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Morphological transformations in a dually thermoresponsive coil–rod–coil bioconjugate†

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Abstract

We report the conformational and assembly behavior of a thermoresponsive triblock biohybrid conjugate under aqueous conditions. The triblock comprises of poly(diethylene glycol methyl ether methacrylate) (PDEGMEMA) conjugated to the ends of a triple-helix forming collagen-like peptide. The circular dichroism (CD) experiment confirms the ability of the collagen-like peptide middle block to assemble as a triple helix in the hybrid conjugate. Above the LCST (~35 °C), the collapse of the thermoresponsive PDEGMEMA polymer at the ends of the peptide domain resulted in a concomitant increase in the conformational stability of the peptide domain towards thermal denaturation. Upon cooling back, the kinetic conformational refolding behavior was still observed for the peptide domain in the hybrid conjugate. Static light scattering (SLS) experiments suggested the formation of supramolecular structures upon increasing solution temperatures to above the LCST. The scattering intensity increased with increasing temperature, until at 75 °C then it was found to decrease. Cryogenic scanning electron microscopy and regular transmission electron microscopy suggested the formation of spherical aggregates that increased in size with increasing temperature up to 65 °C and a morphological transformation into fibrils was also observed at 75 °C. The synergistic effect of dual thermoresponsive behavior from the peptide and the polymer block in the triblock hybrid is suggested for the observed conformational and assembly behaviors.

1. Introduction

The self-assembly of amphiphilic block copolymers offers a very useful tool to create nanostructured materials that have found applications in electronic devices,^{1–4} drug delivery and healthcare.^{5–8} The compositional asymmetry and physiochemical disparity of the covalently linked domains in the block copolymer architecture have been utilized to generate various self-assembled morphologies such as rods, micelles, vesicles or polymersomes and other larger aggregates and interconnected networks.^{5,6} The minimization of the interaction of the solvent molecule with the solvophobic domain often

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guides the assembly. However, external stimuli, commonly pH and temperature,^{7–15} have also been successfully applied to tune the assembly. Several interesting coil–coil as well as rod–coil block copolymer designs have previously been studied.^{16,17} A plethora of possibilities to create new architectures of self-assembling materials has continually progressed our understanding of the assembly mechanisms.

With the recent surge in biohybrid designs containing peptide domains conjugated to synthetic polymers, novel assemblies and network structures have been made possible *via* utilization of the secondary or tertiary conformation of the peptide domain.^{18–21} Thus hierarchically nanostructured bioactive assemblies are now possible. To this end, polymer conjugates with α -helical coiled-coil peptide domains,^{22–25} β -sheet peptide motifs,^{26–31} or peptide motifs derived from structural proteins like elastin^{32,33} have been widely employed. Various polymers, mainly PEG, along with other polymers such as poly(*n*-butyl acrylate), polyisoprene, polybutadiene, and polystyrene, have been used to develop peptide–polymer conjugates.

In this study we have used a thermoresponsive polymer, poly(diethylene glycol methyl ether methacrylate) (PDEGMEMA) conjugated to a triple-helix forming collagen-like peptide, to create a new triblock biohybrid system—PDEGMEMA(coil)–collagen–peptide(rod)–PDEGMEMA(coil),³⁴ as shown in Scheme 1. We anticipated the versatile assembly of such conjugates, owing to the dual thermoresponsiveness resulting from both the polymer and the peptide domains. Although various peptide–polymer hybrid triblock systems^{35–39} have been previously reported, there are several new aspects of the biohybrid design employed in the present study relative to the previous systems. The conjugation of the thermoresponsive polymer to the peptide domain in this study offers temperature as an external stimulus to sequentially modulate the aqueous phase assembly of the hybrids in the absence of any organic solvent. The first level of assembly is achieved *via* association of the collagen peptide to bring together the three triblock chains as a triple-helical unit (Scheme 1). The next level of assembly is achieved by the collapse of the terminal polymer chain ends upon increasing the temperature above the LCST, which we postulated would lead to higher order assembly that could show further morphological transformation with increasing temperatures owing to the thermal unfolding of the peptide domain. In this work we report the synergistic effects, of the two mutually exclusive temperature-dependent transitions from the polymer and the peptide domain, on the conformational behavior of the peptide and the assembly of the conjugate.

2. Experimental

2.1 Triblock synthesis

The synthesis of this triblock hybrid was performed *via* the reaction of a telechelic amine-functionalized collagen-like peptide (containing orthogonally protected side chains) with activated ester-end functionalized PDEGMEMA in DMF at 35 °C in the presence of triethylamine.³⁴ The activated ester end-functionalized PDEGMEMA was synthesized *via* RAFT polymerization described earlier⁴⁰ by utilizing pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) as a functional chain transfer agent. The characterization details of the triblock synthesis can be found in our previous report.³⁴

2.2 Circular dichroic (CD) spectroscopy

Characterization of the secondary structure was conducted *via* circular dichroic spectroscopy (Jasco 810 circular dichroism spectropolarimeter, Jasco, Inc., Easton, MD, USA). First, the triblock hybrid (1 mM) was dissolved in PBS (1 mM, pH 7.4) and incubated at 4 °C for at least overnight to allow triple helix formation. The solution was then stirred at least

overnight at 4 °C open to air, to allow oxidation of the thiol groups on the cysteine residue of the peptide domain to form disulfide bonds and covalently cross-link the trimeric form of the peptide domain.⁴¹ Then a solution of the triblock hybrid (100 μ M) was analyzed *via* CD; the CD spectra were recorded using quartz cells with a 0.1 cm optical path length. The spectra reported are averages of three scans. The scanning rate was 50 nm min⁻¹, with a response time of 4 s. The wavelength scans were obtained from 200 to 250 nm and were recorded every 1 nm with a 1 nm bandwidth. Data points for the thermal unfolding experiments (from 10 °C to 80 °C) were recorded at 227 nm at a heating rate of 10 °C h⁻¹ with an equilibration time of 3 min.

For kinetic refolding studies, temperature-jump experiments were performed. The 100 μ M triblock solution was heated at 75 °C for 30 minutes followed by quenching to 10 °C. (The dead time for the temperature jump was 30 seconds.) The ellipticity at 227 nm as a function of time was then monitored at 10 °C after the temperature jump.

2.3 Static light scattering (SLS)

The change in the intensity of scattering for the triblock hybrid as a function of temperature (from 10 °C to 75 °C) was measured *via* laser light scattering (Brookhaven Instruments, Holtsville, NY) with a laser wavelength of 532 nm and at a 90° scattering angle. The sample was filtered (at 4 °C using 0.45 μ m filter) and a 500 μ L of the filtered sample was placed into a 1.2 cm outer diameter glass test tube for measurements.

2.4 Confocal microscopy

A Zeiss 5 Live Duo Laser Scanning Microscope (Carl Zeiss Inc., Thornwood, New York) was employed to capture the evolution of the assembly of the triblocks in phase contrast mode. The sample (75 μ l) was placed on the top of a glass slide enclosed within a metallic holder and the temperature was controlled using a single channel CL-100 bipolar temperature controller.

2.5 Cryogenic scanning electron microscopy (Cryo-SEM)

Microstructural morphology was characterized by cryo-SEM, using a Hitachi 4700 FE-SEM at an accelerating voltage of 1–3 keV. The sample was heated at 37 °C, 65 °C or 75 °C in Eppendorf tubes in a sand incubator and then transferred to a preheated (at the sample temperature) brass freezer hat (1500 μ m deep) which was then capped to enclose the sample. This process was carefully conducted on a hot plate at the desired temperature, using tweezers incubated at the desired temperature, and in a styrofoam-enclosed setup to minimize any heat loss. The temperature was maintained at the desired value and monitored *via* a thermometer placed on the hot plate. The samples contained in the brass hats were subsequently vitrified in a Leica high pressure freezer (Em PACT) under a stream of liquid nitrogen delivered at >2000 bar over at greater than 10 000 deg s⁻¹. This process vitrifies water without crystallization. The frozen samples were fractured in liquid nitrogen and the exposed surfaces were sublimated at -90 °C to remove water. The etched samples were subsequently coated with approximately 2.5 nm of Au/Pd at -125 °C to minimize charging.

2.6 Transmission electron microscopy (TEM)

Samples for TEM were prepared on a carbon-coated copper grid. The grid was preheated on a hot plate at the desired temperature (37 °C, 65 °C, 75 °C). The sample heated at the desired temperature was drop cast (~8 μ l) on the grid placed on the hot plate and blotted after 20 seconds. For staining, 1% phosphotungstic acid (PTA) as a negative stain was used. Approximately 5 μ l of the PTA solution was drop cast on the grid and blotted after 10 seconds. An additional 8 μ l of doubly distilled water was used to wash the stain, and the

grid was then immediately blotted for 20 seconds. Some non-stained samples were also prepared. The sample was allowed to dry at the desired temperature for 10 minutes and then was air-dried overnight. The temperature of the hot plate was maintained at the desired temperature and monitored as above. TEM images were taken on a Tecnai G2 12 TEM (FEI Company, Hillsboro, OR) at an acceleration voltage of 120 keV.

3. Results and discussion

We have previously reported the synthesis of this hybrid triblock polymer, which was achieved *via* the reaction of a telechelic amine-functionalized collagen-like peptide with a semitelechelic activated ester-functionalized PDEGMEMA in DMF at 35 °C in the presence of triethylamine.³⁴ The activated ester functionalized PDEGMEMA was synthesized as described earlier⁴⁰ *via* RAFT polymerization and utilizing pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) as a functional chain transfer agent. The triblock hybrid was dissolved in PBS (1 mM) and incubated at 4 °C for at least overnight to allow triple helix formation. Subsequently, the thiol group from the C-terminal cysteines on the peptide chain was air oxidized to covalently link the trimer, which imparts additional thermal stability to the triple helical conformation of the peptide domain.⁴¹

CD performed on the triblock (100 μ M in PBS, pH 7.4) shows a local positive maximum around 227 nm (Fig. 1a), indicating the formation of the triple helix. This peak vanishes at 75 °C, indicating the unfolding of the triple helical conformation to random coil. These results suggest that the polymer-conjugated peptide domain is able to assemble into the native collagen triple helical conformation, even when conjugated at both of its termini with a synthetic polymer. The thermal stability of the triple helical domain within the triblock hybrid was monitored as a function of temperature. The thermal stability was monitored *via* the observed decrease in the mean residue ellipticity ($[\theta]_{\text{MRE}}$) at 227 nm with an increase in temperature. Data were plotted as the fraction folded (relative to the ellipticity measured at low temperature) as a function of temperature (Fig. 1b) and compared to data for only the peptide. The results indicate that at ~35 °C, the triblock unfolding profile deviates from the sigmoidal unfolding observed for the unconjugated peptide, with gradual unfolding until the entire triple helical conformation is fully converted to random coil. The LCST of the hybrid, determined *via* SLS (Fig. 1d, described below) is ~35 °C, these data thus suggest that the polymer collapse at the ends of the triple helix constricts the unfolding of the peptide leading to non-two-state thermal transitions in contrast to the transition for the peptide.

This helix-to-coil transition was found to be reversible, thus kinetic refolding studies were carried out *via* temperature-jump experiments (Fig. 1c). In this experiment, the sample was heated to 75 °C and incubated for 30 minutes followed by immediate quenching to 10 °C to allow triple helix refolding. The data presented in Fig. 1c were fit to a first-order exponential rate law, and the derived refolding rate constants for the peptide ($1.0 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$) and the triblock ($1.1 \pm 0.05 \times 10^{-3} \text{ s}^{-1}$) were very similar at 10 °C. These results suggest that the conjugation of the polymer does not adversely affect the kinetics of the refolding at least for the domains that fold. However, the hybrid refolds to only 68% of its original CD intensity, while the peptide refolds to 78%. This result suggests that the PDEGMEMA chains (T_g of PDEGMEA <10 °C), even in their soluble form, provide some steric hindrance to the triple helix refolding, thus reducing the overall extent of refolding under these experimental conditions and times. Future studies exploring changes in the extent of refolding with variations in PDEGMEMA molecular weight will shed more details on the influence of the polymer. In a previous study, the rate of refolding of collagen triple helical peptide was reported to decrease due to the steric hindrance after the hydroxyproline residue of the peptide was modified with the carboxylate moiety linked through the ethyl linker.⁴² Altogether, our CD results indicate that the triple helical form is retained, the thermal

unfolding is sustained, and helix-to-coil transition is reversible in the triblock hybrid context.

To preliminarily assess if any supramolecular structures are formed by the triblock upon heating, the scattering intensity of the sample (100 μM in PBS, pH 7.4) as a function of temperature was monitored *via* static light scattering (SLS, Fig. 1d). Given the dispersity of sizes indicated by the SLS data, we did not estimate sizes of structures from these measurements. The data show that the scattering intensity increases with increasing temperature until 65 $^{\circ}\text{C}$, suggesting the formation of large aggregates. However, the intensity was found to drop at 75 $^{\circ}\text{C}$, despite the observation that the solution remains consistently turbid over the entire temperature regime above the LCST. To rule out the possibility of the sedimentation of the aggregates, the solution was also gently vortexed intermittently to ensure homogeneous mixing of the sample, but resulted in no change in the scattering intensity values. The scattering intensity values were confirmed *via* three experimental repeats and the average value of the scattering intensity was found to be the same in all the experiments at a particular temperature. The scattered intensity was also found to gradually increase with time at temperatures above the LCST, over a period of less than 1 hour, before stabilizing. The reported SLS intensity in Fig. 1d is the mean of the stabilized intensity, a value that remained stabilized for at least 6 hours.

It was further found that after heating the sample to 65 $^{\circ}\text{C}$, and then cooling back to 10 $^{\circ}\text{C}$, the scattering intensity profile was reversible while cooling. Additionally, upon subsequent reheating, the scattering intensity profile was also reproducible. Heating to a temperature of 75 $^{\circ}\text{C}$, however, resulted in the loss of this reversibility and reproducibility. The SLS data thus suggest an irreversible morphological change upon heating to 75 $^{\circ}\text{C}$ (see below). While we report on these morphological changes with heating below, a detailed analysis of morphologies with cooling will be investigated in our future studies.

The LCST of the hybrid determined *via* the SLS plot (as the onset of increase in scattering intensity) was found to be 35 $^{\circ}\text{C}$, suggesting that the hydrophilic collagen peptide domain increases the LCST of the polymer (PDEGMEMA homopolymer LCST $\sim 26^{\circ}\text{C}$). This result also explains the observed deviation in the CD thermal denaturation curve at $\sim 35^{\circ}\text{C}$ for the hybrid and corroborates the role of the collapse of the polymer chain in delaying the unfolding of the collagen triple helix. Similar experiments performed on the collagen peptide at the same concentration (100 μM) resulted in no change in the scattering intensity in the observed temperature regime (Fig. 1d). This suggests that the peptide domain by itself does not form any temperature dependent supramolecular aggregates at the concentration employed in the triblock experiments, although at higher peptide concentration, assembly of the peptide domain is observed.

The observed high intensity of the scattered light upon increasing the temperature suggested the formation of large structures, and hence the morphology of these structures was analyzed *via* a combination of several microscopic techniques—confocal microscopy, cryo-SEM, and TEM. As the temperature is increased above the LCST of the hybrid, this system behaves as (hydrophobic)coil–(hydrophilic)rod–(hydrophobic)coil triblock, in which the polymer block first collapses on the ends, and then eventually the middle collagen peptide block subsequently unfolds. The triple helical form of the collagen peptide has an estimated length of ~ 10 nm, considering the rise/residue of 0.29 nm for the collagen triple helix.^{41,43} The R_g of PDEGMEMA was calculated as 1.9 nm in good solvent (below LCST) and 0.45 nm in bad solvent (above LCST) from previously reported values⁴⁴ and by applying the scaling factor ($R_g \approx N^{\nu}$, where N is the degree of polymerization and $\nu = 3/5$ for a good solvent and $1/3$ for a bad solvent). It is to be noted however, that due to the three PDEGMEMA chains attached to the triple helical collagen peptide domain, the effective volume occupied by the

PDEGMEMA domain would be greater than what is suggested by the R_g value for single chain PDEGMEMA. Given the relative dimension of the two blocks, the hybrid system can be envisioned as a hydrophilic rod with short hydrophobically associating ends (Scheme 1).

Confocal microscopy was performed for *in situ* visualization of the assembling structures (ESI, Movie S1†) in PBS. Upon increasing the temperature from below to above the LCST (37 °C), confocal microscopy revealed the emergence of micrometre-sized spherical aggregates. To more quantitatively analyze these micrometre-sized assembled morphologies under aqueous conditions, cryo-SEM was employed; cryo-SEM can cover larger length scales (up to several micrometres) with high resolution to tens of nanometres.^{45–47} The cryo-SEM images (Fig. 2 and 3) clearly revealed the spherical morphologies formed at temperatures above the LCST of the triblock hybrid. Although the exact quantitative estimation of the dimensions is not possible from the cryo-SEM images due to the thin (~2.5 nm) layer of the Au/Pd deposited on the surface, spheres with diameters of up to approximately 30 µm were observed at 37 °C; these structures also grew to approximately 100 µm in diameter at 65 °C. Freeze-fracture SEM images of the spherical aggregates clearly show fusion of hollow spherical structures (Fig. 3a), entrapment of spheres within larger spherical aggregates (Fig. 3b), multilayered morphology⁴⁶ (Fig. 3c), and unstructured material within the spheres (Fig. 3d).

Interestingly, at 75 °C, a morphological transformation into microfibrils was observed *via* cryo-SEM (Fig. 2c and d); smaller spherical morphologies of less than 200 nm in diameter were also present as a minor component. The length of the fibrils varied and was found to be greater than 10 µm on several clearly discernible areas, while the width of these fibrils varied likely as a result of variation in the number of aggregated species in an individual fibril. Individual fibrils with less than 10 nm width were also observed. CD analysis of samples incubated at 75 °C for several hours (not shown) indicated that there was no detectable β -sheet formation of the samples under the conditions investigated; spectra remained identical to those shown in Fig. 1a.

Cryo-TEM analysis of the samples at high temperatures was found to be very difficult practically and hence non-cryo TEM was performed at different temperatures to probe the morphology of cast film assemblies (Fig. 4 and S1†). Although the TEM grid excludes the larger aggregates visualized *via* cryo-SEM, abundant round structures, with an increasing average size with increasing temperature, were observed. Representative images for samples incubated at 37 °C and 65 °C are shown in Fig. 4a and b, respectively. The sphere-to-fibril morphology transition at 75 °C was also captured in the TEM experiment (Fig. 4c and d and S1d†). These nanoscale morphologies were resolved more clearly in the absence of staining. Fig. S1† shows the representative images of such morphologies—isolated (Fig. S1a†, stained) as well as aggregated (Fig. S1b and S1c†, unstained). Fibrillar assemblies were observed at 75 °C (Fig. S1d†, unstained). Overall, the TEM images further confirmed the temperature-dependence of the association of these triblock conjugates into spherical and fibrillar morphologies. No such structures were observed by TEM for PEG–CLP–PEG conjugates (where CLP indicates the collagen-like peptide used in this study) investigated under the same conditions, corroborating that the thermal responsiveness of the polymer is a critical factor in the association of the observed structures.

The structures and transitions that we observe are similar to those previously reported for other thermoresponsive polymers and their conjugates. As one of the most widely used thermoresponsive polymers, poly(*N*-isopropylacrylamide) (PNIPAM) has been intensively investigated and well characterized,⁴⁸ due to its biocompatibility and its lower critical solution temperature (LCST) of *ca.* 32 °C. Various micro- and nano-structures such as spherical vesicles, micelles and micro-fibrils have been produced *via* self-assembly in

designed hybrid systems by conjugating PNIPAM to other synthetic polymers including polypeptides,⁴⁹ polystyrene,^{50,51} poly(ethylene oxide),⁵² and poly(benzyl ether).⁵³ Similar to the hollow spherical structures formed at 37 °C in our work, Yang *et al.*⁵² observed micron-sized hollow spherical structures from assembly of PEO-*b*-PNIPAM at the same temperature. However, there are fewer reports focused on dual stimuli-responsive hybrid block copolymers, and none to our knowledge in which dual temperature responsiveness is used to effect morphological changes. Chang and Huang⁴⁹ reported PNIPAM-*b*-poly(lysine) as a dual stimuli responsive copolymer with a pH-sensitive poly(lysine) domain and a temperature-sensitive PNIPAM domain. Various well defined particles including micelles, spherical micelles, and vesicles were formed by varying the copolymer compositions or the helicogenic common solvents. Wormlike structures with diameter around 50 nm were also observed when using THF as solvent, similar to the fibrillar structures formed at 75 °C in our work.

The thermally responsive elastin-like polypeptides (ELPs)^{54–58} have also been employed in assembling polymers and block polymers. Above its inverse transition temperature, ELPs alone can form uniform spherical microstructures in aqueous solutions.^{55,58} In studies on block ELPs based on natural tropoelastin, Woodhouse and co-workers⁵⁸ reported self-assembled nanoparticles with a bimodal size distribution of around 500 nm and 4 nm upon coacervation of the elastin exons EP20-244 at 37 °C in 1 M NaCl phosphate-buffered saline (PBS). Poly(ethylene glycol) (PEG) was found to serve as a good cosolvent in reducing the particle size and stabilizing the particles. Of relevance to this reported work, for some ELPs such as tropoelastin^{59,60} and other elastin-like polypeptide sequences,^{58,61–64} similar fibrillar super-structures are formed after aggregation. Tu *et al.*^{59,60} proposed a four-stage process for tropoelastin coalescence during elastin hydrogel formation and found tropoelastin nanoparticles with a diameter of 200–300 nm were early-stage intermediates. Bellingham *et al.*⁶¹ have also reported similar globule-to-fibril transition in shorter ELPs such as EP20-24 and EP20-24-24. After the coacervation of these ELPs, the aggregated nanoparticles with diameters from 25–50 nm were observed to reorganize into compact structures of parallel fibrils with diameters of approximately 3 to 6 nm.

Scheme 2 illustrates the potential molecular organization that might give rise to the structures observed at the various temperatures studied. Flexible polymer based coil–coil–coil triblock architectures with hydrophobic–hydrophilic–hydrophobic composition have been shown to yield flower-like and interconnected micelles or gels.^{65–69} But in the present case, the semiflexible rod-like nature of the collagen peptide domain imposes bending constraints that limit the formation of such micellar structures.

Specifically, since the hydrophobic block in the triblock hybrid is very short compared to the collagen peptide block, this system can be approximated as hydrophilic semi-flexible rod with hydrophobically associating sticky ends. (Recall that the R_g of the PDEGMEMA is estimated to decrease from ~1.9 nm in a good solvent to 0.45 nm in a bad solvent.) Thus the triblock system at this temperature may have dimensions of roughly 0.5 nm–10 nm–0.5 nm. Previous studies, on a triblock system comprising surfactant-coated hydrophilic gold nanorods equipped with short polystyrene chains attached to both the ends, showed that these structures assemble as closed nanorings and nanochains and also as nanospheres, depending on the solvent conditions.^{70,71} In that case, the rigidity of the nanorods predominantly governs the assembly and in systems with differing rigidity the assemblies are expected to be different.^{72,73} For example, Yang *et al.*⁷³ studied a different architecture consisting of a short hydrophobic hexa-*p*-phenylene rod and a hydrophilic poly(ethylene oxide) coil that are fused together into a macrocyclic ring. The flexible nature of the poly(ethylene oxide) chain in the macrocycle allows bilayer stacking of the hydrophobic segment to form barrel-like supramolecular aggregates or tubes with hydrophilic interior and

hydrophilic exterior. In our study the semiflexible nature of the rod-like hydrophilic collagen peptide domain makes our triblock system more similar to the gold nanorod case,^{70,71} consistent with the formation of spherical assemblies where the association of the hydrophobic ends may be possible owing to some flexibility of the collagen peptide domain. While such an arrangement may leave hydrophobic microdomains at the aqueous interface, the multilayer structure and collagen domain rigidity may both serve to stabilize this arrangement.

That these spherical structures could be expected to be multilayered (Fig. 3c and d) is supported by our previous work which demonstrated that the collagen peptide domains assemble into nanorods and microfibrils at high concentration (1.5 wt%).⁴¹ This association, coupled with the hydrophobic collapse of the polymer, might be anticipated to give multilayered structures, as the entropic cost of localizing the chains in such a manner would be offset by the liberation of water molecules from the associated hydrophobic chain ends. The bending energy penalty associated with formation of spheres is likely minimized at least in part by the evolution of very large (micrometre sized) spheres with low curvature.

The increased size of the spherical structures evidenced *via* SLS, cryo-SEM, and TEM may be explained by the conformational change of the collagen peptide domain with increasing temperature (Fig. 2a). Considering the rise per residue along the triple helix axis (0.29 nm) *versus* that for an unfolded extended chain (0.37 nm),⁷⁴ the fully unfolded form of the collagen peptide sequence could theoretically undergo a change in length of up to 2.4 nm. This dimensional change (24%) per molecule of the triblock involved in the assembly could be manifested as a change in the dimension of the spherical aggregates. Also, with the gradual unfolding of the triple helix, the tight triple helical packing (diameter of the collagen triple helix is ~1.5 nm) is relaxed; thus decreasing the packing density of the peptide and potentially leading to the increased exposure of the buried hydrophobic polymer. Therefore, this conformational change associated with the peptide domain is expected to increase the size of the spherical aggregates with increasing temperature.

Related to the present findings, previous reports indicate pH-induced changes in the size, as well as the morphology, of assembled vesicles and micelle structures were associated with an α -helix-to-coil transition.^{75–78} The change in molecular dimensions upon α -helix-to- β -sheet or coil transition^{74,79} from diblock systems containing peptide domains like poly(lysine) or poly(glutamic acids) has also previously been correlated with the change in the size of the assembled structures in solution.

The transition to a fibrillar morphology at 75 °C likely occurs as a result of simple desolvation of the collagen domains, coupled with their increased conformational freedom upon unfolding, which would respectively drive and permit such molecular rearrangement (Scheme 2). It is likely that the collagen peptide segments are located on the exterior of the fibril as indicated in Scheme 2 (although the positioning of the peptide domains cannot be determined). Given the observed dimensions of the fibrils, the data suggest that there are also likely collagen domains located in the interior of the fibrils. The formation of fibrils would also serve to relieve the associated strain with the change in dimension of the collagen domain as mentioned above. The pre-assembled multilayered structure of the spherical aggregates might also facilitate the morphological transition into a superstructured fibrillar network with occasional splaying of individual fibers (Fig. 4c and d and Scheme 2). The intertwining of the fibrils also leads to increase in width of the fibril, and also likely reduces the hydrophobic exposure.

The present studies have evaluated the dual-temperature responsive behavior of a peptide–polymer conjugate in which the transitions of the polymer (LCST) and peptide (unfolding)

are relatively close in value, with initial transitions in the near physiological range. While this behavior is useful for the formation of nanostructures for applications in biology, the range of morphological behavior may be further expanded by changing the temperature responsiveness of the peptide–polymer conjugates. Both polymer composition and length may be used to vary the responsiveness of the polymer block, which for these nonionic polymers is likely to yield greater changes than variations in solution conditions. Future work in this area will permit assessments of the importance of the progression (order) of peptide and polymer transitions in controlling morphologies of these types of responsive materials.

4. Conclusions

In this work, we have studied the assembly of a thermoresponsive triblock hybrid system comprising PDEGMEMA-collagen-like-peptide–PDEGMEMA. The triple helical assembly of the collagen peptide domain guided the initial assembly of the triblock hybrid. The temperature was subsequently used as an external stimulus to collapse the thermoresponsive PDEGMEMA polymer chains on the ends of the triple helical collagen peptide domain, resulting in increased stability of the triple helix against unfolding. Interestingly, increasing the temperature also caused the formation of larger spherical aggregates, which initially form owing to the hydrophobic collapse of the PDEGMEMA, and they increase in size likely due to the unfolding of the triple helical collagen domain. Further unfolding of the collagen peptide domain at even higher temperatures gave rise to a morphological transition from spheres to fibrils, illustrating the utility of dual thermoresponsiveness in manipulating the overall assembly process. Further studies will systematically evaluate the effect of polymer length, composition, architecture and concentration on the assembly process, specifically with respect to varying the temperature ranges over which the polymer collapse and peptide unfolding occur. The biological properties of the collagen peptide⁸⁰ may endow these assemblies with activities that could expand their use as nanostructured biomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Circular dichroism (CD) and static light scattering (SLS) data at 100 μM concentration in phosphate buffered saline (PBS, pH 7.4). (a) CD wavelength spectra for the triblock hybrid at different temperatures. (b) Thermal denaturation curve for the triblock hybrid and the collagen peptide; for the purposes of this analysis, the peptide is considered fully folded at 10 °C, yielding a fraction folded value of 1. (c) Kinetic refolding of the triblock hybrid and the peptide monitored for 90 minutes, after heating the sample to 75 °C and quenching at 10 °C. (d) The scattering intensity for the triblock hybrid and the peptide monitored *via* SLS.

Fig. 2. Cryo-SEM images on the triblock hybrid at different temperatures. Spherical morphologies at (a) 37 °C and (b) 65 °C. Both images show a scale bar of 50 μm. Microfibrils at 75 °C with a scale bar of (c) 2 μm and (d) 500 nm.

Fig. 3. Cryo-SEM images on the triblock hybrid showing (a) fusion of hollow spherical structures, scale bar 5 μm ; (b) entrapment of spheres within larger spherical aggregates, scale bar 10 μm ; (c) multilayered morphology, scale bar 3 μm and (d) unstructured material within spheres, scale bar 1 μm .

Fig. 4. TEM images of the triblock hybrid (stained with 1% PTA) at different temperatures: (a) 37 °C, scale bar 0.5 μm; (b) 65 °C, scale bar 2 μm; (c) 75 °C, scale bar 0.5 μm and (d) 75 °C, scale bar 100 nm.

Scheme 1.

Conjugation scheme showing the synthesis of the triblock hybrid *via* the reaction of the activated ester-terminated PDEGMEMA with telechelic amine-functionalized collagen peptide. The chemical structure of PDEGMEMA and the amino acid sequence of the collagen peptide are shown on the left. The reaction was carried out in dimethylformamide (DMF) in the presence of triethylamine (Et_3N) at 35 °C. The triple helical form of the hybrid was obtained after dissolving the triblock hybrid in phosphate buffered saline (PBS, pH 7.4) for at least overnight, followed by air oxidation of the thiol groups from the cysteine residue on the collagen peptide to cross-link the trimer through disulfide bridges.

Scheme 2.

Schematic illustration of the potential organization of assemblies observed at different temperatures. Spheres formed at 37 °C grow in size at 65 °C due to conformational changes in the peptide domain, and disintegrate at 75 °C leading to the formation of fibrils.