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Proteinase-mediated drastic morphological change of peptide-amphiphile to induce supramolecular hydrogelation

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We report a novel peptide-amphiphile having a simple molecular structure that can gelate an aqueous solution at a remarkably low concentration and can be designed to be responsive to a disease-related enzyme by undergoing a drastic morphological change.

One of the challenging topics in chemistry and biochemistry is to artificially amplify a nanoscale molecular event of a chemical or biochemical reaction to induce a macroscopic event. For example, sequences of chemical and biochemical reactions facilitate a variety of macroscopic life activities in living beings, and this has attracted our interest for a long time now. The detection of a tiny molecular event via various types of amplification is also a significant target in the field of analytical chemistry. The artificial amplification of a molecular event usually involves other chemical and biochemical reactions, spectroscopy and machinery. Gelation is one attractive strategy for amplifying a molecular event because the result is visible. Supramolecular hydrogel, which is formed by the self-assembly of small molecular building blocks (low-molecular-weight gelators), is a new class of hydrogel discovered in the last decade that has attracted much attention owing to being thermoreversible, designable and responsive to external stimuli.[1,2] Since enzymes play essential roles in a broad range of biological processes and disease states, mutual communication between enzyme-responsive artificial materials (hydrogelators) and biological environments brings intriguing properties to hydrogels from the viewpoint of biomedical, food and agrichemical applications.[3] In spite of these studies on enzyme-triggered hydrogelation, there are few reports on enzyme-mediated morphological changes of molecular self-assembly that lead to a macroscopic change in a bulk solution.

Here we report a novel peptide-amphiphile having a simple molecular structure that can gelate an aqueous solution at a remarkably low concentration and can be designed to be responsive to a disease-related enzyme in a way that involves a drastic morphological change of the peptide-amphiphile. Peptide-based supramolecular hydrogelators are expected to provide high biocompatibility,[2b, 4] and appropriate design of the peptide sequence can make the supramolecular hydrogel responsive to enzymes and biological stimuli. In the present study, we adopted matrix metalloproteinase-7 (MMP-7), which is related to various cancers,[5] as a triggering enzyme. We designed the peptide-amphiphile to allow the micelle-to-nanofiber transition of the peptide-amphiphile that leads to gelation to occur through mediation by an MMP-7-catalyzed reaction. The present hydrogelating system can be used for the visual detection of a disease-related enzyme.

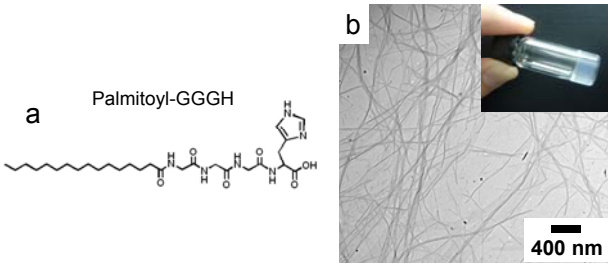


Fig. 1 a) Molecular structure of pal-GGGH. b) TEM image of cryo-dried Pal-GGGH hydrogel and macroscopic image of the hydrogel (0.2 wt%) in pH 7.5 phosphate buffer (inset).

A number of peptide-amphiphiles (especially palmitylated peptides) have been reported to self-assemble in aqueous media and achieve macroscopic hydrogelation by forming three-dimensional networks of nanofibers.[6] The supramolecular self-assembly of a peptide-amphiphile is induced by multiple non-covalent interactions, such as hydrophobic interaction, hydrogen bonding and electrostatic interaction among molecules. As a peptide sequence in a peptide-amphiphile is an important factor in providing intermolecular interaction and maintaining solubility in water, oligopeptides of considerable length are often used for the hydrophilic part of a peptide-amphiphile.[6] To the best of our knowledge, there is no report of a palmitylated peptide composed of less than 5 amino acid residues that can gelate an aqueous solution below a concentration of 0.2 wt%.[6f]

We synthesized various *N*-palmitylated peptides containing a histidine residue employing standard Fmoc chemistry. Of these, *N*-palmitoyl-Gly-Gly-Gly-His (Fig. 1a, pal-GGGH) was found to be an efficient hydrogelator for aqueous solutions, such as pH 7.5 phosphate buffer (inset of Fig. 1b), 1M HCl aqueous solution and an ethanol/water mixture (1:1), even though it had a very simple structure containing a short peptide sequence. Interestingly, a remarkably low concentration (0.03 wt%) of pal-GGGH induced the gelation of pH 7.5 phosphate buffer, which is the lowest critical gelation concentration reported to date for supramolecular hydrogelators.[7] Pal-GGGH was insoluble at weakly acidic pH values (pH 2-6) even with heating of the solution, probably due to its low solubility in water at weakly acidic pH values due to its zwitterions that are present in that pH range. The transmission electron microscope (TEM) image of the hydrogel of 0.1 wt% pal-GGGH showed nanofibers 20 nm in diameter (Fig. 1b). This image suggests that pal-GGGH

Table 1 Gelation properties of the *N*-palmitoyl-tetrapeptides.

pH[a]	Pal-GGGH	Pal-GGHG	Pal-GHGG	Pal-HGGG
0	G	G (0.03)	VS	S
Deionized water	I	I	G (0.05)	G (0.1(
3.0	I	I	G	PG
7.5	G (0.03)	VS	VS	P
10.2	VS	VS	VS	VS

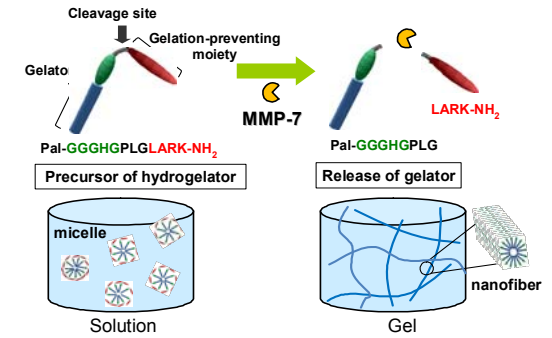
[a] 1M HCl for pH 0, 50 mM acetate buffer for pH 3, 50 mM phosphate buffer for pH 7.5 and 50 mM borate buffer for pH 10.2.
[b] G represents gelation and the value in parentheses represents a critical gelation concentration.
[c] I represents insoluble even if heated.
[d] VS represents viscous solution.
[e] S represents soluble after dissolving a hydrogelator by heating.
[f] PG represents partial gelation.

formed nanofibers in aqueous solution by molecular self-assembly and that a lot of water molecules were retained among the nanofibers.

Of the various palmitoyl peptides synthesized (*N*-palmitoyl-Gly-Gly-His-Gly, *N*-palmitoyl-Gly-His-Gly-Gly and *N*-palmitoyl-His-Gly-Gly-Gly in addition to *N*-palmitoyl-Gly-Gly-Gly-His), the palmitoyl peptide having a histidine residue on its C-terminus showed the highest gelation ability (Table 1). Palmitoyl peptides having a histidine residue at different positions in the peptide sequence formed hydrogels at different pH values. This was probably due to the different solubilities of the palmitoyl peptides at different pH values.

Our newly developed hydrogelator, pal-GGGH, was an amphiphilic structure having a very short peptide that exhibited gelation ability at a remarkably low concentration. These characteristics allowed us to anticipate that the introduction of a substrate peptide to pal-GGGH could easily make it responsive to a peptide-related enzyme, and that a small portion of the product of an enzymatic reaction would result in a remarkable change in the bulk solution via a morphological change derived from the molecular self-assembly of a peptide amphiphile. We then attempted to develop a novel hydrogelator precursor responsive to disease-related proteinase (MMP-7) using an analogue of pal-GGGH (Scheme 1).

N-Palmitoyl-Gly-Gly-Gly-His-Gly-Pro-Leu-Gly-Leu-Ala--Arg-Lys-NH_2 (C-terminal primary amide) was synthesized by solid-phase synthesis on Rink amide MBHA resin[8] and purified by reverse-phase HPLC with an ODS column. The obtained palmitoyl-Gly-Gly-Gly-His-Gly-Pro-Leu-Gly-Leu-Ala-Arg-Lys-NH_2 was used as a precursor of a hydrogelator. The peptide sequence -Pro-Leu-Gly-Leu- is the substrate sequence of MMP-7.[9] This precursor has a long hydrophilic moiety and a curved structure based on Pro to prevent the formation of nanofibers and to allow micelle formation due to the sterically bulky peptide sequence (Scheme 1). The precursor was indeed soluble in aqueous solution and the dynamic light scattering (DLS) measurement indicated the formation of a micelle-like self-assembly with a diameter of approx. 10 nm. It should be noted that no significant change in the macroscopic state was observed for 2 days after the dissolution of the precursor at 0.2 wt% in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4), indicating that gelation was prevented by the long hydrophilic peptide sequence attached to pal-GGGH. When MMP-7 (2 μg/ml) was



Scheme 1 Schematic illustration of MMP-7-mediated hydrogelation of a precursor solution.

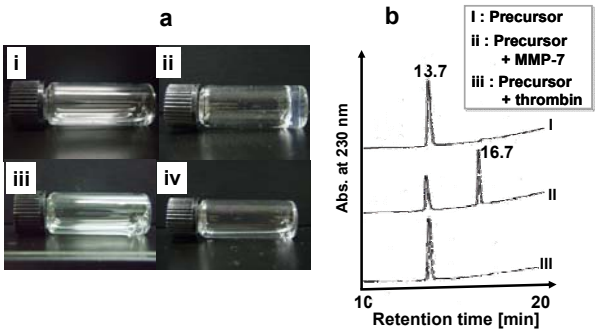


Fig. 2 Optical images (a) and corresponding HPLC charts (b) of the 0.2 wt% precursor solution in TBS buffer (i), the hydrogel obtained after adding MMP-7 (2 μg/ml) to the solution (reaction time of 2 h) (ii), and precursor solutions containing 5 μg/ml BSA (iii) or 5 μg/ml thrombin (iv).

added to the precursor solution (0.2 wt%), the solution was completely converted to a translucent hydrogel within 2 h (Fig. 2). HPLC analyses revealed that approx. 40% of the precursor was hydrolyzed by MMP-7 after 2 h of reaction (Fig. 2b). The hydrolysis product was collected by HPLC and analyzed by MALDI TOF−MS (ESI). The mass spectrum showed that the molecular mass of the hydrolysis product $(M+H)^+$ was 889.2 m/z, indicating that the precursor was hydrolyzed between the Gly and Leu of -Pro-Leu-Gly-Leu- which is identical to an MMP-7 cleavage site.

When bovine serum albumin (BSA) or thrombin (5 μg/ml each) instead of MMP-7 was added to the precursor solution, no hydrogelation was observed and the solution state was maintained for more than 24 hours. HPLC analyses showed that no hydrolysis of the precursor occurred in the presence of BSA or thrombin. These results mean that the precursor was

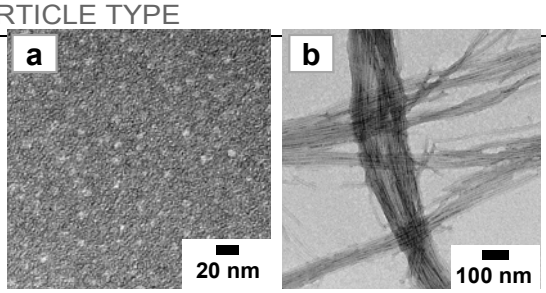


Fig. 3 TEM images of the cryo-dried 0.2 wt% precursor solution (a) and hydrogel (b) obtained after adding 1 μg/ml MMP-7 to the solution. Both were stained by 2 % uranyl acetate.

not degraded in the absence of a certain biocatalyst and also that unexpected interaction between the precursor and proteins did not cause the hydrogelation. To confirm the hydrogelation triggered by MMP-7, the hydrogelation of the precursor solution was studied in the presence of MMP-7 together with the MMP inhibitor II,[10] and no hydrogelation took place in this case. These results suggested that MMP-7 specifically catalyzed the hydrolysis of the precursor peptide and triggered the hydrogelation of the precursor solution.

Finally we investigated the morphology of the precursor self-assembly and the resultant hydrogel by TEM. In the precursor solution, the precursor molecules formed nanometer-sized spherical aggregates (like micelles) approx. 10 nm in diameter, which was also confirmed by the DLS measurement. On the other hand, a drastic morphological change was observed in the precursor solution after the MMP-7-catalyzed reaction. The hydrogel prepared by MMP-7 (1 μg/ml) showed nanofibers 10 nm in width and their bundles in the TEM image, which would play an important role in forming the hydrogel. These results concluded that the transition from solution to gel was induced by the morphological change in the precursor solution due to the enzymatic hydrolysis of the sterically bulky peptide of the precursor as illustrated in Scheme 1.

In conclusion, we have presented a novel acylated short peptide, *N*-palmitoyl-Gly-Gly-Gly-His, as a supramolecular hydrogelator. Despite its very simple molecular structure, this newly developed hydrogelator forms a hydrogel at a remarkably low concentration (0.03 wt%) in buffer solution at strongly acidic, neutral and weakly basic pH values. A major advantage of this hydrogelator is that it is possible to introduce a functional peptide sequence into its molecular structure. Using *N*-palmitoyl-Gly-Gly-Gly-His, we designed a peptide sequence for the hydrogelator precursor and succeeded in obtaining a drastic morphological change in the precursor solution triggered by MMP-7-catalyzed proteolysis that resulted in the sol-gel phase transition. Possible applications of this MMP-7-sensitive peptide-amphiphile are a diagnostic sensor to detect MMP-7-related diseases by macroscopic observation and a medicine to selectively kill cancerized cells.[2c]

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental section and results on the effect of the acyl chain on hydrogelation (Table S1). See DOI: 10.1039/b000000x/

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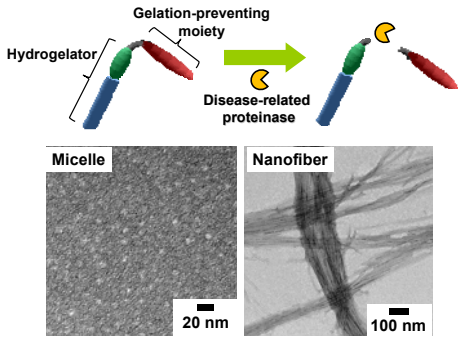

Graphical and textual abstract

We report a novel peptide-amphiphile that form supramolecular hydrogel at a remarkably low concentration and can be designed to be responsive to a disease-related enzyme through a drastic morphological change.

Electronic Supplementary Information: Proteinase-mediated drastic morphological change of peptide-amphiphile to induce hydrogelation

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Experimental

Materials: Fmoc amino acids, MBHA-rink amide resin, amino acid-preloaded Barlos resin, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU), trifluoroacetic acid (TFA) and triisopropyl silane (TIPS) were purchased from Watanabe Chemical Industry (Hiroshima, Japan). Diisopropylethylamine (DIEA) was purchased from Tokyo Chemical Industry (Tokyo). Palmitic acid and dimethylformamide (DMF) were purchased from Kishida Chemical Industry (Osaka). Matrix metalloproteinase-7 (MMP-7) as matrilysin human recombinant, acetonitrile and diethylether were purchased from Wako Pure Chemicals Industries (Osaka). Kaiser reagents for ninhydrin tests were purchased from Kokusan Chemical (Tokyo). MMP inhibitor II (N-hydroxy-1,3-di-(4-methoxy-benzenesulfonyl)-5,5-dimethyl-[1,3]-piperazine-2-carbo xamide)[10] was purchased from Merck. BSA (bovine serum albumin) and thrombin from bovine were purchased from Sigma.

Solid-phase syntheses of N-acylated peptides: Palmitylated peptides were synthesized by the standard fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis protocols.[1] Fmoc protected amino acid (3 equiv) were coupled to 0.3 mmol amino acid-preloaded Barlos resin, using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) and 1-hydroxybenzotriazole (HOBt) as the coupling agents in the presence of diisopropylethylamine (DIEA) in dimethylformamide (DMF). N-termini of the peptides synthesized on the resin were subjected to N-acylation with palmitic acid (3 equiv). Qualitative ninhydrin tests were used to confirm the completion of each coupling reaction. Deprotection and cleavage of the palmitylated peptides were performed in a mixture of trifluoroacetic acid (TFA), triisopropyl silane (TIPS) and water at a ratio of 95:2.5:2.5 for 2 h at room temperature. Palmitylated peptides in the cleavage mixture

were precipitated with water or diethylether, collected by filtration, washed with water and dried under vacuum. Resultant products were washed with acetonitrile and identified by a matrix-assisted laser desorption ionization−time of flight mass spectrometer (MALDI-TOF-MS) and 1H NMR (supporting information).

Preparation of hydrogel: Palmitylated peptides were dissolved in water or buffer solution at given concentrations in a glass microtube by heating and then slowly cooling down to room temperature to form a hydrogel. Gelation was confirmed by inverting the glass microtube containing the solution.

Enzymatic reactions: After 1 mg of precursor was dissolved in 400 μl Tris-HCl buffer (50 mM, pH = 7.4), the solution was heated and cooled down to room temperature, followed by the addition of 100 μL of MMP-7 (2 μg/ml) solution. After incubation at room temperature, gelation was confirmed by inverting the glass vial containing the solution. For the inhibition study, MMP inhibitor 2 was added to the precursor solution before the addition of MMP-7. Enzymatic reaction solutions were analyzed by reverse phase HPLC equipped with an ODS column.

HPLC analysis: An inertsil ODS-3 (10×250 mm) column was used to quantify MMP-7-catalyzed hydrolysis. The gradient was 20% acetonitrile in water containing 0.1% TFA with a linear gradient of 20 to 50% acetonitrile in water containing 0.1% TFA over 10 min and then to 100% acetonitrile containing 0.1% TFA at 20 min. Isocratic 100 % acetonitrile was kept for 10 min. The eluted compounds were observed by detecting absorbance at 230 nm.

TEM observation: Hydrogels and precursor solutions were prepared for TEM observation. Carbon coated copper grids were dipped in the hydrogel samples, followed by staining with 2 % uranyl acetate and drying under vacuum to remove solvent. Solution samples were dropped onto the TEM grids, followed by the same process. TEM experiments were performed by using a Hitachi H-7000 TEM (acceleration voltage of 120 kV).

H NMR assignments

N-Palmitoyl-GGGH: MS calc. M^+ = 565.4 [m/z], obsvd. $(M+1)^+$ = 565.4 [m/z]

^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.17(m, 1H), 7.18(s, 1H), 4.49(m, 1H), 3.71(m, 6H), 3.44-3.43(m, 2H), 3.07-2.95(m, 2H), 1.47(m, 2H), 1,23(m, 24H), 0.85(m, 3H)

N-Palmitoyl-GGHG: MS: calc. M^+ = 565.4 [m/z], obsvd. $(M+1)^+$ = 565.4 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.26(m, 1H), 7.08(s, 1H), 4.34(m, 1H), 3.69(m, 6H), 3.44-3.40(m, 2H), 3.05-2.91(m, 2H), 1.47(m, 2H), 1,23(m, 24H), 0.85(m, 3H)

N-Palmitoyl-GHGG: MS [m/z]: calc. M^+ = 565.4 [m/z], obsvd. $(M+1)^+$ = 565.5 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.16(m, 1H), 7.02(s, 1H), 4.35(m, 1H), 3.68(m, 6H), 3.43-3.40(m, 2H), 2.99-2.91(m, 2H), 1.46(m, 2H), 1,23(m, 24H), 0.85(m, 3H)

N-Palmitoyl-HGGG: MS calc. M^+ = 565.4 [m/z], obsvd. $(M+1)^+$ = 565.4 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.26(m, 1H), 7.08(s, 1H), 4.34(m, 1H), 3.69(m, 6H), 3.44-3.40(m, 2H), 3.06-2.91(m, 2H), 1.47(m, 2H), 1,23(m, 24H), 0.85(m, 3H)

N-Myristoyl-GGGH: MS calc. M^+ = 537.3 [m/z], obsvd. $(M+1)^+$ = 537.4 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.16(m, 1H), 7.11(s, 1H), 4.48(m, 1H), 3.71(m, 6H), 3.45-3.43(m, 2H), 3.02-2.94(m, 2H), 1.47(m, 2H), 1,23(m, 20H), 0.85(m, 3H)

N-Stearoyl-GGGH: MS calc. M^+ = 593.4 [m/z], obsvd. $(M+1)^+$ = 593.4 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.16(m, 1H), 7.12(s, 1H), 4.48(m, 1H), 3.70(m, 6H), 3.44-3.43(m, 2H), 3.03-2.94(m, 2H), 1.47(m, 2H), 1,23(m, 28H), 0.86(m, 3H)

N-Palmitoyl-GGGHGPLGLARK-NH_2: MS calc. M^+ = 1356.7 [m/z], obsvd. $(M+1)^+$ = 1356.1 [m/z]
^{1}H-NMR, (DMSO-d_6, 500MHz)δ 8.21(m, 1H), 7.06(s, 1H), 4.16(m, 1H), 4.04(m, 1H), 3.88-3.72(m, 13H), 3.57(m, 1H), 3.37-3.29(m, 7H), 3.08(m, 2H), 2.11(t, 2H), 1.91(m, 2H), 1.77(m, 1H), 1.63-1.47(m, 17H), 1.23(m, 24H), 0.86(m, 9H)

Results for the effect of the acyl chain length

We investigated three different types of acyl chains (myristoyl, palmitoyl and stearoyl peptides) and found that the palmitoyl peptide showed the highest gelation ability (Table S1). The balance of hydrophilicity and hydrophobicity in a peptide-amphiphile would play a critical role in gelation.

Table S1 Gelation properties of acylated GGGH having different fatty acids.

pH	Myristoyl-GGGH	Palmitoyl-GGGH	Stearoyl-GGGH
0	G (0.2)[b]	G (0.03)	PG
Deionized water	PG[c]	I[c]	VS
3.0	G (0.1)	I	I
7.5	PG	G (0.03)	G (0.2)
10.1	S	VS	VS

Solutions at various pH values were the same as those in Table 1.

Abbreviations are the same as those in Table 1.

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