Enzymatically triggered self-assembly of poly(ethylene glycol)-attached oligopeptides into well-organized nanofibers†

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A unique and programmable peptide self-assembling system has been fabricated by using poly(ethylene glycol)-attached amphiphilic oligopeptide, which shows rapid self-assembly into well-organized β-sheet nanofibers in response to an enzymatic reaction.

Advances in molecular self-assembly provide strong incentives to fabricate novel nanostructured materials. Peptides and proteins are versatile building blocks for constructing well-defined three-dimensional (3D)-architectures on the scale between nanometres and submicrons; examples of their use as various scaffolds are found in nature. Several self-assembling peptide systems that form micelles,[1] vesicles,[2] networks,[3] and a variety of other morphologies[4] have already been developed. In particular, the biological β-sheet motif has attracted much attention because of its association with neurodegenerative diseases,[5] such as Alzheimer's and Creutzfeldt–Jacob's, and as a building block in the design of supramolecular nanofibers.[6] Such unique structures are expected to have potential as novel biological materials with diverse applications like nano-scaffolds and nano-templates. Recently, we have reported that fine tuning of peptide sequence, stereochemistry, and solution pH enables the nanostructure control of 3D-architectures, including nanofibers, by controlled self-assembly of 16-mer peptide building units.[6h,6i] In addition to such structural control, it is also important to establish facile and practical guidelines to control spontaneous peptide self-assembling process, that is temporal control, in order to advance a bottom-up nanobiotechnology (programmable peptide self-assembly). A few previous reports have achieved the construction of stimuli-responsive (temperature, light) self-assembling systems into nanofibers.[7] More recently, Mutter's group used enzymes to trigger an acyl migration that allowed a conformational transition and self-assembly of various peptides.[8]

In the present study, we describe a facile and unique self-assembling system using poly(ethylene glycol) (PEG)-attached amphiphilic oligopeptide that forms β-sheet nanofiber architectures rapidly in response to enzymatic triggering. PEG is an important biocompatible polymer because of its non-toxicity and non-antigenic activity. Such properties of the PEG unit, as well as its high solubility in various solvents, are convenient for engineering bio-related nanomaterials by structurally- and temporally-controlled peptide self-assembly. Furthermore, our approaches may have significance as an amyloid model system because the amyloidogenic Aβ(1–40/42) peptides are also derived from the 695 amino acid long type-I transmembrane protein, known as the Aβ precursor protein, by the action of the β- and γ-secretases.[9]

A novel amphiphilic block peptide (**1**), in which a β-sheet forming peptide ($L_4K_8L_4$) was conjugated with the relatively hydrophilic PEG segment through a thrombin-cleavable site (VPRGS), was designed and employed as building block for self-assembly (Fig. 1). A control peptide, with a scramble sequence for thrombin, $L_4K_8L_4$–SGRPVL–PEG (**2**), was also employed for comparison. These peptides were easily prepared by standard solid phase peptide synthesis (SPPS) using Fmoc chemistry on PAP Tenta-Gel (Rapp Polymere) (see ESI†). Cleavage by TFA and deprotection yielded a linear PEG (av. M_w: 3000–3300) covalently bound to the C-terminus of the designed peptide. We demonstrated recently that the triblock-type $L_4K_8L_4$ sequence was a stable β-sheet foldamer and could be hierarchically self-assembled into amyloid-like nanofibers upon partial neutralization of the charge of Lys residues at around pH 9.[6h]

To test the biological activity, the peptides **1** and **2** were first digested by thrombin at pH 9.0. Thrombin, a known endopeptidase, selectively hydrolyzes the Arg–Gly peptide bond, and has been often used in artificial protein synthesis by recombinant DNA methods for the cleavage of fusion proteins. Both peptides were dissolved in Tris/HCl buffer (containing 5% TFE, pH 9.0, 40 μM peptide) and exposed to thrombin (0.8 NIH unit) for 30 min at room temperature. The reaction was analyzed by MALDI-TOF MS spectroscopy (Fig. 2). After digestion for 30 min, two peaks were newly observed at *ca.* 3500 (broad) and 2302 (sharp) in the case of peptide **1**, along with complete disappearance of the broad peak at *ca.* 5800 from the starting compound (Fig. 2b). This result

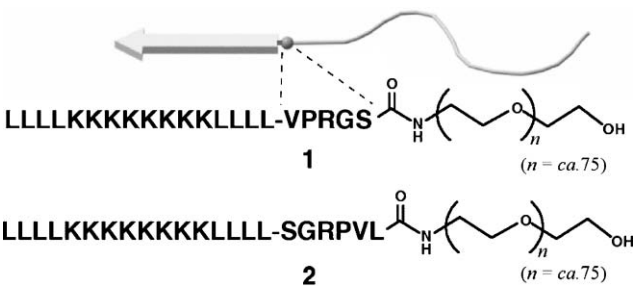

Fig. 1 Molecular structures of poly(ethylene glycol)-conjugated β-sheet peptides, $L_4K_8L_4$–VPRGS–PEG (**1**) and $L_4K_8L_4$–SGRPVL–PEG (**2**), which were used as building blocks for self-assembly.

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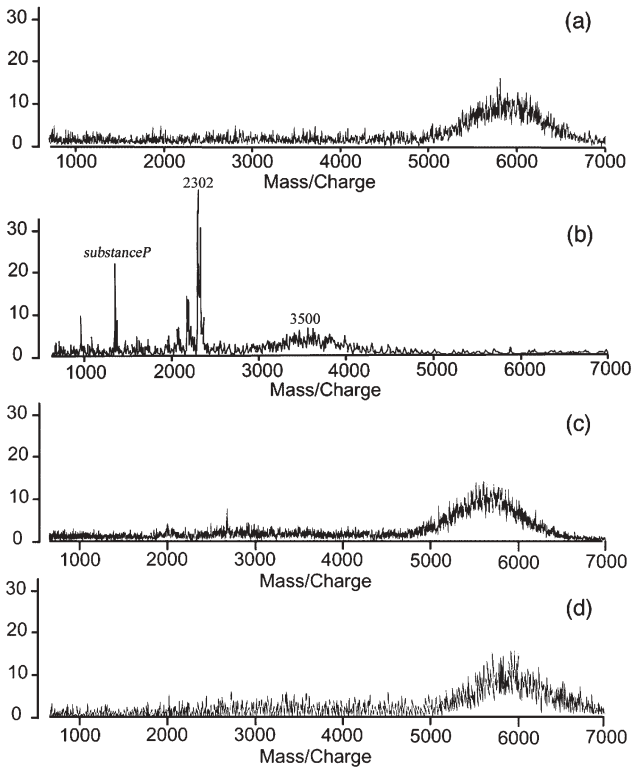

Fig. 2 Matrix-assisted laser desorption ionization-time of flight mass (MALDI-TOF MS) spectra of peptide **1** (a and b) and **2** (c and d) before (a and c) and after (b and d) exposure to thrombin (0.8 NIH unit) for 30 min at pH 9.0, room temperature. Note that the substance P (H_2N–MLGFFQQPKPR–COOH), which was observed in spectrum (b), was added into the sample and used as a standard peptide to check a mass value for digestion analysis.

shows that the site-specific digestion of **1** took place smoothly at the R–G bond within 30 min and that, as a result, the $L_4K_8L_4$–VPR peptide ($[M + H]^+_{theory}$: 2302.1) was generated by detaching the PEG-based unit. On the other hand, such digestion was not observed for peptide **2** (Fig. 2d), demonstrating the high specificity of enzyme for the sequence of cleavage site.

The conformational properties of **1** and **2** in response to the enzymatic reaction were subsequently investigated by means of circular dichroism (CD) spectroscopy. Fig. 3A (left) shows the time-dependence of CD spectra for **1** at pH 9.0. With freshly prepared sample solution, the CD spectrum gave mixed patterns of α-helix and random coil structures with two negative maxima, at 222 nm and 204 nm. This α-helical conformation (containing random coil structure) of **1** was maintained even after incubation for 8 h, although a slight decrease in the molar ellipticity was observed at 215 nm. It seems that the PEG-based hydrophilic tail attached to the β-sheet formable peptide acts to regulate the conformation by increasing solubility in water and/or by creating a steric block, and prevents the marked aggregation of **1** into a β-sheet structure. Interestingly, the addition of thrombin was found to cause remarkable α-to-β structural transition of **1** (Fig. 3A (right)). As plotted in Fig. 3B(a), the molar ellipticity differences at 215 nm, $\Delta[\theta]_{215}$ values, decreased immediately without a significant lag phase after thrombin addition (arrow), and reached a constant value within 3 h. In contrast to **1**, the control peptide **2** showed no significant change of $\Delta[\theta]_{215}$ values upon the addition of thrombin

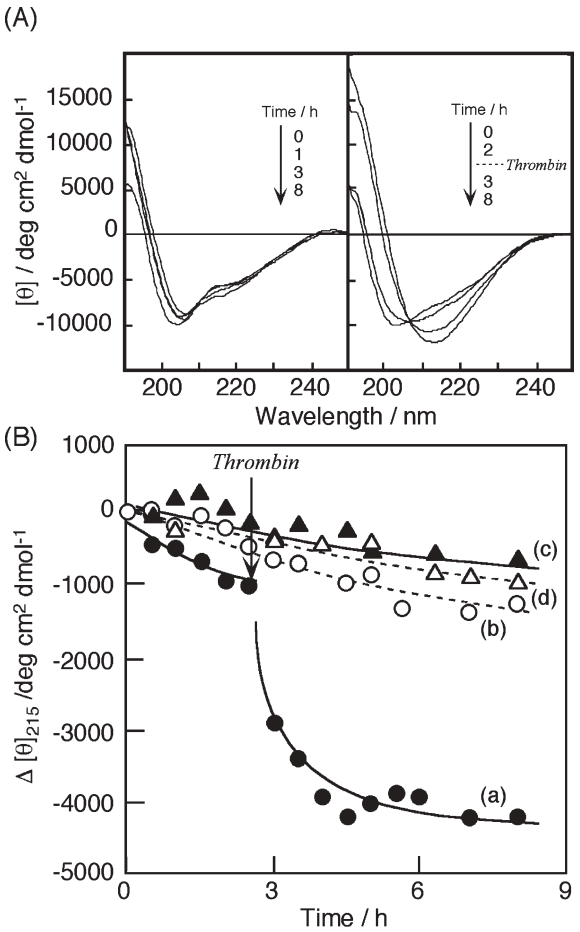

Fig. 3 (A) CD spectral changes of **1** in the absence (left) and presence (right) of thrombin in Tris/HCl buffer (containing 5% TFE) at pH 9.0. The peptide was incubated at room temperature for the time indicated (0–8 h), and the thrombin was added into the **1**-solution after 2.5 h incubation. [**1**] = 40 μM. (B) Time dependencies of $\Delta[\theta]_{215}$ for **1** (a and b) and **2** (c and d) with (solid lines; a and c) and without (dashed lines; b and d) the addition of thrombin under above condition. The arrow indicates the time at which thrombin was added in the **1**- or **2**-solution. [**1**] = [**2**] = 40 μM.

(Fig. 3B(c)), demonstrating the conformational change was not triggered by non-specific interaction with protein surfaces. Therefore, the observed enzyme-triggered self-assembly of peptide **1** into a β-sheet structure would be driven by the detaching of GS-PEG unit from **1**, permitting attractive hydrophobic and van der Waals forces among the resultant $L_4K_8L_4$–VPR peptides to dominate. In fact, spontaneous and quick β-sheet formation was observed for the independently and chemically synthesized $L_4K_8L_4$VPR peptide under the same condition (see ESI, Fig. S1†). In this case, the control $L_4K_8L_4$VPR peptide showed a drastic α-to-β conformational transition within 3 h through relatively similar transition kinetics to that of the enzymatically produced-peptide.

The self-assembled nanostructures induced by thrombin addition were also characterized by atomic force microscopy (AFM). Fig. 4 shows the tapping-mode AFM images (3 μm × 3 μm) of **1** and thrombin digested **1** at 24 h. Note that the digestion of **1** was also carried out after 2.5 h incubation. The AFM image of **1**, in which the peptide segment took mainly an α-helical and random coil form, revealed amorphous aggregates (Fig. 4A). These

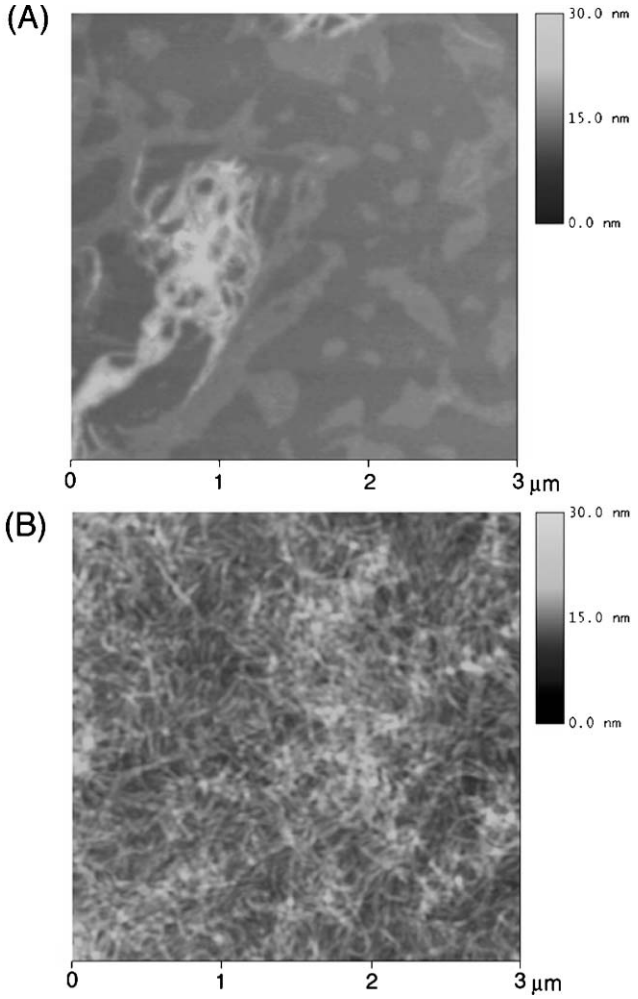

Fig. 4 Tapping-mode AFM images (3 μm × 3 μm) of (A) **1** and (B) thrombin digested **1** at 24 h, pH 9.0. Note that the digestion of **1** by thrombin was carried out after 2.5 h-incubation. *z*-Scale: 30 nm. [**1**] = 40 μM.

amorphous aggregates contain partly fibrous form and globular (plate) form, but their shapes and sizes, namely height and width, are not uniform. Therefore, these aggregates are probably due to a non-specific hydrophobic interaction between peptide segments and/or a small amount of β-sheet conformation of **1**. On the other hand, well-organized nanofiber structures with nearly uniform diameters of *ca.* 5–6 nm were observed from thrombin digested **1** (Fig. 4B). Such dimensions were in fair agreement with those of authentic amyloids.[10] These results clearly show the enzymatically triggered self-assembly of **1** into β-sheet nanofibers, and also agree well with the results of CD studies.

In this communication, we have shown a unique method for controlling the self-assembly of PEG-attached amphiphilic oligopeptides into nanofibers through an enzymatic reaction. Such an approach should be useful in establishing a programmable molecular self-assembling system for the fabrication of designed 3D-nanoarchitectures at controlled timing, as well as for our understanding of the peptide fibrillogenesis. Most notably, our approach permits an easy manipulation of the sequences of both self-assembling peptide moiety and enzyme-recognition site only by a conventional SPPS method. Further studies involving the fabrication of various nanostructured materials through peptide self-assembly in response to the specific enzyme are underway.

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