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Peptide precursors that acquire denatured collagen-hybridizing ability by O-to-N acyl migration at physiological pH†

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Here, we report peptide probes with either single or cyclic double stranded collagen-like sequences that spontaneously acquire collagen-hybridizing ability at physiological pH. These peptides have ester bonds derived from O-acyl isopeptide units that are converted to amide bonds via intramolecular O-to-N acyl migration by a pH shift. The peptides that do not require pre-treatment for disassembly will be useful as prodrugs in theranostic treatments targeting unfolded collagen.

Collagen is the main component of the extracellular matrix and forms a triple-helical structure composed of polypeptides with Xaa-Yaa-Gly triplet repeats, in which the Xaa and Yaa positions are frequently occupied by Pro and 4-hydroxyproline (Hyp) residues, respectively. Denatured collagen with unfolded polypeptide chains has been reported to accumulate in several diseases, including cancer^{1,2} and osteoarthritis.³

Synthetic collagen-mimetic peptides (CMPs) consisting of Xaa-Yaa-Gly-repeating sequences can hybridize with denatured collagen by their triple helix-forming ability.⁴ CMPs have been used for *in vitro* imaging of articular tissue from osteoarthritis patients,⁵ and *in vivo* imaging of xenografted tumors in mice.^{6,7} Recently, we have developed cyclic CMPs (cCMPs), which are composed of two parallel CMPs by linking the N- and C-termini.⁸ The cCMPs showed binding affinity that was two orders of magnitude higher than the conventional single-stranded CMPs. However, CMPs, including cCMPs, have a common problem of self-assembly, which competes with the hybridization with denatured collagen (Fig. 1a). CMPs self-assemble at room temperature and need heating just before use for disassembly. To avoid this unfavorable self-assembly, a CMP harboring a photocleavable protecting group was developed. This CMP acquired binding activity after UV-induced de-

protection.⁶ However, pre-treatments are inconvenient for *in vivo* CMP administration and can interfere with quantitative analysis. Therefore, several strategies to suppress the self-assembly of CMPs have been reported. Introduction of a bulky fluorescent dye into the central region of a CMP suppressed the self-assembly.⁹ Replacing the Pro and Hyp residues in CMPs with (2*S*,4*S*)-4-fluoroproline and (2*S*,4*R*)-4-fluoroproline, respectively, resulted in suppression of the self-assembly.^{10,11} Recently, our group developed cCMPs whose self-assembling property was minimized by introducing charge repulsion and a deformation in the cCMPs' backbone structure.¹² Although such *ad hoc* strategies for suppressing unfavorable self-assembly worked well, the development of a more versatile strategy applicable to a variety of CMPs would be beneficial.

Herein, as a general approach for avoiding the self-assembly, we have developed CMP precursors that contain O-acyl isopeptide units (Fig. 1b). Under neutral or slightly basic pH, the ester bond in the O-acyl isopeptide unit is converted to an amide bond via O-to-N acyl migration.^{13,14} Previous reports have demonstrated that O-acyl isopeptide units could mask the aggregation properties of amyloid β peptide 1–42 and islet amyloid polypeptide through disrupting the secondary struc-

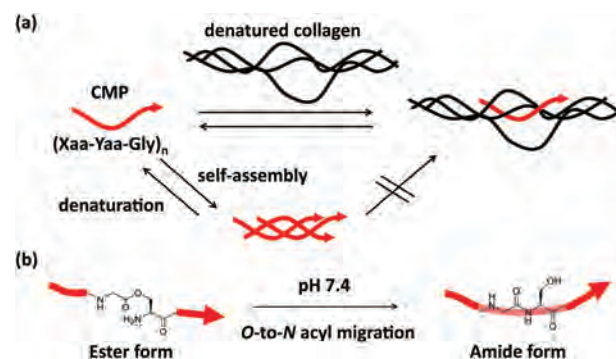


Fig. 1 Concept of this study. (a) Hybridization of CMPs with denatured collagen and self-assembly of the CMPs. (b) Conversion of the ester bond in an O-acyl isopeptide unit to an amide bond at slightly basic pH.

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ture formed by hydrogen-bond networks.^{15–18} The secondary structure folding was then recovered by *O*-to-*N* acyl migration. Thus, it was expected that the CMP precursors would not self-assemble under acidic conditions, while at physiological pH, the CMPs would spontaneously acquire collagen-binding ability by *O*-to-*N* acyl migration.

We first investigated *O*-to-*N* acyl migration of a short model peptide with Xaa-Yaa-Gly-repeating sequences (peptide **1**; Fig. 2a, S2 and Table S1†). The *O*-acyl isopeptide unit was introduced using Boc-Ser(Fmoc-Gly)-OH as a building block. Treatment of **1** with 0.1 M phosphate buffer (pH 7.4) shifted the retention time from that observed for **1** in acidic solution (Fig. 2b). Its retention time and *m/z* value were consistent with the corresponding amide form **2** (Fig. 2b and S3†). FT-IR analysis supported that **1** was converted to **2** by *O*-to-*N* acyl migration (Fig. S4†). **1** showed a stretching band around 1760 cm^{−1} which was assigned to an ester carbonyl group, while this band was disappeared for **2**.^{19,20} In addition, the structures of **1** and **2** were confirmed based on NMR spectrometry (Fig. S5–S18 and Tables S2, S3†). The HMQC analysis showed an upfield shift of H β of Ser from 4.62 and 4.41 ppm (**1**) to 3.82 ppm (**2**), which was consistent with the previous result obtained using a peptide containing a similar *O*-acyl isopeptide unit (Fig. S19†).²¹

Next, we designed and synthesized a CMP precursor with a longer peptide chain (peptide **3**; Table 1). The corresponding amide form **4** was also prepared independently (Table 1). By treatment with phosphate buffered saline (PBS) or fetal bovine

Table 1 Structures of single-stranded CMPs and precursors

R-(Pro-Hyp-Gly) ₃ -Pro-Hyp-X-Hyp-Gly-(Pro-Hyp-Gly) ₄ -Tyr-NH ₂		
Peptide	R	X
3	Ac	
4	Ac	
5	Biotin-(PEG) ₄	
6	Biotin-(PEG) ₄	

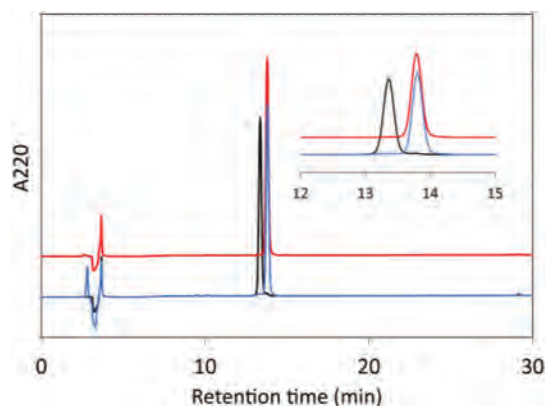


Fig. 3 HPLC profiles of compound **3** before (black) and after (blue) treatment with PBS at RT for 10 min; and compound **4** (red).

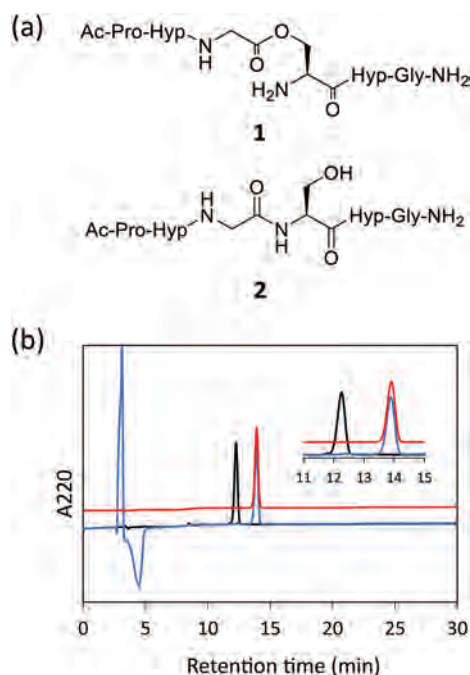


Fig. 2 Model experiment using short peptides with Xaa-Yaa-Gly-repeats. (a) Structures of **1** and **2**. (b) HPLC profiles of **1** before (black) and after (blue) treatment with 0.1 M phosphate buffer (pH 7.4) at RT; and **2** (red).

serum, **3** (ester) converted to the corresponding amide form which was identical to authentic **4** (Fig. 3, S20, S21 and Table S1†). This conversion was almost quantitatively processed within 10 min. FT-IR analysis showed the disappearance of a stretching band around 1755 cm^{−1} by the conversion (Fig. S22†). Similar results were also observed using a peptide **7** with a Gly-Thr isopeptide unit (Fig. S1, S23 and Table S1†).

We compared the conformational properties of **3** and **4** using circular dichroism (CD) spectroscopy. **3** and **4** showed negative CD signals around 200 nm and positive signals around 225 nm, indicating that they had a polyproline-II helix structure (Fig. 4a). Because the absolute values of $[0]_{200}$ and $[0]_{225}$ for **3** were less than those for **4**, the polyproline-II helix content of **4** was greater than that of **3**.²² The $[0]_{225}$ value of **4** cooperatively decreased with increasing temperature, while that of **3** linearly decreased (Fig. 4b). These results indicated that the polyproline-II helix of **3** was single-stranded²³ and that of **4** composed a triple-helix.²⁴ The melting temperature of the triple helix of **4** was estimated to be 55 °C (Fig. 4b).

We next analysed the kinetics of the *O*-to-*N* acyl migration of **3** and the triple-helix formation of **4**. The conversion was quantitatively processed within 2 min in phosphate buffer (pH

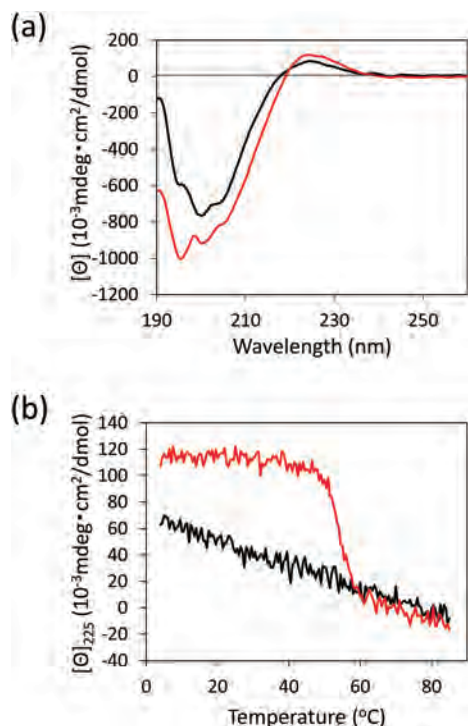


Fig. 4 CD profiles of 0.5 mg mL⁻¹ compounds **3** and **4**. (a) CD spectra recorded in 0.05% TFA/H₂O at 4 °C. (b) Thermal melting curves of the triple helices. The black and red lines represent compounds **3** and **4**, respectively.

7.4) at 37 °C (Fig. S24†). The conversion rates were calculated from the peak areas of **3** and **4** by RP-HPLC and plotted versus time (Fig. 5). The triple-helical folded fraction of **4** (1.0 mg mL⁻¹, 382 μM) was estimated from the [θ]₂₂₅ values in the CD

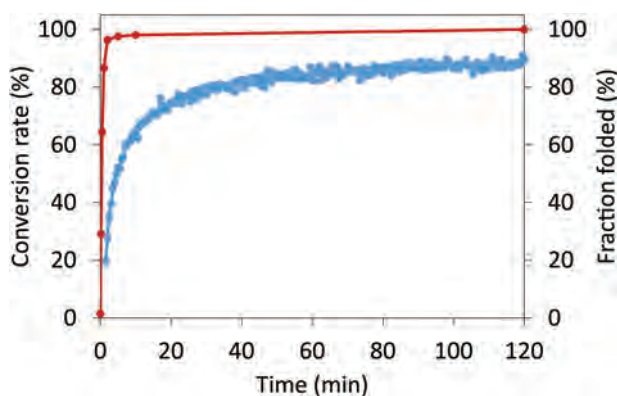


Fig. 5 Kinetics of *O*-to-*N* acyl migration and triple-helix formation. *O*-to-*N* acyl migration was performed in phosphate buffer (pH 7.4) at 37 °C. The conversion rate was estimated by the peak areas of compounds **3** and **4** by RP-HPLC. For estimating the kinetics of triple helix formation, compound **4** was preheated at 85 °C, followed by incubation at 37 °C and observation of the [θ]₂₂₅ in the CD spectrum. Complete folding (100%) was defined as the [θ]₂₂₅ value of compound **4** after annealing at 4 °C overnight. The red and blue lines represent the conversion rate and fraction folded, respectively.

spectra (Fig. 5). The plots for the conversion rate and triple-helical folded fraction were well fitted to first-²⁵ and third-order kinetics,²⁶ and the rate constants were estimated to be 2.0 min⁻¹ and 8.3 × 10⁵ M⁻² min⁻¹, respectively. The reported concentration of CMPs for *in vivo* use is approximately 40 μM which is lower than the concentration used in this measurement.⁶ Considering that there was sufficient time between converting and forming triple-helices even in this experiment (Fig. 5) and their kinetics orders, **3** could be quickly converted to **4** and exist as a monomer *in vivo* before hybridizing with denatured collagen.

We evaluated the binding of **3** and **4** to heat-denatured collagen I at 37 °C by an enzyme-linked immunosorbent assay (ELISA) with biotin-labeled peptides (the biotin-labeled derivatives of **3** and **4** were named as **5** and **6**, respectively; Table 1). The ester-type CMP **5** was dissolved in 1 mM AcOH/H₂O (pH 4.4). **5** did not exhibit binding activity under acidic conditions. In contrast, significant binding was observed after *in situ* conversion to **6** by shifting to pH 7.4, and the binding activity was almost the same as that of authentic compound **6** after disassembly by heating (Fig. 6 and S25†). These results demonstrated that the CMP precursor containing an *O*-acyl isopeptide unit allowed the detection of denatured collagen at physiological pH without pre-heating.

Our concept was further evaluated using cCMPs. We prepared the cCMP precursor **8** in which isopeptide units were introduced into both peptide chains (Table 2). The RP-HPLC retention time and *m/z* value of **8** after treatment with PBS were the same as those of **9**, which had been independently prepared (Tables 2, S1 and Fig. S26†). In FT-IR analysis, **8** showed a band of an ester carbonyl group, but this band disappeared for **9**. It was consistent with the result obtained using the single-stranded CMPs (Fig. S27†). The kinetics of conversion from **8** to **9** were almost the same as that of the single-stranded **3** (Fig. S28†). CD analysis indicated that **8** con-

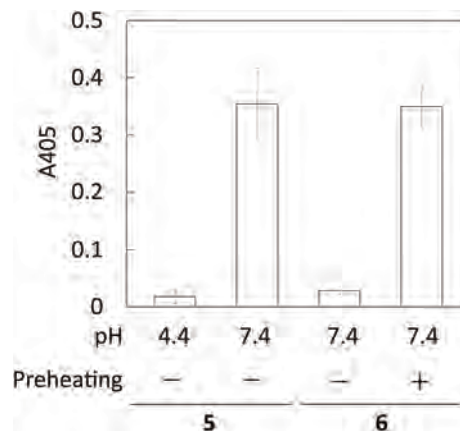
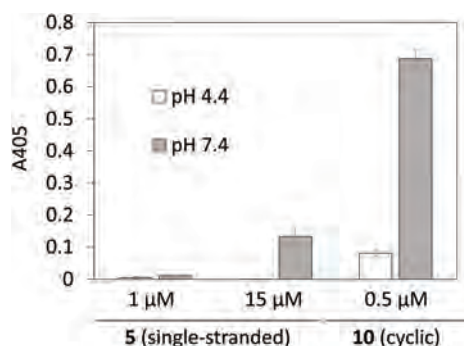


Fig. 6 Binding of CMP derivatives to denatured collagen determined by ELISA. Binding of 16 μM single-stranded CMP derivatives to heat-denatured collagen at 37 °C, mean ± SD (*n* = 3). ABTS is converted to its oxidized form by HRP in the presence of H₂O₂. The oxidized product showed absorption at 405 nm.

Table 2 Structures of cCMPs and precursors

Peptide	R	X
$\text{Ac-Cys-(Pro-Hyp-Gly)}_2\text{-Pro-Hyp-X-Hyp-Gly-(Pro-Hyp-Gly)}_4\text{-Lys-Tyr-Lys-NH}_2$		
8	H	
9	H	
10	Biotin-(PEG) ₄	

Fig. 7 Comparison of binding to denatured collagen between single-stranded and cyclic CMP derivatives at 37 °C, mean $\pm$ SD ($n = 3$).

tained a single-stranded polypyrrolone-II helix, and **9** had triple-helical forming activity (Fig. S29†). These conformational properties were similar to the properties of the respective single-stranded compounds.

We investigated the binding to denatured collagen using a biotin-labeled cCMP precursor (biotin-labeled **8** was named as compound **10**; Table 2). CMP precursors **5** and **10** were dissolved in 1 mM AcOH/H₂O. Compound **10** showed much higher activity at 0.5 μM at physiological pH, compared with the single-stranded counterpart **5** even at 15 μM (Fig. 7 and S30†). These results indicated that the cCMP precursors containing *O*-acyl isopeptide units are more promising probes for denatured collagen than the single-stranded analogs, in terms of binding-ability.

In conclusion, we have developed CMP precursors that are free from self-assembling properties. The introduction of an *O*-acyl isopeptide unit to the Pro-Hyp-Gly repeated sequences allowed these compounds to acquire hybridizing ability at physiological pH. The precursors were converted to the activated forms *via* *O*-to-*N* acyl migration at physiological pH. This strategy using cCMP precursors enabled us to detect denatured collagen more sensitively than the single-stranded counterparts, without pre-heating. An *in vivo* application study of the cCMP precursors for targeting denatured collagen is underway.

Conflicts of interest

There are no conflicts to declare.

Note added after first publication

This article replaces the version published on 25 Mar 2020, which contained errors in Table 1 and Table 2.

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