short peptides would be highly desirable for the formation of versatile nano-objects.

Herein, we describe the direct and selective synthesis of fluorescent peptide nanocapsules and free-standing peptide thin films with extraordinary mechanical strength. The method entails one-step photopolymerization of a peptide by dityrosine crosslinkages. A short peptide with an YYAYY sequence was designed to have symmetrical tyrosine pairs at both ends. This arrangement provides a high density of covalent bonds with adjacent tyrosines in proximate peptides (Figure 1). The morphology of the resulting nanostructure was controlled by the simple choice of the reaction medium without any template: water for the formation of nanocapsules and methanol for the formation of lamella film. The self-assembled peptide films formed by the dityrosine crosslinks exhibited superior mechanical and conformational durability compared to the non-covalent peptides. This simple method could lead to applications in the development of self-assembly technologies and could provide scalable solutions to mechanically robust nanostructures.

For the synthesis of the hollow nanocapsules, the peptides were self-assembled directly by UV irradiation of short YYAYY peptides in pH 10 buffer for 20 h, followed by dialysis (Figure 2). The UV treatment yielded a clear yellow solution (Supporting Information, Figure S1). According to dynamic light scattering (DLS), the average diameter of the peptide nanocapsule in the wet state was 155.1 ± 46.13 nm (Figure 2a). In high resolution transmission electron microscopy (TEM) images, the dried nanocapsules were spherical

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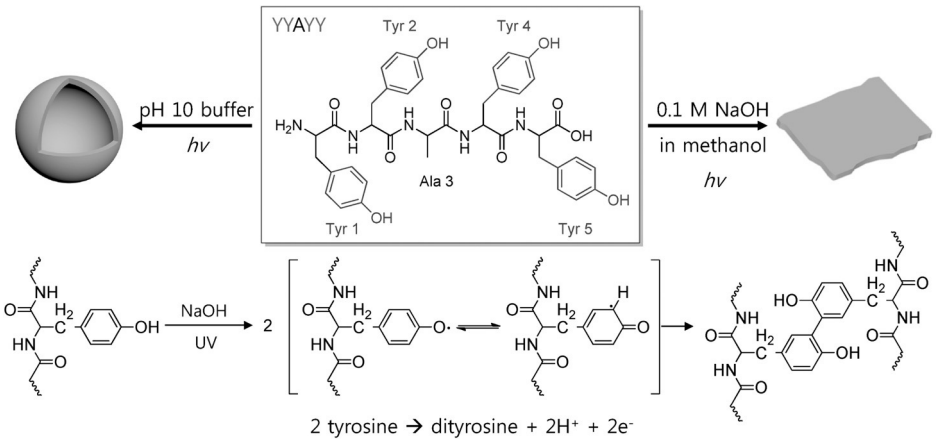

Figure 1. Synthesis of fluorescent peptide hollow nanocapsule and free-standing thin lamella film by tyrosine–tyrosine UV crosslinking.

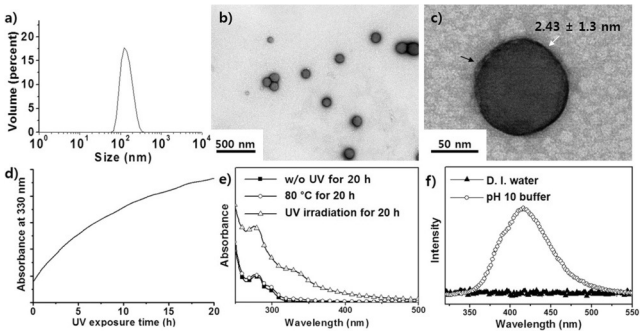

Figure 2. Morphology of self-assembled peptides synthesized from 1 mg mL^{-1} of YYAYY in pH 10 buffer. a) Size distribution and b, c) TEM images of hollow nanocapsule obtained by UV irradiation for 20 h. Arrows indicate thickness (white) of shells (black) on the nanocapsule. d) Time-dependent UV absorption (330 nm) during dityrosine formation upon prolonged UV exposure (300 nm). e) UV/Vis absorption spectra of peptides treated under different conditions. f) Fluorescence emission spectra of peptide in deionized (D.I.) water (after heating at 80 °C for 20 h) and covalently assembled peptide nanocapsule (pH 10 buffer and UV irradiation for 20 h) under 330 nm excitation.

without deformation or crumple, with a hollow interior surrounded by several layers of shells, with an average total thickness of 2.43 ± 1.3 nm (Figure 2c). The AFM line profile image of a dried peptide nanocapsule showed a flattened sphere with a diameter of 137 ± 28 nm and a height of 10 ± 1.1 nm (Figure S2).

Various spectroscopic and imaging techniques were used to investigate the crosslinking of tyrosine. The dityrosine bond is known to emit an intrinsic blue fluorescence at wavelength $\lambda \approx 410$ nm and to absorb an UV at $\lambda \approx 330$ nm.[7] Originally, the model short peptide YYAYY is poorly soluble in neutral water, but became soluble at high pH. UV irradiation ($\lambda \approx 300$ nm) in pH 10 buffer stimulated the dityrosine-crosslinking reaction and caused a continuous increase in the absorption intensity at $\lambda \approx 330$ nm, and the emission of blue fluorescence at $\lambda \approx 410$ nm, while heating/cooling of the peptide in neutral water did not cause blue fluorescence (Figure 2d–f). In the absence of UV irradiation, this reaction did not occur even when the peptide dissolved in pH 10 buffer was kept at 80 °C or room temperature for 20 h. UV irradiation is well known to initiate the formation of dityrosine bonds by generation of a solvated electron (e^-_{aq}), and a radical cation (Tyr-OH$^{\bullet+}$), then deprotonation to form a tyrosyl radical that undergoes radical isomerization, diradical reaction, and enolization (Figure 1).[7c,e] It is known that the density of tyrosyl radicals increases with higher pH and ionic strength,[7e] and it is expected that this enhanced radical density highly activates photopolymerization to tightly crosslink the nanocapsule texture. As a result, the nanocapsule size dramatically decreased to an average size of 26.3 ± 8.9 nm, when an aqueous 0.1 M NaOH (pH 13) was used instead of the pH 10 buffer (Figures S5, S6). The crosslinking during UV irradiation was also monitored through changes in the ^{1}H NMR spectra (Figure S7, S8). In general, the formation of dityrosine crosslinks and new linkages increased the complexity of the polymeric structure. In comparison to the monomer, the peaks of the peptide polymer broadened and the peak shifted slightly; these changes are typical when the magnetic environments of both *meta* and *para* protons of tyrosine moieties change. However, dityrosine crosslinks formed only under UV exposure in the reaction medium with pH ≥ 10 (Figure 2e,f and Figure S9).

Furthermore, the step-wise formation of the self-assembled nanocapsule was observed by TEM during photopolymerization (Figure S10). Initially, no organized structure occurred, but after 3 min of UV exposure, imperfect spheres with pinholes appeared. After 15 min, semi-capsules formed, and the remaining tyrosine residue on the curved shell surface formed additional dityrosine bonds, thereby becoming highly crosslinked and closely covering the surface. After 1 h, the hollow nanocapsule morphology finally emerged. We hypothesize that the photopolymerized crosslinks anchored the peptide network, and contributed to the growth of lateral assembly of peptides into an oligomeric sheet. Further reactions between curved patches generated a loosely crosslinked hollow sphere, and eventually the dityrosine network entirely bridged the self-assembled neighboring patches in the shell to form the highly crosslinked peptide capsules (Figure 2c). This approach led to the one-step self-assembly of short peptides by covalent bonding between tyrosines without the use of templates or multistep processes.

The reaction medium can strongly influence the morphology of the self-assembled product. It is known that low bending rigidity of building blocks and a poor solvent encourage the generation of curvature in the intermediates, whereas high bending rigidity and a good solvent allow lateral growth of intermediates without bending.[8,9] Reaction of the YYAYY peptide dissolved in methanol, a good solvent, with

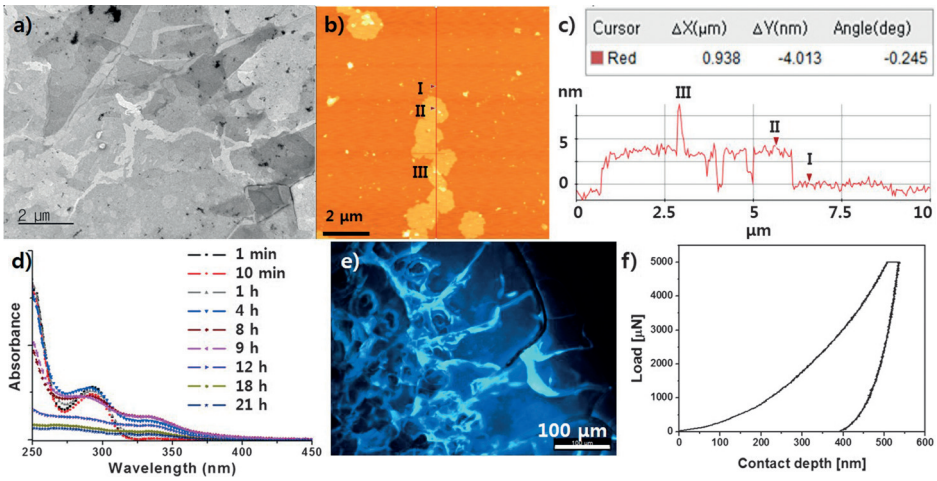

Figure 3. a) TEM image of free-standing peptide thin films synthesized from 1 mg mL^{-1} of YYAYY in methanol with 0.1 M NaOH after UV irradiation for 20 h. b) AFM image of peptide films coated on Si wafer after UV reaction for 3 h. c) Line profile obtained from b (site I: Si wafer, II: peptide film, III: films overlapped). d) Time-dependent UV/Vis spectra of dityrosine formation under UV irradiation. e) Fluorescence image of self-assembled YYAYY sequence peptide film dispersion solution roughly coated on quartz after complete drying. f) Representative load versus depth curve of peptide films measured by nanoindentation.

0.1M NaOH produced free-standing peptide thin films after UV exposure (Figure 3a–c). Pale yellow product formed in the methanol solution, and precipitated when the solution was left undisturbed. The peptide films were wrinkle-free, several micrometers long, and ≈4 nm thick (Figure 3a,b). Time-dependent UV/Vis spectra during self-assembly of the peptide film confirmed the formation of dityrosine and the occurrence of blue fluorescence (Figure 3d,e). Methanol may have dominated the lateral growth of the peptide networks by forming dityrosine-crosslinks under UV irradiation. The resultant assemblage expanded to form 2D peptide patches and then overlaid to form films when the conditions for peptide sheet/solvent contact were favorable.[8,9] Extending the reaction time yielded peptide films that were several tens of micrometers in length (Figure 3a,b and Figure S11).

To investigate the mechanical strength of this covalently self-assembled peptide film, nanoindentation measurements were made. The measurements revealed the outstanding mechanical properties of the peptide film, yielding an elastic modulus of 29.88 ± 1.43 GPa and hardness of 740 ± 0.12 MPa (Figure 3f). This modulus is superior to the elastic modulus (8.4 GPa) of the tyrosine-mediated peptide film assembled by non-covalent interactions,[10] and also superior to several biological proteinaceous and nonproteinaceous materials.[5d,11] These peptide films maintained their morphology even after exposure to 80 °C for one week or to 1M HCl for one day (Figure S12), whereas the non-covalent peptide nanostructures disassembled under similar conditions.[7] These results are explained by the presence of dityrosine-crosslinks that can cause protein chains to be mechanically and conformationally stable, elastic, and resistant to proteolysis.[5–7] This simple and scalable approach to producing large crosslinked peptide thin films with strong durability may have applications in membrane separation and water desalination.

We also used the approach to generate microemulsions of peptidosomes, which constitute a new class of microcompartments that have various biochemical applications, such as protocells and artificial microreactors.[12] To test the ability of Pickering-type emulsion to assemble peptide nanocapsules at the interface, a water-in-oil peptide microemulsion was produced by introducing the pre-synthesized peptide nanocapsule solution, as the dispersion phase, and the hexadecane mixed with 1 wt% Span-80, as the continuous phase, into a microfluidic device based on a polyimide film (Figure 4a). The size of the peptide emulsions were ≈50 μm in diameter (Figure 4b).

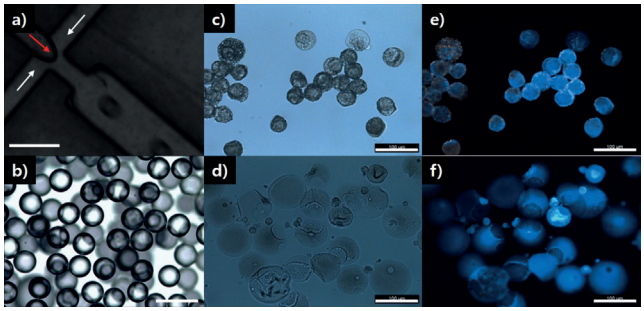

Figure 4. Peptide emulsions produced by a microfluidic device. a) Photograph of flow-focusing part in the polyimide-film-based microfluidic device to generate peptide emulsion. Arrows: flow direction of pre-synthesized peptide nanocapsule solution (red) and hexadecane with 1 wt% of Span-80 (white). Scale bar: 200 μm. b–f) Optical and fluorescence images of covalently self-assembled nanocapsule-entrapped emulsions: b) initial; c, e) semi-dried retaining the spherical emulsion shape; d, f) completely dried and flattened emusion, obtained by evaporating solvent passively in air at room temperature. Scale bars in (b–f): 100 μm.

The use of pre-synthesized peptide nanocapsule solutions allowed formation of a stable boundary by spontaneous self-assembly at the interface without leakage of peptide nanocapsules. The enclosed membrane of peptide emulsion did not lose compositional integrity, even after drying (Figure 4c,d). The dried emulsions had deflated, but the membranes did not coalesce. Corresponding fluorescence microscopy images indicated the presence of dityrosine-crosslinked peptides in the intact microcompartments (Figure 4e,f). This observation indicated that the structural component of the membrane was composed of self-assembled peptide nanocapsules. To the best of our knowledge, this is the first production of

peptidosomes from covalently self-assembled peptide nanocapsules.

In summary, UV-initiated covalent dityrosine-crosslinking of short peptides in pH 10 water or methanol solution yielded selective self-assembly of nano-objects without requiring templates. This one-step crosslink and simultaneous self-assembly generated hollow fluorescent peptide nanocapsules (100–200 nm diameter), and robust free-standing thin films with elastic modulus of ≈30 GPa and hardness of ≈740 MPa, which are mechanically stronger and conformationally more stable than previously reported non-covalent peptides. Pre-synthesized peptide nanocapsules were used to form enclosed membranes in peptide emulsions. These peptidosomes constitute a new class of microcompartments with compositional integrity. This simple and scalable approach would contribute to the development of methods for self-assembling mechanically robust nanostructures.

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