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Improvement of Temperature-responsive Drug Release from Collagen-mimic Dendrimers

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Gelatin, a thermally denatured collagen, forms a temperature-dependent gel, in which the efficiency of the collagen-like triple helix is dependent on temperature. In this study, (proline–hydroxyproline–glycine)$_n$ peptides were modified at the dendrimer to produce a type of collagen-mimic dendrimer. These dendrimers worked as a drug carrier with thermosensitive release capabilities and could retain rose bengal molecules stably.

Collagen is the most abundant protein in mammals and has been classically used as a biomaterial. For example, collagen gels are useful for long-term slow release drug delivery applications.[1] However, the release from collagen gels is generally uncontrollable, and thus functional collagen materials are desired. Thermally denatured collagen, commonly called gelatin, forms a gel on cooling and can be dissolved by heating.[2] Therefore, collagen (gelatin) materials are a potential temperature-dependent biomaterial. Collagen is mainly composed of glycine–proline–hydroxyproline (GPP or GPO) repeats that form a triple helix,[3] which is dependent on temperature. Because the efficiency of the triple helix formation depends on the peptide sequence and the peptide length,[3] design of a proper collagen model peptide is indispensable for temperature-dependent drug delivery.

Dendrimers have highly controllable size and surface properties, which have been used for drug and gene delivery.[4] Dendrimers also have multifunctional groups at the surface, which plays a role as a scaffold for functional molecules such as (antigen) peptides, imaging probes, and temperature-sensitive moieties.[4] Some collagen model peptides have also been conjugated to the dendrimer to produce artificial collagen materials.[5–9] We have designed a collagen-mimic dendrimer by combining collagen model peptides, PPG5, with a generation 4 (G4) polyamidoamine (PAMAM) dendrimer. The dendrimer formed the collagen-like triple helix, dependent on temperature. Interestingly, the temperature-dependent release of a model drug, rose bengal (RB), from the collagen-mimic dendrimer was shown. However, the dendrimer rapidly released RB at 37 °C[6] and, therefore, required improvement for applications. We have prepared various types of collagen-mimic dendrimer based on different generation dendrimers and different length PPG*n* peptides.[7,8] The dendrimer generation did not affect the efficiency of triple helix formation.[7] Although PPG10-den exhibited a thermally stable triple helical structure, it was aggregated at temperatures lower than 40 °C and thus was not suitable as a drug carrier.[8] More hydrophilic collagen model peptides, (Pro–Hyp–Gly)$_n$ (POG*n*), can be used to synthesize a novel type of collagen-mimic dendrimer. Previously, POG5-den and POG10-den were prepared and physicochemically characterized because POG5 and POG10 peptides are commercially available.[9] In this study, we synthesized various POG*n*-dens in addition to POG5-den and POG10-den. POG2 and POG8 peptides were synthesized through Fmoc-based solid-phase synthesis. These peptides were acetylated and attached to a generation 4 (G4) PAMAM dendrimer. The dendrimers were characterized. The higher order structures of POG*n*-attached dendrimers were estimated from circular dichroism (CD) spectrometry at different temperatures. Finally, a model drug, RB, was encapsulated in these dendrimers, and the release profiles were examined at different temperatures.

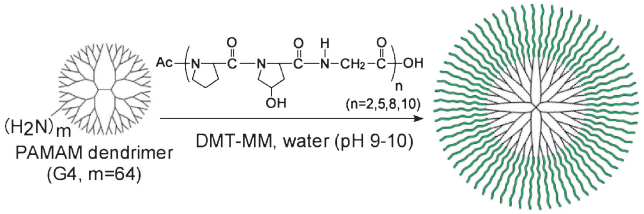

Figure 1. Synthetic pathway of POG*n*-den.

POG*n*-attached dendrimers were synthesized according to Figure 1. Synthesis of POG5-den and POG10-den was reported in our previous report.[9] Since POG2 and POG8 were not purchased, they were synthesized by Fmoc-based solid-state synthesis and acetylated in situ with acetic anhydride. Detailed experimental procedures were shown in the Supporting Information.[10] The purity was estimated by HPLC to be more than 90%. The subsequent conjugation to the dendrimer was performed using a water-soluble condensation agent, DMT-MM, under alkali conditions. Because it is reported that DMT-MM selectively forms amide bonds rather than ester bonds, unprotected peptides were used in the reaction. Even if an ester bond were to be formed, it would be degraded in the alkali solution. The synthesized dendrimers were characterized by ^{1}H NMR. Figure 2 indicates ^{1}H NMR spectra of POG2-den and POG8-den. The spectra contain signals derived from POG*n* (around 1.8–2.3 and 3.2–4.7 ppm) and the PAMAM dendrimer (around 2.4, 2.7, 2.9, and 3.3 ppm). The integral ratio of the bound peptide (around 2.0 ppm) to the dendrimer (2.9 ppm) indicates the number of bound POG*n* adducts, as described in our previous report.[9] The numbers of POG2 and POG8 bound were 67 and 63, respectively. Because the PAMAM dendrimer has 64 terminal amino groups, these indicate that essentially every amino group of the dendrimer was conjugated with the peptides. Therefore, POG2-den and POG8-den were modified with POG*n* peptides at essentially every terminal, like POG5-den and POG10-den. It was reported that POG*n* peptides form the triple helical structure, which affected the NMR signals.[11] The different patterns in the NMR spectra may come from the different efficiency of the triple helix formation.

Figure 3A shows the CD spectra of POG*n*-dens and the peptides themselves. PPG5-den is shown as a control.[10] The

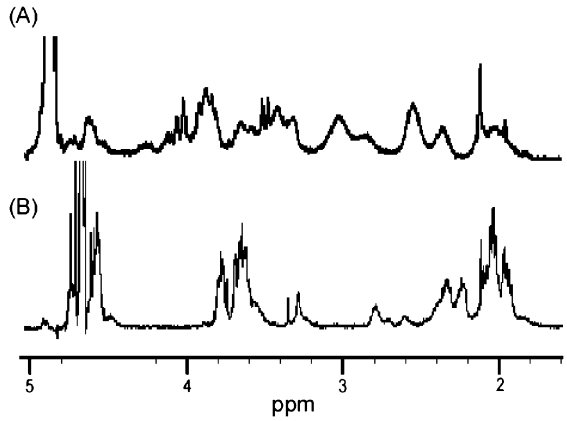

Figure 2. ^{1}H NMR spectra of POG2-den (A) in D_2O at room temperature and POG8-den (B) in D_2O containing NaOD at 50 °C.

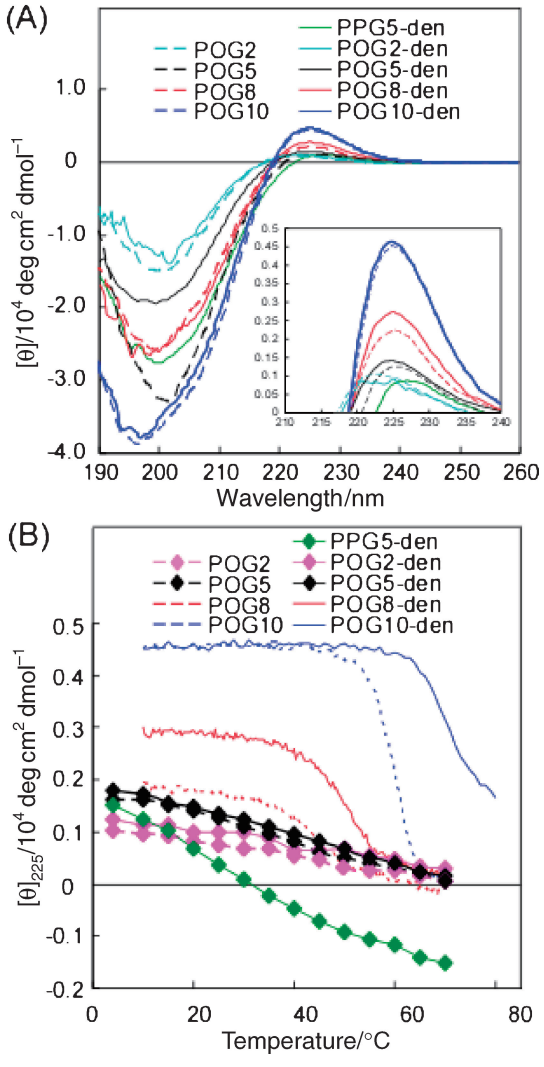

Figure 3. (A) CD spectra of POG*n*-dens (solid lines) and the POG*n* peptides (dotted lines). (B) Thermal denaturation of POG*n*-dens (solid lines) and the POG*n* peptides (dotted lines). PPG5-den is also shown as a control.[6]

Table 1. Parameters of POG*n*-den estimated by CD spectrometry

Sample	R_{pn}	T_m/°C
Collagen	0.11	42
PPG5-den	0.03	ND
POG2	0.07	ND
POG2-den	0.06	ND
POG5	0.05	ND
POG5-den	0.07	ND
POG8	0.08	44
POG8-den	0.10	50
POG10	0.12	60
POG10-den	0.12	ca. 75

positive cotton effect at 225 nm, which corresponds to a collagen-like triple helix, was observed in these spectra.[6–9] Larger peaks were observed for the longer peptides and the longer peptide-modified dendrimers, indicating that the longer peptides induced collagen-like triple helical formation efficiently. The ratio of positive and negative peaks (R_{pn}) is a standard measure of the efficiency of the collagen-like triple helical structure.[7–9] The R_{pn} of these collagen-mimic materials is listed in Table 1. A higher R_{pn} was obtained for the longer peptides and the dendrimers. In addition, the values for POG5-den and POG8-den were greater than for the peptides themselves, but the values for POG2-den and POG10-den were not. This suggests that the POG5 and POG8 peptides formed a collagen-like triple helical structure more efficiently at the surface of the dendrimer but that the POG2 and POG10 peptides did not. It is possible that a dendrimer clustering effect did not affect triple helix formation by POG2 and POG10 because the peptides were too short and too long, respectively.

The molar ellipticities at 225 nm of collagen decrease at high temperature to become gelatin. The temperature dependency of the collagen-mimic compounds is affected by the peptide length and the sequences.[3] We next investigated the thermal stability of the collagen-mimic dendrimer (Figure 3B).[10] Even though the molar ellipticities of POG2-den and POG5-den gradually decreased with increasing temperature, those of POG8-den, POG10-den, and the corresponding peptides markedly decreased. As described in our previous reports, PPG5-den showed a gradual decrease in the molar ellipticity at 225 nm by heating.[6] The thermal stability of PPG5-den was lower than that of the dendrimers modified with POG5, POG8, or POG10, as shown in Figure 3. The melting temperature (T_m) of these compounds are estimated,[12] listed in Table 1. The T_m became higher with increasing peptide length. In addition, the T_m of POG*n*-dens was higher than that of the POG*n* peptides. Altogether, longer peptide chains, hydroxyproline, and cluster effect may play an important role in stabilizing the triple helical structure.

Our previous work indicated that PPG5-den showed a temperature-dependent drug release and that the amount of triple helical structures may influence the drug release behavior.[6] It is expected that POG*n*-dens with more thermally stable triple helical structures would improve the controlled release properties. We investigated the release of a model drug, RB, from POG5-den and POG10-den at different temperatures in phos-

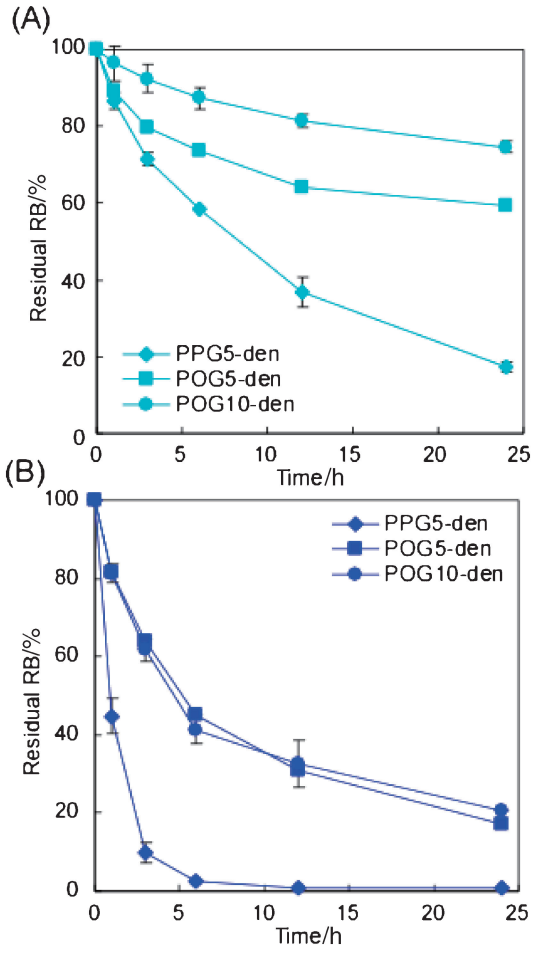

Figure 4. Release profiles of RB from POG5-den, POG10-den, and PPG5-den at 4 (A) and 37 °C (B) in PBS (2.5 μM dendrimer). The data for PPG5-den were sourced from ref 6.

phate buffer saline (PBS) (Figure 4).[10] Ten equivalents of RB were encapsulated in the collagen-mimic dendrimers. A dialysis release experiment was then performed to examine the release behaviors, following the method reported in our previous report. Because the dialysis membrane allows permeation of small drug molecules but not large dendrimers, the absorbance in the inner phase was monitored over 24 h.[6,13] POG5-den and POG10-den retained more RB at 4 °C than 37 °C. This indicates that both POG*n*-dens exhibited temperature-dependent drug release, similar to PPG5-den. The release of RB from both POG*n*-dens was suppressed even at 37 °C, compared to PPG5-den. This improvement may come from enhanced triple helix formation by the POG*n*-dens. However, the release profiles of POG5-den and POG10-den were similar at 37 °C, although the amount of the triple helical structure was quite different, as shown in Figure 3 and Table 1. This suggests that there are other factors that influence the release behavior from POG*n*-dens in addition to the efficiency of the triple helical structure. POG*n*-dens contain many hydroxy groups in the side chain of hydroxyproline. Hydrogen bonds from hydroxyproline may also contribute to drug loading. It is possible that POG5-den has sufficient hydrogen-bonding sites for drug encapsulation.

In conclusion, we synthesized and characterized a type of collagen-mimic dendrimer in which POG*n* fully modified the termini of the dendrimers. POG*n* induced a triple helix formation in the collagen-mimic dendrimers more efficiently, and longer peptides were particularly effective. The thermal stability of the triple helical structure was also improved by the attachment of longer POG*n* peptides to the dendrimer. The POG*n*-dens showed temperature-dependent drug release, like conventional PPG5-den, but the retention of the model drug in the POG*n*-den at 37 °C was much improved. Therefore, POG*n*-den is more suitable for controlled release applications. We also reported that PPG10-den and POG10-den formed temperature-dependent hydrogels in high concentration (15 wt %).[8,9] The former gel was dissolved at 45 °C by heating,[8] but the latter gel was formed at 40 °C by heating.[9] The POG10-den based hydrogels might be also useful for temperature-dependent drug delivery.

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