Formation of supramolecular nanostructures via in situ self-assembly and post-assembly modification of a biocatalytically constructed dipeptide hydrazide

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The in situ self-assembly of a biocatalytically constructed dipeptide hydrazide gives rise to supramolecular hydrogels, consisting of networks of supramolecular nanoarchitectures, under mild aqueous conditions. Moreover, the post-assembly modification via hydrazone bond formation enables the decoration of the prefabricated supramolecular architectures.

Keywords: biocatalysts • helical nanoribbon • peptides • post-assembly modification • self-assembly • supramolecular hydrogels
Abstract
Aqueous self-assembly of short peptides has attracted growing attention for the construction of supramolecular materials for various bioapplications. Herein, we describe that the thermolysin-assisted biocatalytic construction of a dipeptide hydrazide from an N-protected amino acid and an amino acid hydrazide leads to the formation of thermally stable supramolecular hydrogels. In addition, we demonstrate the post-assembly modification of the in situ constructed supramolecular architectures tethering hydrazide groups as a chemical handle by means of fluorescence imaging.

Introduction
The aqueous self-assembly of short peptides consisting of two or three amino acids has attracted growing attention for the construction of supramolecular soft nanomaterials due to their potential biocompatibility and the facile accessibility and synthesis of the constituent amino acids or peptide derivatives.[1] In this context, we have recently investigated the aqueous self-assembly of dipeptides bearing a hydrazide group at the C-terminal (hereafter referred to as dipeptide hydrazides).[2] The hydrazide group enables the decoration and functionalization of prefabricated supramolecular architectures under aqueous conditions with functional molecules bearing an aldehyde group via hydrazone bond formation on the basis of adaptive or dynamic combinatorial chemistry.[3] Nevertheless, we found that potentially robust self-assembling dipeptide hydrazides such as phenylalanine–phenylalanine (FF) exhibited limited aqueous dispersibility even at elevated temperature (e.g., at the boiling point of an aqueous dispersion).[2] which hampered the construction of thermodynamically or kinetically favorable supramolecular nanoarchitectures and supramolecular hydrogels via simple thermal (heating–cooling) treatment or solvent exchange protocols.[4] To circumvent the solubility issue, we conjugated the hydrazide group with saccharides[2a] or a hydrophilic benzaldehyde[2d], which enabled the modulation of the water solubility and the realization of stimuli-responsiveness. In addition, we investigated a simple molecular design for the construction of one-dimensional fibrous or two-dimensional planar supramolecular architectures by adjusting the number and position of the phenyl group in a dipeptide hydrazide scaffold.[2e]

From the viewpoint of practical application and for the spatiotemporally controlled construction of targeted supramolecular architectures, the in situ construction of a self-assembling molecule from non (or weak)-self-assembling ones triggered by a specific cue and subsequent autonomous self-assembly to produce programmed supramolecular architectures is a valuable approach. In fact, the use of biocatalytic triggers such as phosphatases, esterases, and proteases for the self-assembly of peptide derivatives has expanded rapidly.[5] For example, the peri-, intra-, and even subcellular self-assembly of peptide derivatives triggered by intrinsic or overexpressed enzymes has enabled the control of the targeted cell fate via the formation of a variety of supramolecular nanostructures in a spatiotemporally controlled manner.[5a, 6] Apart from these biological
applications, intriguing adaptive supramolecular materials have been constructed by performing a dynamic selection with the assistance of proteases, for example, to develop morphology at the nanoscale and conductive property. In this context, we herein describe that the biocatalytic construction of a dipeptide hydrazide (a self-assembling dipeptide derivative) from an N-protected amino acid and an amino acid hydrazide (two non-self-assembling amino acids derivatives) with the aid of thermolysin leads to the simultaneous formation of supramolecular hydrogels via reversed hydrolysis, that is, peptide bond formation, as depicted in Figure 1A. This is, to our knowledge, the first example of thermolysin-triggered supramolecular hydrogelation through the in situ construction of a peptide hydrazide, which could extend the molecular toolbox for programmable supramolecular nanostructures guided by protease-instructed self-assembly and subsequent dynamic combinatorial chemistry based on hydrazone formation (Figure 1B,C).

**Figure 1.** (A) Thermolysin-assisted biocatalytic construction of a dipeptide hydrazide (NPmoc-FF-NHNH₂) from an N-protected amino acid (NPmoc-F) and an amino acid hydrazide (F-NHNH₂). (B) Post-assembly modification of the self-assembled dipeptide hydrazide via dynamic combinatorial chemistry. (C) Schematic illustration of the formation of a supramolecular hydrogel with a helical nanoribbon network triggered by thermolysin-mediated ligation and subsequent post-assembly modification to modulate the properties and functions of the supramolecular hydrogel. A chemically synthesized peptide hydrazide (NPmoc-FF-NHNH₂).
showed no stable hydrogel formation ability via self-assembly by conventional thermal treatment (heating–cooling) due to its limited aqueous solubility.\(^\text{[2e]}\)

**Results and Discussion**

*Biocatalytic construction of a dipeptide hydrazide and in situ supramolecular hydrogel formation.* Typically, to an aqueous solution (200 µL) of NPmoc-F\(^\text{[10]}\) (20 mM, 0.71 wt%) and F-NHNH\(_2\) (4.0 equiv. against NPmoc-F) in 100 mM phosphate buffer (pH 8.0; optimal pH for thermolysin\(^\text{[9a]}\)), was added an aqueous solution of thermolysin (1.0 mg/mL, 50 µL). Hydrogel formation was observed within 25 min (around 23 min) after the addition of thermolysin, and the resultant opaque gel (Figure 2A\(_\text{(i)}\)) became gradually turbid while the gel state was maintained even after 1 day. In contrast, no hydrogel formation was confirmed even after 24 h in the absence of thermolysin under the same conditions. As shown in Figure 2A\(_\text{(ii)}\), a control experiment using F-NH\(_2\) instead of F-NHNH\(_2\) afforded similar but significantly faster hydrogel formation (within 3 min). This result is consistent with reports on Fmoc and amino acids having an amide group at the C-terminal.\(^\text{[11]}\) Nevertheless, the NPmoc-F/F-NH\(_2\) system gave more turbid hydrogels than NPmoc-F/F-NHNH\(_2\).

To evaluate the viscoelastic properties of the NPmoc-F/F-NHNH\(_2\) and NPmoc-F/F-NH\(_2\) hydrogels, rheological measurements were conducted. As shown in Figure 2B, the presence of a linear viscoelastic region at low frequency was confirmed. The storage moduli (\(G'\)) of both hydrogels were larger than the loss moduli (\(G''\)) as a result of their viscoelastic property. In more detail, the tan \(\delta\) (\(G''/G'\)) values at 1.0 rad s\(^{-1}\) for NPmoc-F/F-NHNH\(_2\) and NPmoc-F/F-NH\(_2\) were estimated to be 0.18 and 0.12, respectively, which are comparable to those reported for this type of low-molecular-weight supramolecular hydrogels.\(^\text{[12]}\) Next, the macroscopic gel-to-sol transition temperature (\(T_{\text{gel}}\)) of the NPmoc-F/F-NHNH\(_2\) hydrogel was evaluated. Intriguingly, no heat-induced gel-to-sol transition was observed even upon heating the hydrogel in an oil bath at 100 °C (Figure S1). Such a high \(T_{\text{gel}}\) can be ascribed to the low solubility of NPmoc-FF-NHNH\(_2\) in aqueous media, as we previously reported.\(^\text{[2c]}\) It should be noted that the conventional thermal treatment (heating–cooling) of a mixture of a chemically synthesized NPmoc-FF-NHNH\(_2\)\(^\text{[2c]}\) in a similar aqueous buffer did not lead to the formation of a stable hydrogel (Figure 1C).
Figure 2. Thermolysin-mediated biocatalytic formation of the NPmoc-F/F-NHNH₂ and NPmoc-F/F-NH₂ hydrogels. (A) Photographs before and after the addition of thermolysin. Left: (i) NPmoc-F/F-NHNH₂, Right: (ii) NPmoc-F/F-NH₂. Sol: solution, pGel: partial gel, oGel: opaque gel, tGel: turbid gel. (B) Rheological properties of the NPmoc-F/F-NHNH₂ and NPmoc-F/F-NH₂ hydrogels under frequency sweep (1.0 rad/s for strain sweep). Conditions: [NPmoc-F] = 16 mM (0.56 wt%), [F-NHNH₂]/[NPmoc-F] = [F-NH₂]/[NPmoc-F] = 4.0, [thermolysin] = 200 µg/mL (5.8 µM) in 100 mM phosphate buffer (pH 8.0), room temperature for gelation tests, 25 °C during rheology measurements.

Then, ¹H nuclear magnetic resonance (NMR) spectroscopic measurements were conducted to evaluate the time course of the reversed hydrolysis, which is the conversion from NPmoc-F to NPmoc-FF-NHNH₂ in the presence of an excess amount of F-NHNH₂ catalyzed by thermolysin (see Supporting information for details). In brief, the NPmoc-F/F-NHNH₂ hydrogels prepared under the same conditions except using deuterated phosphate buffer were dissolved by adding DMSO-d₆ at designated time periods to measure the ¹H NMR spectra. As shown in Figure 3A (i), several peaks assignable to the NPmoc-F moiety were shifted (i.e., b/bᵢ, c/cᵢ, and d/dᵢ), which is consistent with the conversion from NPmoc-F to NPmoc-FF-NHNH₂ (the conversion was supported by the electrospray ionization-mass spectra depicted in Figure S2). The conversion (%) from NPmoc-F to NPmoc-FF-NHNH₂ was evaluated according to the integration values of the d and dᵢ peaks; the obtained time course is shown in Figure 3B (i, red plot). The conversion reached 67% 20 min after the addition of thermolysin and was almost saturated at this point. The conversion from NPmoc-F to NPmoc-FF-NH₂ was evaluated in a similar manner, and the corresponding time course is shown in Figure 3B (ii, green plot, Figure S3), which clearly indicates that the conversion was significantly faster for NPmoc-F/F-NH₂ than for NPmoc-F/F-NHNH₂, whereas the saturated conversion (%) was comparable (i.e., 77% and 79% after 40 min for NPmoc-F/F-NH₂ and NPmoc-F/F-NHNH₂, respectively). These results can be ascribed to the fact that the more biorelevant F-NH₂ is
kinetically more favorable as a substrate for thermolysin than F-NHNH$_2$. In addition, as can be extracted from the fact that the conversion reached above 60% after 3 and 20 min for NPmoc-F/F-NHNH$_2$ and NPmoc-F/F-NHNH$_2$, respectively, the hydrogel formation was faster for NPmoc-F/F-NH$_2$, which can be attributed to a faster ligation to give the corresponding dipeptide derivatives. It has been already reported that a peptide hydrazide can act as a substrate for the hydrolysis of thermolysin.$^{[13]}$ Nevertheless, to our knowledge, this is the first demonstration of an amino acid hydrazide acting as a substrate for thermolysin-catalyzed ligation (reversed hydrolysis) to give a self-assembling dipeptide hydrazide for simultaneous hydrogel formation via self-assembly, as illustrated in Figure 1A.

Figure 3C displays the circular dichroism (CD) spectra of NPmoc-F/F-NHNH$_2$ before and after the formation of the hydrogel (unfortunately, the NPmoc-F/F-NHNH$_2$ hydrogel was too turbid to measure its CD spectra). As shown in Figure 3C, a rather broad but significantly enhanced negative CD signal at around 300 nm, which could be attributed to the nitrophenyl moiety,$^{[2e, 14]}$ appeared after the hydrogel formation. This CD signal suggests the chiral orientation and $\beta$-type aggregation of the nitrophenyl moiety in the self-assembled structures,$^{[15]}$ since the absorption band shifted to longer wavelength compared with that of an aqueous mixture of NPmoc-F and F-NHNH$_2$ before hydrogel formation (Figure S4). The nitrophenyl moiety of the NPmoc group, which can function as a reduction-responsive unit (Figure S5)$^{[14, 16]}$ as well as provide distinct signals in the $^1$H NMR and CD spectra, allowed the detailed assessment of the self-assembly process.$^{[2e]}$
To gain insight into the self-assembled structures at the nanoscale, transmission electron microscopy (TEM) observations were conducted. As shown in Figure 4A i–iv, helical nanoribbons with an average diameter of 36 ± 5 nm and their networks were found for the NPmoc-F/F-NHNH₂ hydrogel. Atomic force microscopy (AFM) observations revealed the presence of right-handed helical nanoribbons with an average pitch of 65 nm and a height of 18 nm, as shown in Figure 4A ii, iv. Such supramolecular helical architectures are consistent with the enhanced CD signal suggesting a slipped stacking (J-type aggregation) of the nitrophenyl moiety in the self-assembled structures (vide supra, Figure 3B). The relatively smaller height in the AFM images compared with the average diameter estimated using the TEM images could be due to tip-induced deformations of the self-assembled structures. Meanwhile, the TEM and AFM images (Figure 4B i, ii) of the NPmoc-F/F-NH₂ hydrogel revealed the presence of rather flat and wider ribbons and their networks, which would be consistent with the highly turbid macroscopic appearance of the hydrogel.
Figure 4. Representative (i) transmission electron microscopy and (ii) atomic force microscopy (height, tapping mode) images of the (A) N\text{Pmoc}-F/F-NHNH\textsubscript{2} and (B) N\text{Pmoc}-F/F-NH\textsubscript{2} hydrogels. In panel A, magnified images (A\textsubscript{iii,iv}) and a cross-section profile along the white line inside the rectangle in panel (A\textsubscript{ii}) are presented.

Post-assembly modification of supramolecular nanofibers through dynamic combinatorial chemistry. Tubulins, which are dynamic, intracellular fibrous architectures and the constituent monomers of microtubules, are involved in a range of post-translational modifications including acetylation, (de)tyrosination, glycylation, and polyglutamylation.\textsuperscript{[19]} The tubulin code promotes the involvement of microtubules in diverse functions ranging from development, differentiation, and cell cycle in organisms. For example, a recent study based on super-resolution microscopy revealed the presence of two different microtubule populations in neurons, i.e., one type of microtubules that are acetylated but rarely tyrosinated in the center of the neuronal dendrite and another tyrosinated but rarely acetylated type at the dendrite periphery. This kind of opposite polarity for microtubules
gives rise to two types of transport: retrograde with kinesin-1 and anterograde with kinesin-3. To emulate such sophisticated properties and functions of fibrous bio-supramolecular architectures, controlled postmodification of artificial supramolecular architectures is a promising approach.

To explore the post-assembly modification via hydrazone bond formation of the in situ generated supramolecular nanofiber network inside the NPmoc-F/F-NHNH$_2$ hydrogel, as depicted in Figure 1C, confocal laser scanning microscopy observations were conducted. Upon the addition of a hydrophobic fluorescent probe (Nile red, Figure 5B) to the NPmoc-F/F-NHNH$_2$ hydrogel, an aggregated fibrous morphology (Nile red: magenta pseudo-color channel) was visualized as shown in Figure 5A_i,S6, which is consistent with previous reports. Then, to remove the remaining F-NHNH$_2$, which can interfere with the desired hydrazone bond formation between NPmoc-FF-NHNH$_2$ and the added aldehyde-containing molecules, the pH of the aqueous solution was carefully changed from pH 8.0 (optimal for thermolysin-catalyzed ligation) to pH 5.7 (suitable for hydrazone ligation, fluorescence of fluorescein can be weakened but detected) by adding the corresponding aqueous buffer on the top of the hydrogel (12.75 mL against 250 µL of the hydrogel) and exchanging the supernatant five times (see Supporting information for details). It should be noted that the NPmoc-FF-NHNH$_2$ hydrogel seemed stable after the solution exchange process because the initial gel volume was almost unchanged (Figure S6). Then, after the addition of a fluorescein derivative bearing an aldehyde group (FA, Figure 5B), followed by incubation at room temperature for 60 min, an aggregated fibrous morphology appeared in the green channel (fluorescein: green pseudo-color channel) as shown in Figure 5A_iii. The merged image (Figure 5A_iv) between the green and magenta channels became almost white and the Pearson’s correlation coefficient (PCC) estimated using the image was 0.78, indicating the high degree of colocalization of the two fluorophores. In contrast, upon the addition of pristine fluorescein without an aldehyde group instead of FA under the same conditions, no fibrous morphology appeared in the green channel (Figure 5A_vi) even in the presence of the fibrous morphology seen in the magenta channel (Figure 5A_v), and a low PCC value of 0.01 was obtained. This can be ascribed to the lack of accumulation of the hydrophilic fluorescein molecule on the fibrous supramolecular architectures without hydrazone bond formation. In addition, solution exchange without pH shift resulted in no accumulation of FA along fibrous morphology, most probably due to low ligation efficiency under rather alkaline pH 8.0 (Figure S6). Overall, these results clearly demonstrate that the in situ fabricated supramolecular nanofiber network consisting mainly of NPmoc-FF-NHNH$_2$ in hydrogel state can be decorated with an aldehyde-appended molecule via post-assembly modification.
Figure 5. (A) Representative confocal laser scanning microscopy images for the postmodification of self-assembled NPmoc-FF-NHNH$_2$ (i) before and (ii–iv) after the addition of a fluorescein derivative bearing an aldehyde group (FA) and (v–vii) after the addition of a pristine fluorescein without aldehyde group. See Supporting information (Figure S6) for experimental details and additional images. (B) Schematic illustration of the plausible post-assembly modification process and chemical structures of FA, Nile red, and fluorescein. Conditions: [Nile red] = 11 µM, [FA] or [fluorescein] = 19 µM, 100 mM MES(2-Morpholinoethanesulfonic acid)-NaOH buffer (pH 5.7), 23 ºC.

Conclusions

In summary, we demonstrated that the thermolysin-catalyzed construction of a dipeptide hydrazide from an N-protected amino acid and an amino acid hydrazide enables the in situ formation of self-assembled nanofiber networks and a supramolecular hydrogel simultaneously. The use of enzymatic triggers for the construction of self-assembling peptide derivatives, such as that demonstrated in this study, can be envisaged as a mild and sustainable approach for in situ and simultaneous construction of supramolecular architectures. Nevertheless, as previously reported,$^{[5-8]}$ the substrates applicable to this approach depend on the selectivity of enzymes used as triggers, which may limit the construction of a library of dipeptides capable of forming supramolecular architectures with different morphologies. In fact, low efficient ligation was observed between NPmoc-F and A-NHNH$_2$ (A: alanine, data not shown), which is consistent with a previous report.$^{[5]}$ To address these issues, pursuing new methodology for the aqueous ligation of amino acid and peptide derivatives would be required.$^{[23]}$
In addition, we demonstrated the post-assembly modification capability of the in situ constructed supramolecular architectures tethering hydrazide groups as a chemical handle. To emulate the sophisticated properties and functions of bio-supramolecular architectures such as microtubules, post-assembly modification of artificial supramolecular architectures and investigation of the resulting properties is an attractive subject.[20]

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Conflict of Interest

The authors declare no conflict of interest.

References


