Supramolecular Chemistry

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Mixing Biomimetic Heterodimers of Nucleopeptides to Generate Biocompatible and Biostable Supramolecular Hydrogels**

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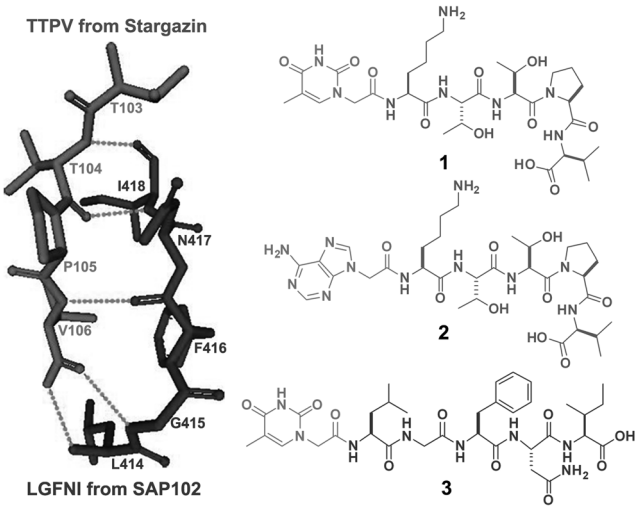
Abstract: *As a new class of biomaterials, most supramolecular hydrogels formed by small peptides require the attachment of long alkyl chains, multiple aromatic groups, or strong electrostatic interactions. Based on the fact that the most abundant protein assemblies in nature are dimeric, we select short peptide sequences from the interface of a heterodimer of proteins with known crystal structure to conjugate with nucleobases to form nucleopeptides. Being driven mainly by hydrogen bonds, the nucleopeptides self-assemble to form nanofibers, which results in supramolecular hydrogels upon simple mixing of two distinct nucleopeptides in water. Moreover, besides being biocompatible to mammalian cells, the heterodimer of the nucleopeptides exhibit excellent proteolytic resistance against proteinase K. This work illustrates a new and rational approach to create soft biomaterials by a supramolecular hydrogelation triggered by mixing heterodimeric nucleopeptides.*

Supramolecular hydrogels,[1] formed by small molecules (referred to as hydrogelators) in aqueous phase, share certain properties (e.g., soft, polymorphic, and bioactive) with extracellular matrices in tissue, and have attracted considerable attention as emerging biomaterials for applications such as stem cell delivery,[2] antibacterial,[3] cancer cell inhibition,[4] scaffolds for regenerative medicine[5] and cell cultures,[6] and media for electrophoresis.[7] Among the building blocks for generating supramolecular hydrogels, peptides[1c,8] are the most explored ones. The current strategies for designing peptide hydrogelators are to introduce a long alkyl chain,[8a,9] aromatic groups,[8c,10] or residues of opposite charges.[11] Each of these strategies can effectively generate hydrogels, but not without limitation. For example, it is difficult to choose the length of the alkyl chain,[9b] too many aromatic groups may inhibit cells in some cases,[4,12] too strong charge interactions results in poor reversibility.[13] Thus, it is necessary and beneficial to explore new strategies to complement the existing ones for supramolecular hydrogels.

One attractive approach is to learn from nature. Intriguingly, most of the cytosol proteins exist as protein assemblies with different symmetry,[14] yet the most common form of protein assemblies are dimers (e.g., 38% of proteins in *E. coli* exist as dimers[14]). The formation of heterodimers implies rather strong noncovalent interactions at the interface of two proteins, thus one should be able to take those complementary sequence to generate hydrogels. This approach, in fact, has been explored by a few groups by using peptides to bind with proteins.[15] For example, the specific TPR–peptide,[15a] TIP1–peptide,[15b] and heparin–VEGF interaction,[15c] allows the formation of polymeric hydrogels. One drawback of this approach is the use of proteins being high-priced and susceptible to proteolysis. Interestingly, this approach has yet to be explored for the use of nucleopeptides[16] for creating supramolecular hydrogels. Based on this principle, our work on supramolecular hydrogels made of homonucleopeptides by pH changes or enzymatic reactions,[16a] and the biostability of nucleopeptides,[16a,17] we decided to explore the use of heteronucleopeptides to generate hydrogels by simple mixing of two structurally distinct nucleopeptides that bind with each other.

We choose a nucleobase (thymine or adenine) to connect with short peptide sequences (Scheme 1) from the binding interface of two well-characterized proteins,[18] calcium channel protein (stargazin[19]) and synapse-associated protein 102 (SAP102[20]). While the homonucleopeptides themselves are unable to self-assemble to form molecular nanofibers that result in a hydrogel, the mix of heteronucleopeptides, **1**+**3** and **2**+**3**, results in self-assembly to form supramolecular



Scheme 1. Molecular structures of the nucleopeptides containing the epitopes from stargazin or SAP102.

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nanofibers/hydrogels. Furthermore, the nucleopeptides show excellent cell compatibilities, and the hydrogels of the heterodimers exhibit enhanced biostability. As the first report of supramolecular hydrogels formed by mixing heterodimeric nucleopeptides, this work illustrates a facile and rational methodology for creating nucleopeptides that act as a new class of supramolecular hydrogelators for developing sophisticated soft materials according to the need of various applications.

We choose thymine and adenine as the complementary nucleobases for producing the nucleopeptides because of their application in designing supramolecular materials.[21] Among many available heterodimeric proteins that have well-characterized structures, we select a pentapeptidic sequence, leucine–glycine–phenylalanine–asparagine–isoleucine (LGFNI) from the binding loop of PDZ domain,[22] which is a common modular domain for protein–protein interactions in many organisms.[22,23] To match with the LGFNI sequence, we use another pentapeptide, lysine–threonine–threonine–proline–valine (KTTPV) for generating the nucleopeptides because the recent crystal structure of the binding of TTPV with a PDZ domain[18] has provided atomistic details of the noncovalent interactions (e.g., hydrogen bonding (shown in Scheme 1)) between LGFNI and KTTPV, which offers the molecular base that warrants adequate binding between the designed heterodimeric nucleopeptides. We choose to connect the nucleobase at the N-terminal of the peptides because the attachment of nucleobase at the C-terminal of small peptides unlikely would result in effective molecular self-assembly.[24] According to these nucleobases and pentapeptides, we plan to examine the gelation properties of **1**, **2**, **3**, and their mixtures (Scheme 1).

After their synthesis and characterization, we tested the ability of the nucleopeptides to form hydrogels. The dissolution of **3** (12 mg) in PBS (1 mL) gives a clear solution of **3** (16.4 mM and pH 6.2), so we also prepared the solutions of **1** and **2** in PBS (pH 6.2) at 16.4 mM. The simple mixing of **1** (or **2**) with an equal volume of **3** affords the mixture, **1**+**3** (or **2**+**3**), with each component to be 8.2 mM in concentration. After 48 h at room temperature, the mixture of **1**+**3** (or **2**+**3**) transforms from a clear solution to a transparent hydrogel (Figure 1), whereas the stock solutions of **1**, **2**, and **3** (at 16.4 mM; Figure S4) as well as the mixture of **1**+**2** (Figure S6) remain transparent solutions. Rheometry shows that dynamic storage moduli (G') dominate the dynamic loss moduli (G'') for the mixture of **1**+**3** (or **2**+**3**), confirming that **1**+**3** (or **2**+**3**) forms a hydrogel. On the contrary, the G' values overlap with the G'' values for the solution of **1**, **2**, or **3** (Figure S5), confirming that **1**, **2**, or **3** itself at 16.4 mM remain dissolved. Moreover, the maximum storage modulus of the hydrogel of **2**+**3** ($G' = 8928$ Pa) is about an order of magnitude higher than that of the hydrogel of **1**+**3** ($G' = 942$ Pa). This result is consistent with the structure difference between **1** and **2** and indicates that the interactions between the Watson–Crick base pair (from adenine in **2** and thymine in **3**) results in higher mechanical strength of the heterodimeric nucleopeptides than the interactions between thymines (from **1** and **3**) do.

We use transmission electron microscopy (TEM) to characterize the hydrogels of the heterodimeric nucleopeptides. The hydrogel of **1**+**3** contains long and aligned bundles that consist of thin nanofibers with an average width of about 4 ± 2 nm (Figure 1 A). The TEM image of the hydrogel of **2**+**3** shows long, flexible, and entangled nanofibers with an average width of about 9 ± 2 nm (Figure 1 B). The extensive entanglement of the nanofibers in the hydrogel of **2**+**3** agrees with its higher storage moduli compared to that of the hydrogel of **1**+**3**. This result suggests that the interactions in A–T pairs and the interactions in T–T pairs provide quite different interfibrillar interactions in the hydrogels. There are hardly any ordered nanostructures in the solutions of **1**, **2**, and **3** (Figure S4), agreeing with the character of a solution.

To investigate the interaction between **1** (or **2**) with **3**, we examined the mixture of **1**+**3** (or **2**+**3**) from 0.13 mM to 8.2 mM by circular dichroism (CD). The CD spectra of **1**+**3** display a decrease of the intensity of the peak originating from a π–π^* transition (around 200 nm) with increasing concentration of the mixture (Figure 2 A), indicating that the association of the heterodimer of **1** and **3** results in antiparallel dipole interactions to reduce the CD signals. The negative peaks shift from 204 nm to 234 nm from 0.13 to

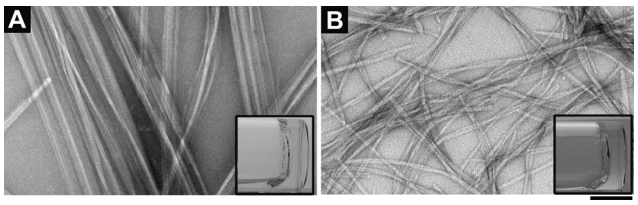

Figure 1. TEM images of A) hydrogel of **1**+**3**; B) hydrogel of **2**+**3**. All compounds are dissolved in PBS buffer (pH 6.2, and [**1**] = [**2**] = [**3**] = 8.2 mM). Insets: optical images of the hydrogels. Scale bar = 100 nm.

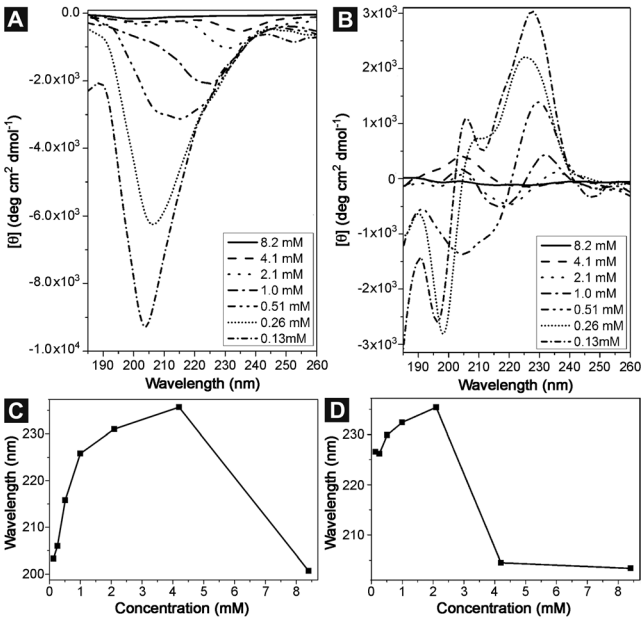

Figure 2. Circular dichroism (CD) spectra of A) **1**+**3** and B) **2**+**3** from 0.13 mM to 8.2 mM. Relationship between wavelength and concentration in CD spectra of C) **1**+**3** and D) **2**+**3**.

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4.1 mM, which is consistent with the fact that the increase of dimerization places the π–π* transition in less aqueous environment.[25] The sudden peak-shift back to 200 nm at 8.2 mM agrees with the phase transition due to hydrogelation, implying that the weak peaks at 200 nm likely originate from small amounts of monomer **1** or **3** (Figure 2C). For the mixture of **2** + **3**, the π–π* transitions also exhibits a redshift (227 nm to 236 nm) from 0.13 to 2.1 mM (Figure 2B), indicating an increase of dimerization. However, the sudden blueshift back to around 200 nm occurs at 4.1 mM (Figure 2D), which is consistent with the fact that the interaction between **2** and **3** is stronger than that between **1** and **3** to favor self-assembly at lower threshold concentration. The most notable feature of the CD signals of the mixture of **1** + **3** or **2** + **3** is the α-helix- and random-coil-like character of the heterodimeric self-assembly in the hydrogels, which differs from the β-sheet-like features usually observed in other types of supramolecular hydrogels.

We examined the FTIR of the solutions of **1**, **3**, and **1** + **3** in DMSO containing different ratios of D_2O (Figure S8, Table S1). After mixing of **1** and **3**, the weak N–H stretch vibration peaks turn to a broadened peak, and the broadened peak expands more with increasing D_2O content. The strong C=O stretch vibration peaks of **1** and **3** also disappear in **1** + **3**, showing two weak peaks at 1687 cm^{-1} and 1674 cm^{-1}. The weak peaks shift with the addition of different amounts of D_2O. In addition, the N–H bending vibration was weaker in **1** + **3** compared to that of **1** and **3**, and weaker with higher contents of D_2O. These changes of the amide vibrations suggest the formation of hydrogen bonds between **1** and **3**.

To assess the biostability of the nucleopeptides (**1**, **2**, and **3**), we incubate **1**, **2**, **3**, **1** + **3**, and **2** + **3** with proteinase K, a powerful protease, for 24 h at 37 °C. As shown in Figure 3A, **1** or **2** resists protease K (96.5 ± 1.8 % of **1** or 91.5 ± 1.9 % of **2**

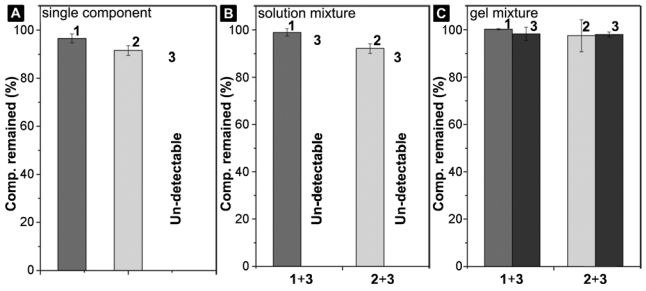

Figure 3. Compounds remained after incubating with proteinase K (3.2 U mL^{-1}) for 24 h at 37 °C. A) Single component of **1**, **2**, or **3** at the concentration of 0.2 mg mL^{-1} in HEPES buffer (pH 7.4); B) solution mixture of **1** + **3** or **2** + **3** in PBS buffer ([**1**] = [**2**] = [**3**] = 4.1 mM, pH 6.2); C) gel mixture of **1** + **3** or **2** + **3** in PBS buffer ([**1**] = [**2**] = [**3**] = 8.2 mM, pH 6.2).

remain after 24 h), and **3** is hydrolyzed completely after 24 h. Since the pentapeptide (KTTPV) resists proteolysis (Figure S9), the proteolytic stability of **1** and **2** likely originates from the KTTPV. For the solution mixture of **1** + **3** (or **2** + **3**), 98.9 ± 1.6 % of **1** (or 92.1 ± 1.0 % of **2**) remains, but **3** still degrades completely after 24 h (Figure 3B). Contrary to the properties of the solution mixtures, the hydrogelation of the heterodimers drastically improves the proteolytic stability of these nucleopeptides, and 98 % of **3** remains in the hydrogel of **1** + **3** or **2** + **3** after the incubation with proteinase K for 24 h (Figure 3C). This result suggests that the strong interactions between nucleopeptides in the gel phase significantly improve the biostability of **3**, thus providing a new approach to enhance the resistance of small-peptide hydrogels against proteolysis.

We also investigate their biocompatibility by incubating **1**, **2**, **3**, **1** + **3**, or **2** + **3** with two types of mammalian cells, HeLa cells (24 h doubling time[26]) for 3 days and PC12 cells (92 h doubling time[27]) for 7 days. As shown in Figure S9, at the concentration range of 20–500 μM, the cell viabilities of HeLa are about 100 % after the treatment of **1** + **3** or **2** + **3** for 3 days; **1** + **3** is innocuous to PC12 cells; **2** + **3** hardly inhibits PC12 cell proliferation (Figure S10). In addition, **1**, **2**, or **3** shows no toxicity to HeLa cells and PC12 cells (Figures S11 and S12). These results indicate that these nucleopeptides are cell-compatible.

In conclusion, this work illustrates a novel and rational method to produce supramolecular hydrogels that integrates known noncovalent interactions between heteropeptide motifs of proteins and the interactions between nucleobases. The well-established reversibility of the interactions between nucleobases also warrants the reversibility of the interaction between the heterodimeric nucleopeptides. The facile synthesis, biostability, and non-β-sheet secondary structures of nucleopeptides in the gel phase render the supramolecular hydrogels of nucleopeptides to have many unexplored features that warrant further development. For example, our result indicates that the use of nucleopeptides promises the development of the supramolecular hydrogels that require the preservation of α-helical and random-coil motifs to exhibit specific functions, which is a direction of our ongoing research.

Keywords: biocompatibility · biostability · hydrogels · nucleopeptide · supramolecular chemistry

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