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Enzyme-Triggered Self-Assembly of Peptide Hydrogels via Reversed Hydrolysis

Sophie Toledano, Richard J. Williams, Vineetha Jayawarna, and Rein V. Ulijn*
School of Materials & Manchester Interdisciplinary Biocentre, Grosvenor Street, The University of Manchester, Manchester M1 7HS, UK

Received September 23, 2005; E-mail: R.Ulijn@manchester.ac.uk

Self-assembly of macroscopic materials from small-molecule building blocks provides a route to designed molecular biomaterials.[1-3] The ability to control the assembly of these structures *on demand* by application of an external stimulus is of value, especially in biomedical contexts. For example, in minimal invasive surgery for tissue repair, a liquid precursor is mixed with cells and injected into the body to form a gel scaffold in situ for tissue regrowth.[2] Stimuli that have been used to trigger self-assembly include a variety of chemical and physical means, such as changes in ionic strength, pH, and temperature, and addition of certain chemical entities.[2-4] An alternative approach is to exploit enzyme-catalyzed reactions as selective biological stimuli to trigger hydrogel assembly. This approach has a number of advantages because enzymes (a) are uniquely chemo-, regio-, and enantioselective; (b) work under mild conditions (aqueous, pH 5−8, 37 °C); (c) play key roles as selective catalysts in cell pathways and disease states. Work in the area of enzyme-assisted assembly was reviewed recently[5] and includes the use of protein cross-linking enzymes to trigger assembly of peptide conjugates,[6] the use of enzymatic (de-)phosphorylation to control β-sheet assembly,[7] hydrolysis of L-diphenylalanine peptide nanotubes,[8] dephosphorylation to trigger gelation,[9] enzymatic (dis)-assembly of a DNA/gold nanoparticle system,[10] enzyme-triggered intramolecular acyl migration in modified peptides,[11] thermal/pH-activated disassembly followed by an enzymatic step,[12] protease-catalyzed hydrolysis of cross-linked hydrogels.[13]

We describe a conceptually novel approach by using proteases, enzymes that normally *hydrolyze* peptide bonds in aqueous medium, to perform the reverse reaction (i.e., peptide *synthesis* or *reversed hydrolysis*) to produce amphiphilic peptide hydrogelators that self-assemble to form nanofibrous structures (Figure 1). The main advantage of exploiting reverse hydrolysis is that no byproducts are formed other than an equivalent of water for each peptide bond. In different contexts[14] it is well-known that proteases can be made to work in reverse by providing the appropriate environment to favor thermodynamically the reverse direction. For example, organic (co-) solvents,[14a] highly concentrated substrate suspensions,[14b] and solid-phase methods with immobilized reactants[14c] have been used to shift the reaction equilibrium from hydrolysis to synthesis. In each of these examples, the favored direction of the reaction is reversed by thermodynamic stabilization of the peptide reaction product relative to its precursors.[14] We hypothesized that self-assembly of a peptide into a higher-order structure may provide an alternative means of stabilizing the peptide reaction product and may therefore also be used as a driving force to favor peptide synthesis and consequent formation of supramolecular hydrogels.

The self-assembly mechanism that is exploited in the current work builds on recent reports by Xu et al. that demonstrated that a number of *N*-(fluorenylmethoxycarbonyl) (Fmoc)-modified small-molecule amphiphiles self-assemble into nanofibrous structures driven by π-stacking (Figure 1C) of the highly conjugated fluorenyl group further stabilized by formation of helical structures.[4] Fmoc is widely used as a protecting group in peptide chemistry and when coupled to amino acids is known to have anti-inflammatory properties.[15] We hypothesized that proteases may be used to link nongelling Fmoc-amino acid and dipeptide starting materials to form amphiphilic Fmoc-tripeptides. Normally, low concentrations of Fmoc-tripeptide would be expected because thermodynamic equilibrium lies strongly toward hydrolysis in aqueous media (Figure 1, $K_{eq,1} < 1$). However, in cases where a further equilibrium favors self-assembly to higher-order structures ($K_{eq,2} > K_{eq,1}$), a driving force is provided to trigger further enzymatic peptide synthesis, ultimately resulting in enzyme-triggered gelation (Figure 1). Thermolysin from *Thermoproteolyticus rokko* was selected as a suitable model enzyme to test this hypothesis. This enzyme has been used in reverse hydrolysis reactions before[14] and has a well-known preference for hydrophobic/aromatic residues on the amine side of the peptide bond, whereas it is nonspecific for carboxylic acid residue. $(Phe)_2$[16] was used as the dipeptide nucleophile, and a small library of Fmoc-amino acids with a range of hydrophobicities (Figure 1) was chosen (Gly (**a**), Ala (**b**), Val (**c**), Leu (**d**), Phe (**e**)) in addition to Pro (**f**), that was selected to investigate the need for linearity in the peptide backbone. In each case equimolar amounts of 40 μmol of the Fmoc amino acid and $(Phe)_2$ were mixed to give a suspension that was dissolved by addition of concentrated NaOH 0.5 M. This was followed by a gradual lowering of pH to a value of 7 using a concentrated 0.1 M HCl solution which resulted in formation of a fine suspension with <1% w/w precursors (0.12 μmol/g). Gelation was not observed prior to enzyme addition in these reaction mixtures.

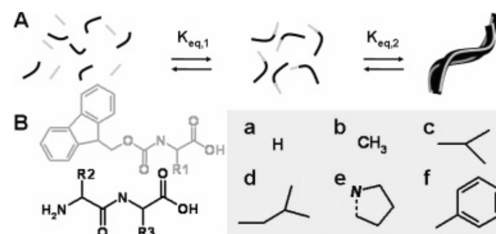

Figure 1. (A) Proposed mechanism: Fmoc amino acids (gray) are enzymatically coupled to dipeptides (black) by a protease to form Fmoc-tripeptides that self-assemble to higher-order aggregates driven by $\pi-\pi$ interactions between fluorenyl groups. $K_{eq,1}$ represents the equilibrium constant for peptide synthesis/hydrolysis, $K_{eq,2}$ for self-assembly. (B) Chemical structures of Fmoc-amino acids, dipeptide precursors and amino acid side chains: **a** Gly, **b** Ala, **c** Val, **d** Leu, **e** Pro, **f** Phe.

Upon addition of 2 mg of enzyme gelation was observed (Figure 2A) within minutes for several combinations of Fmoc-amino acids and peptides (Table 1, entries **2**−**5**), while no obvious macroscopic changes took place for entries **1**, **6**, **7** after a period of up to 2 weeks. HPLC analysis of the reaction mixtures revealed that for entries **1**−**6** and **8** Fmoc-tripeptides were formed in varying yields. Macroscopically observed hydrogel formation correlated well with formation of Fmoc-tripeptide with non-gelling entries **1**, **6**, **7**

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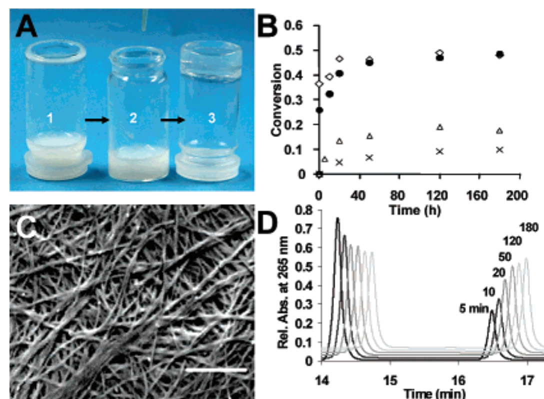

Figure 2. (A) 1: Suspension of Fmoc-Phe and $(Phe)_2$. 2: addition of 0.5 mg of thermolysin. 3: Inversion demonstrates self-supporting gel formation. (B) Effect of enzyme amount on the rate of Fmoc-$(Phe)_3$ formation. From top to bottom the data represent 2, 0.5, 0.1, 0.05 mg of thermolysin in a total volume of 3.4 mL. Conversions are based on the HPLC peak areas at 256 nm. (C) Cryo-SEM micrograph of enzymatically prepared Fmoc-$(Phe)_3$ hydrogel obtained from 40 μmol of Fmoc-Phe and $(Phe)_2$ and 0.5 mg thermolysin. The scale bar represents 0.5 μm. (D) HPLC chromatograms of peptide synthesis in the course of time in the presence of 0.5 mg thermolysin. Peaks shown represent Fmoc-Phe and Fmoc-$(Phe)_3$.

Table 1. Fmoc-Amino Acid/Dipeptide Combinations in Enzyme-Triggered Hydrogel Formation[a]

entry	Fmoc-X	% conversion	gel formed?[e]
1	Gly	<8	no
2	Ala	27	yes
3	Val	64	yes
4	Leu	51[b]	yes
5	Phe	54, 55[c]	yes
6	Pro	<8	no
7	Pro	<0.1[d]	no
8	Leu	16[f]	yes

[a] Conditions were 22 °C, pH 7, 40 μmol Fmoc-amino acid and dipeptide, 2 mg of enzyme powder in a total volume of 3.4 mL. [b] Fmoc-pentapeptide also formed [c] 60 μmol starting material [d] Chymotrypsin instead of thermolysin [e] Self-supporting gel was assessed by inversion of the reaction vial [f] $(Phe)_2$ was replaced with $(Leu)_2$.

showing low conversions of <8%. Yields of peptide formed increased with increasing hydrophobicity with hydrophobic amino acids Fmoc-Val (**3**), Leu (**4**), and Phe (**5**) giving rise to significantly higher yields compared to Fmoc-Ala (**2**) and Fmoc-Gly (**1**). The <8% conversion that was observed with Fmoc-Gly as the acyl donor appears to be below the critical gelation concentration, therefore not providing a sufficient thermodynamic driving force to trigger self-assembly under these conditions. However, it is possible that the tripeptide self-assembled, but was not capable of gelation through the formation of stable intermediary structures. Entry **6** (Fmoc-Pro) also does not give a gel, and only a small amount (<8%) of Fmoc-Pro-Phe-Phe was formed, which was thought to be related to the nonlinear nature of the peptide thus preventing formation of hydrogen bond/π-stacking stabilized assemblies. In entry **4** a mixture of Fmoc-tri- and pentapeptides was observed, formed by further coupling of $(Phe)_2$ to Fmoc-tripeptides. $(Phe)_2$ has been identified as a key peptide sequence in amyloid fibril-forming peptides.[8] To verify whether gel formation was dependent on aggregation of $(Phe)_2$ molecules we tested its replacement by $(Leu)_2$ (**8**) where Fmoc-tripeptide formation and gelation were also observed. Overall, the results demonstrate that thermolysin can selectively trigger hydrogel formation via reversed hydrolysis.

Cryo-SEM analysis of the resulting Fmoc-$(Phe)_3$ gel revealed interwoven fibers of approximately 10−20 nm in diameter (Figure 2C) similar in dimension to that of fibrous structures observed by Xu et al.[4,9] To study the minimal amount of enzyme required for significant peptide formation it was varied between 0.05 and 5 mg (Figure 2, B and D). For up to 2 mg an increase of the enzyme quantity resulted in a steady increase of the reaction rate up to 3.2 μmol/min/mg, which is in line with previously reported reaction rates for this enzyme.[17] It is hypothesized that the asymptotic reduction of enzyme activity at 0.005 and 0.01 mg enzyme was caused by entrapment of enzyme molecules within small peptide aggregates that prevented further synthesis. For these experiments no gelation was observed up to 2 weeks. To put the experiments into a biomedical context, we observed that enzyme-triggered gelation of entries **5** (Table 1) occurred in 0.15 M phosphate buffered saline solution and in a tissue culture medium at 37 °C.

In summary, we have demonstrated that a protease can be used to selectively trigger the self-assembly of peptide hydrogels via *reversed* hydrolysis. This conceptually novel method may have implications for in situ formation of nanofibrous hydrogel scaffolds for cell culture.

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