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The design, synthesis, and characterization of a PAMAM-based triple helical collagen mimetic dendrimer

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Abstract—The synthesis and characterization of a collagen mimetic dendrimer composed of the Gly-Pro-Nleu sequence is described. The dendrimer is built on a 'first generation' poly(amidoamine) core and is synthesized in 38% yield. This dendrimer exhibits a melting temperature of 25 °C, which is in between previously studied analogous molecules of identical sequence and length.

1. Introduction

Collagen is the most abundant protein in vertebrates comprising roughly one-third of all proteins in animals[1] and is an integral component of the extracellular matrix upon which tissues are built. The extracellular matrix promotes the differentiation and proliferation of many cell types. For example, fibroblasts within the dermis form a complex three-dimensional network while in tissue culture fibroblasts cease to proliferate once a two-dimensional monolayer is formed.[2] When collagen biosynthesis was stimulated in vitro by a vitamin C derivative the dermal fibroblasts formed a three-dimensional tissue similar to that of natural skin.[3] The biosynthesis of collagen and other extracellular matrix proteins is vital for the differentiation of cells in a developing fetus. The formation of cartilage is one of the earliest morphogenetic events in the development of an embryo. The ultimate transformation of this cartilage into skeletal tissue is brought about by the biosynthesis of various collagens. A signaling pathway is initiated that causes the vascularization of the cartilage. The resulting blood flow infuses osteoblasts into this site and the process of replacing the cartilage with mineralized bone begins.[4]

Collagen functions as the major load-bearing constituent of the connective tissues that comprise teeth, bone, skin, cartilage, and tendons among others. Much of collagen's high tensile strength is derived from its triple helical structure. Three polypeptide chains, each in a left-handed polyproline II type helix, coil around one another about a common axis to form a right-handed triple helix. The sterically hindered environment created by this triple helical conformation results in a repeating Gly-Xaa-Yaa sequence where Xaa and Yaa are frequently populated by imino acids.[5]

Collagen represents a nearly ideal target for the preparation of biomaterials due to its ubiquity, low cytotoxicity, low immunogenicity, and high durability. Natural collagen has been utilized for cartilage replacement, surgical sutures, hemostatic agents, and tissue replacements for blood vessels and valves to name a few applications.[6–9] However, there are problems associated with the use of natural collagen as a biomaterial. Many of the methods used to purify and sterilize natural collagen results in the disruption of its structural integrity. Also, the chemical methods utilized to crosslink the collagen may result in cytotoxicity upon implantation. Frequently, collagens are prepared from bovine sources, which can cause allergic and inflammatory responses.[10] If the collagen is obtained from a human cadaver, there are concerns of disease transmission. The issues stated above underscore the need for the preparation of artificial and synthetic collagen-based biomaterials.

Inspired by the enormous significance of collagen in nature and its remarkable biological and physiological properties, we sought to develop new artificial collagens. We hypothesized that such compounds could be tuned to have the desired biological properties and be devoid of any side effects related to the use of natural collagen. With this idea in mind, we developed a collagen–dendrimer conjugate research program. The use of dendrimers in biomaterial and therapeutic applications is becoming increasingly significant.[11–13] Dendrimers represent a relatively new class of polymer with a more defined and monodisperse molecular structure than their traditional linear polymeric analogues. Because dendrimers are considered to be spherical, they have a distinct periphery where a multitude of functional groups can be displayed. Researchers have derivatized these groups with various structures that drive the assembly of

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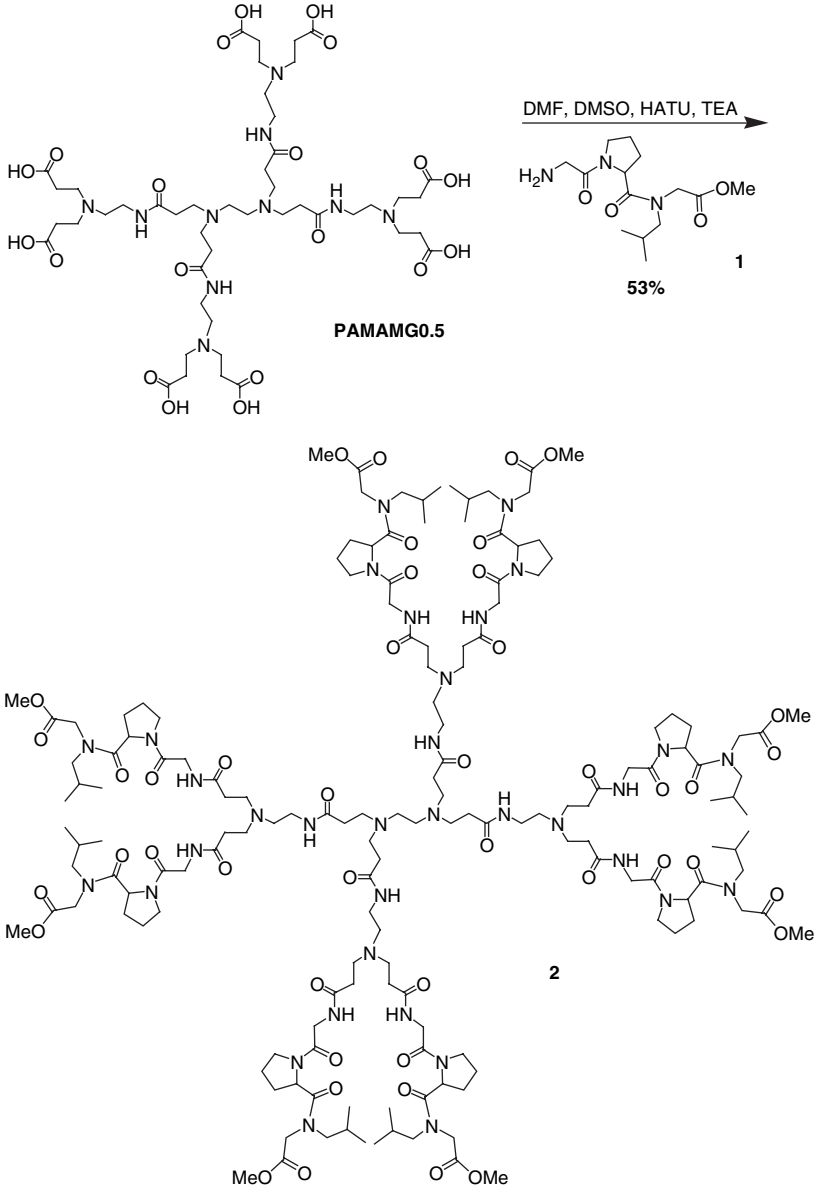
dendrimers to fashion nanostructures[14] and nanodevices[15] having applications as biomaterials.[16] For example, Higashi and Niwa utilized a third generation poly(amidoamine) (PAMAM) dendrimer to assemble synthetic α-helical peptides into a closely packed array. The densely packed protein-like environment induced by the dendritic structure resulted in a nearly 50% enhancement in α-helical character of the peptides. It is known that collagen triple helices associate with one another to form collagen fibrils. Therefore, we chose to use Higashi's approach in our collagen mimetics program. In this prototype the PAMAM dendrimer will act not only as a scaffold to promote intramolecular triple helical formation but also may act as a nucleation source for the interaction of intramolecular triple helical arrays.

PAMAM dendrimers represent the first dendrimer family to be made commercially available. Consequently, PAMAM dendrimers are widely utilized in the biomedical field. Applications range from immunodiagnostics,[17,18] gene transfection,[19–21] drug delivery,[18,22–24] and magnetic resonance imaging contrast agents.[25–28]

Previously we reported the synthesis of collagen mimetic dendrimers based on a trimesic acid (TMA) core structure and a tris-based scaffold.[29] In this publication we describe the preparation and characterization of a collagen mimetic dendrimer based on a PAMAM dendrimer core. The collagen–PAMAM conjugates were synthesized to prepare collagen mimetic dendrimers with increased functionality with respect to the TMA-based structures and to study the effect of different dendritic architecture on collagen mimetic melting transitions.

2. Results and discussion

The synthesis of PAMAM–collagen conjugate structures was undertaken by utilizing the first generation PAMAM

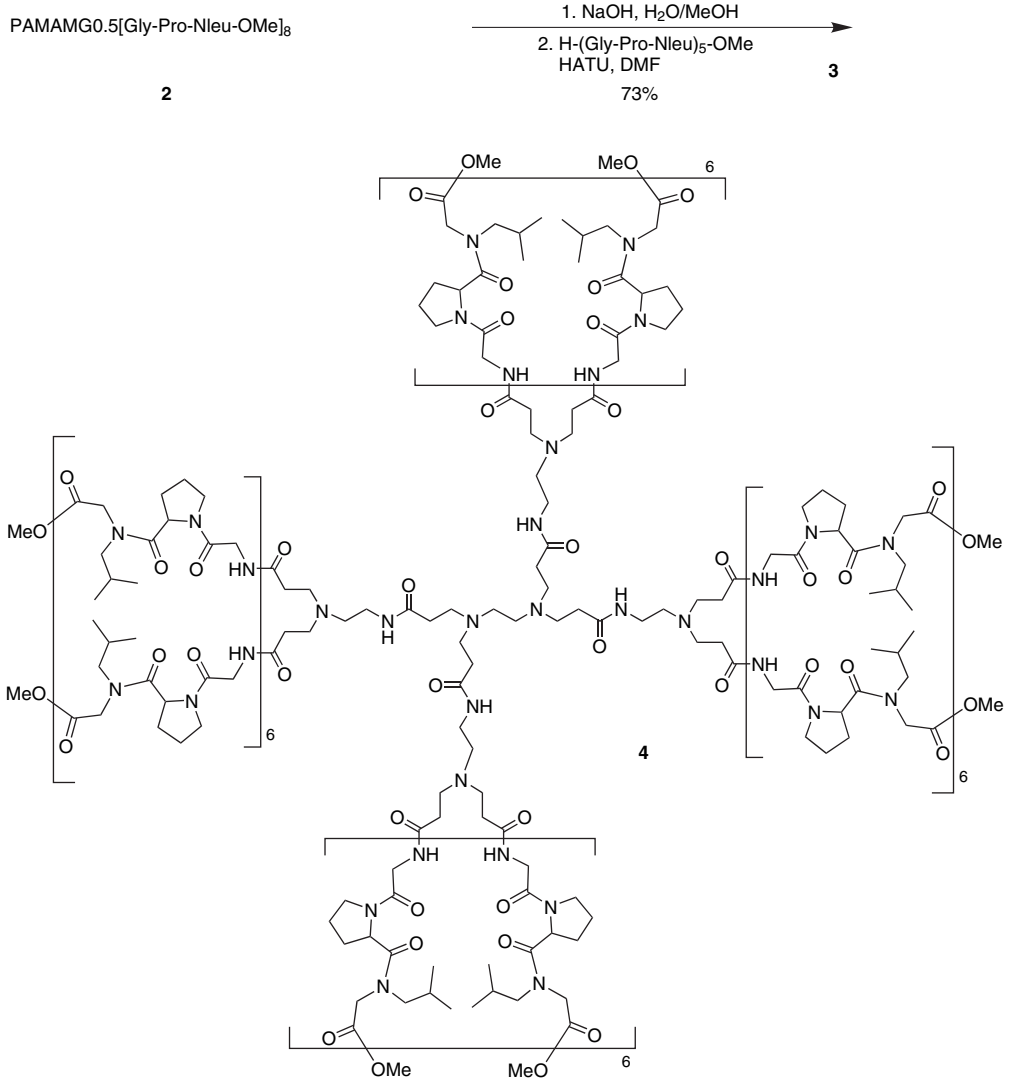


Scheme 1.

dendrimer containing eight sodium carboxylate groups (PAMAMG0.5) as shown in Scheme 1. The PAMAMG0.5 dendrimer was converted to the free acid by the addition of TFA in methanol. The salts were removed by GPC and the solvent was removed under reduced pressure. The reaction was then carried out according to Scheme 1 where the PAMAMG0.5 dendrimer was allowed to react with an excess of H-Gly-Pro-Nleu-OMe (**1**) in the presence of HATU. The product, PAMAMG0.5[Gly-Pro-Nleu-OMe]$_8$ (**2**), was obtained after purification by anion exchange chromatography in the carbonate form and gel permeation chromatography (GPC) in a yield of 53%. The product was characterized by analytical HPLC and mass spectrometry.

The PAMAM–collagen conjugate composed of eight discrete tripeptide units of Gly-Pro-Nleu (PAMAMG0.5[Gly-Pro-Nleu-OMe]$_8$) (**2**) was allowed to react with aqueous sodium hydroxide in methanol (Scheme 2). The reaction was followed by analytical C4 RP-HPLC. The reaction mixture was acidified to pH 0 and the sodium salts were removed by GPC. The conjugate, which contained eight carboxylic acids, obtained in 95% yield, was allowed to react with the free amine pentadecapeptide ester H-(Gly-Pro-Nleu)$_5$-OMe in the presence of the coupling reagent HATU as shown in Scheme 2. The product (**4**) was purified by anion exchange and GPC as describe above and isolated in 77% yield. The core PAMAM dendrimer structure possesses six tertiary amines. This resulted in facile ionization and characterization of the desired product by mass spectrometry. The desired product was identified by analytical HPLC and MALDI-TOF mass spectrometry.

The characterization of the triple helical structure of the PAMAM–collagen mimetic conjugate consisted of circular dichroism (CD) spectroscopy and thermal denaturation monitored by optical rotation. Triple helical collagen-like structures exhibit a unique CD spectrum consisting of a minimum, crossover, and maximum around 197, 213, and 220 nm, respectively.[30,31] When the triple helical structure of collagen mimetics composed of the Gly-Pro-Nleu sequence is denatured, a characteristic blue shift is observed in the above spectral positions. In addition, when observed by temperature dependent optical rotation, the disruption of triple helicity is coincident with a cooperative melting



Scheme 2.

transition. The results of the biophysical characterization for the triple helical PAMAM–collagen chimera are shown in Figures 2 and 3. For comparison, Figures 2 and 3 also display the characterization of the TMA dendrimer and its constituents, which was reported previously (Fig. 1).[29] The molecules studied were incubated in H_2O (0.1–0.2 mg/mL) at 4 °C for 1–7 days prior to characterization. The scaffold-assembled and dendritic collagen structures were incubated for 24 h prior to analysis because the intramolecular association is rapid, while the single chain collagen mimetic (**5**) was incubated for 7 days at high concentration (10 mg/mL) to facilitate intermolecular strand association.[32] This solution was diluted to 0.2 mg/mL 1 day prior to analysis. Figure 2A and B show the CD spectra of compounds **4**–**7** at 8 and 22 °C, respectively. At 8 °C the single chain collagen structure **5** is not triple helical as shown by its blue shifted positive peak at 215 nm. The other three mimetics are triple helical and exhibit nearly superimposable CD spectra with maximum, crossover, and minimum near 220, 213, and 199 nm, respectively. When the spectra are reacquired at 22 °C (Fig. 2B) the single chain **5** remains non-triple helical as expected but the tris-assembled collagen mimetic **6** is nearly completely denatured. The PAMAM (**4**) and TMA (**7**) collagen mimetic dendrimers remain triple helical in structure. While the tris-assembled structure clearly possesses a melting temperature (T_m) below room temperature, an aspect of the dendritic architecture of compounds **4** and **7** impart an added thermal stability which allows for triple helical structure above room temperature.

Optical rotation measurements of compounds **4** through **7** are shown in Figure 3. The single chain compound (**5**) shows no melting transition over the temperature range tested and is therefore not triple helical in H_2O. The tris-assembled mimetic (**6**) has a T_m of 19 °C, thus explaining the denatured CD spectrum of this molecule at 22 °C. Melting transitions for the PAMAM (**4**) and TMA (**7**) collagen mimetic dendrimers are shown in Figure 3 with T_m of 25 and 28 °C,

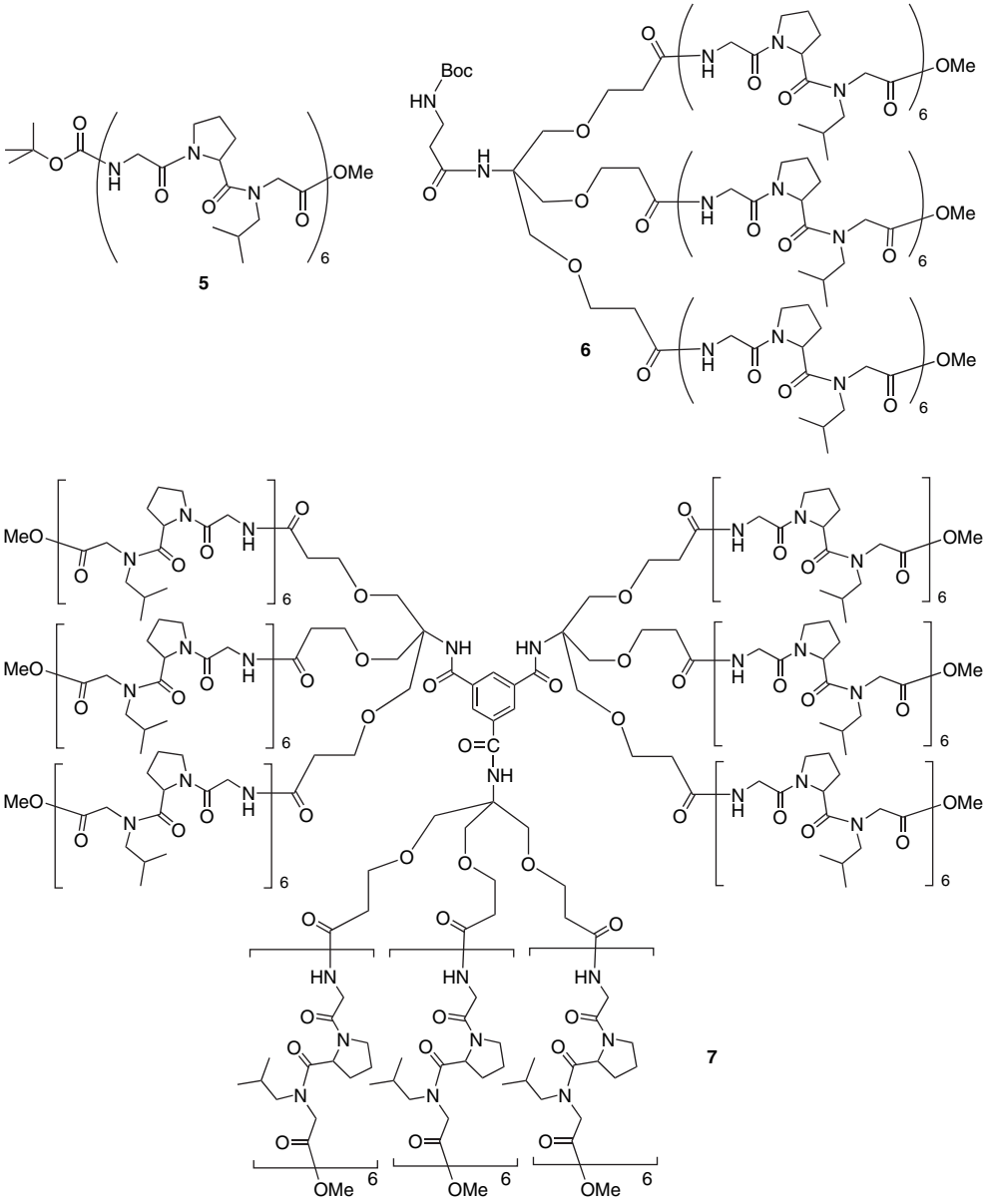


Figure 1. Boc-(Gly-Pro-Nleu)$_6$-OMe (**5**), Boc-βAla-tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$ (**6**), and TMA[tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$]$_3$ (**7**).

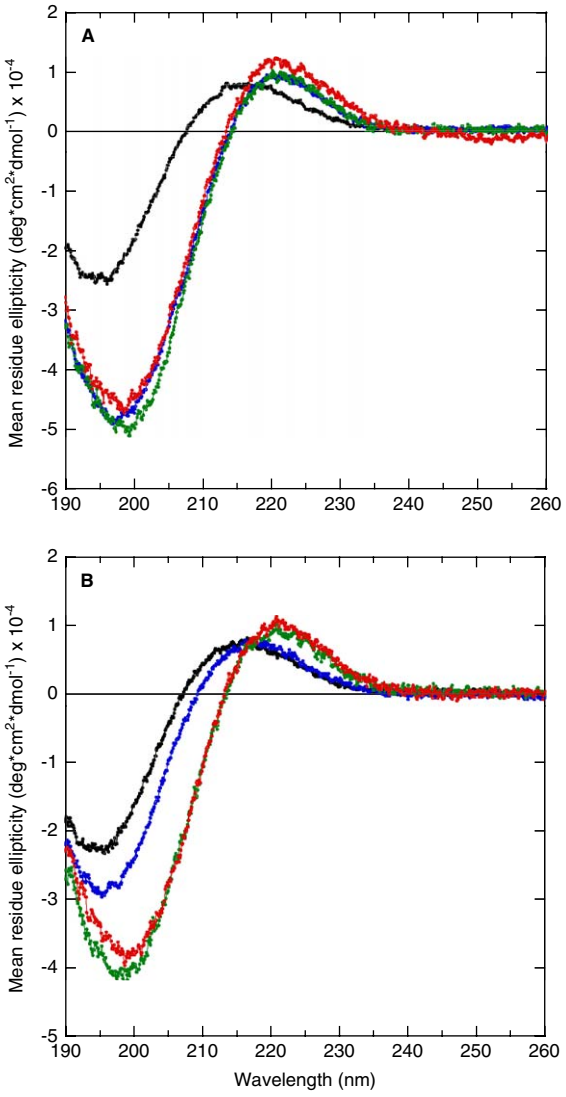


Figure 2. CD spectra at 8 °C (A) and 22 °C (B) of Boc-(Gly-Pro-Nleu)$_6$-OMe (*black*, 0.2 mg/mL), Boc-βAla-tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$ (*blue*, 0.2 mg/mL), TMA[tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$]$_3$ (*green*, 0.1 mg/mL), and PAMAMG0.5[(Gly-Pro-Nleu)$_6$-OMe]$_8$ (*red*, 0.1 mg/mL). Measurements were carried out in H_2O.

respectively. Results obtained from the CD studies and the optical rotation measurements are consistent with each other.

3. Conclusion

It is interesting to note that the T_m of the PAMAM–collagen conjugate PAMAMG0.5[(Gly-Pro-Nleu)$_6$-OMe]$_8$, at 25 °C, is between that of the tris-assembled structure Boc-β-Ala-tris[(Gly-Pro-Nleu)$_6$-OMe], at 19 °C, and the TMA dendrimer TMA[tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$]$_3$, at 28 °C. In a previous publication we asserted that the TMA dendrimer (**7**) possesses a higher melting temperature due to an intramolecular clustering of triple helices.[29] This results in a packed structure that excludes solvent from the interior of the dendrimer and imbues a higher triple helical thermal stability. The clustered ensemble responsible for the enhanced thermal stability of the triple helices of the TMA

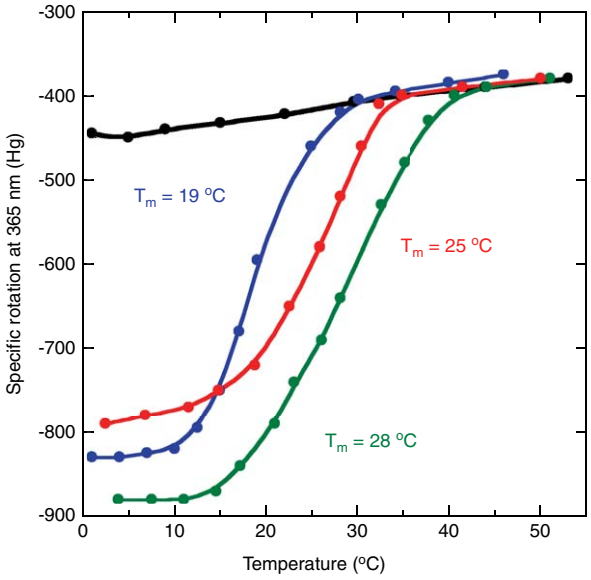


Figure 3. Thermal denaturation monitored by optical rotation of Boc-(Gly-Pro-Nleu)$_6$-OMe (*black*, 0.2 mg/mL), Boc-βAla-tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$ (*blue*, 0.2 mg/mL), TMA[tris[(Gly-Pro-Nleu)$_6$-OMe]$_3$]$_3$ (*green*, 0.1 mg/mL), and PAMAMG0.5[(Gly-Pro-Nleu)$_6$-OMe]$_8$ (*red*, 0.1 mg/mL). Measurements were carried out in H_2O.

dendrimer also may be present in the PAMAM–collagen conjugate. However, the conjugate structure has eight peptidomimetic chains and can form only two complete triple helical arrays. This results in a different intramolecular structure that less effectively shields the triple helices from solvent invasion, hence a lower melting temperature relative to the TMA-assembled dendrimer. It appears that the thermal stability of collagen mimetic dendrimers can be attenuated by inhibiting the interaction between the triple helical arrays or by decreasing the number of triple helical arrays that associate with each other.

PAMAM dendrimers have been shown to complex a variety of transition metals, particularly Cu^{2+} and Ni^{2+}, by the donation of amine and amide electrons.[33–35] Copper(II) is an important enzyme cofactor in lysyl oxidase, an enzyme that carries out the oxidation of lysine and hydroxylysine to the corresponding aldehyde for the crosslinking of fibril collagens. This crosslinking is vital for the structural integrity of connective tissues.[36] In addition, many biological events involving collagen such as wound healing and bone mineralization are facilitated by metal cofactors.[37,38] Therefore, the synthesis of collagen–PAMAM conjugates and their subsequent complexation with biologically relevant metals may lead to compounds with novel biological properties.

4. Experimental

4.1. General

All amino acids used were of L-configuration unless otherwise specified. Protected amino acids were purchased from Novabiochem. The chemicals HOAt and HATU were purchased from Perseptive Biosystems. Reagent grade and HPLC-grade solvents were purchased from Fisher Scientific with DCM, THF, and TEA being distilled before use when

appropriate. All other reagents were purchased either from Aldrich or Acros.

Reactions carried out in solution were monitored by HPLC. Both preparatory and analytic HPLC were carried out on two instruments. One was a Waters (two Waters 510 pumps and 2487 Dual λ Absorbance detector) system. The other was a Waters Millennium 2010 system (715 Ultra WISP sample processor, 996 photodiode array detector, and two Waters 510 pumps) with a NexStar PC compatible computer interface. The solvents used in HPLC were (A) water with 0.1% TFA and (B) acetonitrile with 0.1% TFA. The flow rate for preparatory purification was 10 mL/min (Vydac, C-18 and C-4, 25×2.2 cm) and 1 mL/min (Vydac, C-18 and C-4, 25×0.46 cm).

Circular dichroism (CD) measurements were carried out on a Cary 61 spectropolarimeter, which was modified by replacing the original Pockel cell with a 50 kHz photoelastic modulator (Hinds International FS-5/PEM-80). The original Cary linear polarizer was replaced with a MgF_2 linear polarizer supplied by AVIV Inc. An EGG Princeton Applied Research model 128A lock in amplifier was used to integrate the phase-detected output of the original end-on photomultiplier tube and preamp. System automation, multiple scan signal averaging, and base line subtraction were accomplished with an AT286 PC interfaced directly to both the Cary 61 and the 128A amplifier. The system software and custom hardware interfaces were designed by Allen Micro-Computer Services Inc. and the UC San Diego, Department of Chemistry and Biochemistry Computer Facility. The CD spectra were obtained using a 0.02–0.5 cm path length cell by signal averaging 10 scans from 190 to 300 nm at a scan speed of 0.8 nm/s.

Optical rotations were measured with a Perkin–Elmer 241 Polarimeter equipped with a Model 900 isotemp refrigerator circulator (Fisher Scientific). Data were collected at 365 nm (Hg). The solutions were stored at 4 °C for at least 24 h for equilibration of triple helix formation. Before recording an optical rotation, the sample was equilibrated 30 min at the initial temperature. At each subsequent temperature point, the samples were allowed to equilibrate for 30 min.

Mass spectra were obtained at UC San Diego or the Scripps Research Institute. Fast atom bombardment (FAB), electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) methods were used to determine product mass.

4.2. Synthesis

4.2.1. PAMAMG0.5[Gly-Pro-Nleu-OMe]$_8$ (2). The PAMAMG0.5 sodium salt starting material was converted to the free acid using TFA. The inorganic salts were removed by GPC. The PAMAMG0.5 free acid was concentrated and used in the coupling reaction. To a solution of PAMAMG0.5 (43.5 mg, 0.0343 mmol), H-Gly-Pro-Nleu-OMe (**1**) (138 mg, 0.411 mmol), and HOAt (23.3 mg, 0.171 mmol), stirred under Ar gas in a solution of DMSO/DMF/DCM (3:3:2, 800 μL) was added HATU (130.3 mg, 0.3428 mmol). After 2 min TEA (142 mg, 1.405 mmol) was added and the reaction was allowed to continue for 12 h. The solvents were removed under reduced pressure and the resulting residue was dissolved in MeOH (3 mL) and passed through an anion exchange column. This solution was then passed through a gel permeation column (Sephadex LH-20) twice to effect purification. The fractions containing the desired product were combined and the solvent was removed under reduced pressure. The product was obtained as a white solid (61 mg, 53%). MS-ESI (*m*/*z*) calculated for $C_{158}H_{264}N_{34}O_{44}$ 3342.0, found 1115.9 $[M+3H]^{3+}$, reconstruction 3345.9 [M+3H]. Analytical C4 RP-HPLC t_R=20.85 min (20–80% solvent B over 30 min).

4.2.2. PAMAMG0.5[Gly-Pro-Nleu-OH]$_8$. To a solution of **2** in MeOH (0.2 mL) was added NaOH (2.75 M, 0.2 mL) solution in water. The reaction was allowed to stir for 1 h. The reaction mixture was acidified to pH 0 by using 2 N HCl. The solution was passed down a GPC column (LH-20). The fractions containing the product were concentrated to obtain a white solid (60 mg, 95%). This compound was used without further purification or characterization.

4.2.3. PAMAMG0.5[(Gly-Pro-Nleu)$_6$-OMe]$_8$ (4). To a solution of PAMAMG0.5[Gly-Pro-Nleu-OH]$_8$ (60 mg, 17.4 μmol), **3** (293 mg, 209 μmol), and HOAt (19 mg, 139 μmol), stirred under Ar gas in DMF (800 μL) at 50 °C was added HATU (66 mg, 174 μmol). After 2 min TEA (77 mg, 765 μmol) was added and the reaction was allowed to continue for 12 h. The solvents were removed under reduced pressure and the resulting residue was dissolved in MeOH (3 mL) and passed through an anion exchange column. The remaining material was passed down a gel permeation column (Sephadex LH-60) twice to effect purification. The fractions containing the desired product were combined and the solvent was removed under reduced pressure. The product was obtained as a white solid (187 mg, 77%). MALDI-TOF (*m*/*z*) calculated for $C_{678}H_{1104}N_{154}O_{164}K$ 14,076, found 14,077 $[M+K]^+$. Analytical C4 RP-HPLC t_R=26.0 min (nontriple helical peak) and 27.4 min (triple helical peak) (20–90% solvent B over 35 min).

The syntheses of compounds **1**, **3**, **5**, **6**, and **7** have been previously reported.[29]

Acknowledgments

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