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A Versatile Grafting-to Approach for the Bioconjugation of Polymers to Collagen-like Peptides Using an Activated Ester Chain Transfer Agent

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Biohybrid materials consisting of synthetic polymers and biological moieties have gained more and more interest in the recent years.[1−10] The combination of these two material classes on the molecular scale offers not only the opportunity to overcome the limitations of the single building blocks but also the chance to design new materials that show improved or emergent properties based on the individual physicochemical and biological properties of the components. Of particular interest are block copolymers combining advantageous features of synthetic polymers, i.e., flexibility in the design of architecture and functionality,[11−14] solubility, processability, and biocompatibility as well as stimuli-responsive behavior, with advantageous features of peptides and polypeptides,[15] i.e., monodispersity and defined primary structure, controlled secondary structures, programmed assembly, and bioactivity, to yield materials that can interact with biology.[16−20] Moreover, such biohybrid polymers offer myriad opportunities to exert control over nanoscale structure; thus, study of their self-assembly and stimuli-responsive behavior may increase our understanding of molecular processes in complex biological systems.[21−24]

Modern polymerization techniques, such as controlled radical polymerization methods, enable the design of well-defined synthetic polymers. The recent development in the synthesis of numerous functional initiators or chain transfer agents for these controlled radical polymerization techniques provides a versatile toolbox for the synthesis of peptide-reactive polymers with well-defined architecture.[25−31] Recently, Theato and co-workers reported the synthesis of a functional chain transfer agent (CTA) for reversible addition−fragmentation chain transfer (RAFT) polymerization containing a single activated ester end-group.[32] This CTA could be used for the controlled polymerization of a wide range of monomers, and the end-groups of the resulting polymers could easily be functionalized via conversion of the activated ester with different amines. Kiick and co-workers have designed and synthesized a novel collagen-like peptide that is capable of forming thermally stable triple-helical structures as well as higher order assembled structures, under mild conditions.[33,34]

In this Communication, we present the use of the RAFT CTA for the covalent conjugation of the thermally responsive polymer, poly(diethylene glycol methyl ether methacrylate) (PDEGMEMA),[35,36] to the collagen-like peptide equipped with amine groups at both the N- and C-termini. The use of these two building blocks was motivated by our interests in preassembly of thermally responsive triblock polymers through the biologically active collagen-like peptide domain prior to collapse of the polymer domain. After deprotection of the peptide sequence, the synthesized triblock structure shows expected assembly into collagen-like triple helices in aqueous solution, as indicated by circular dichroic (CD) spectroscopy.

The stimuli-responsive polymer, PDEGMEMA, was synthesized via RAFT polymerization using pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) as CTA following a standard polymerization procedure as described previously.[32] The polymer with a molecular weight of M_n = 5600 g/mol and with a molecular weight distribution of M_w/M_n = 1.26 featured as a reactive α-end-group the pentafluorophenyl ester, which can be reacted with an amine-terminated peptide sequence or the amine group of a lysine residue. Hence, no postpolymerization functionalization is necessary to convert the polymer α-end-group into a reactive end-group. However, the ω-dithioester end-group resulting from the RAFT process is known to be labile toward aminolysis and would cause the loss of one equivalent of the amino-functionalized species per synthetic polymer block. To avoid the undesired loss of 1 equiv of peptide per polymer chain used, the dithioester end-group was radically substituted beforehand with an isobutyronitrile group via the conversion of the RAFT polymer with a 20-fold excess of AIBN in dioxane at 80 °C for 2.5 h following the procedure by Perrier et al.[37]

The collagen-like peptide was synthesized via automated Fmoc solid-phase peptide synthesis (SPPS) in *N*,*N*-dimethylformamide (DMF) carried out on a 2-chlorotrityl chloride resin (CLTR); the resin was prefunctionalized with 1,3-diaminopropane in order to obtain a peptide sequence with reactive amine groups at both chain ends. Selective protection of an internal lysine in the sequence was achieved by taking advantage of the higher stability of the *tert*-butyloxycarbonyl (Boc) protecting group compared to the linkage of the peptide to the CLTR resin. After the SPPS, mild cleavage of the peptide with 20% 1,1,1,3,3,3-hexafluoro-2-propanol in dichloromethane (2 h at room temperature) from the resin was performed, yielding a fully protected collagen-like peptide[38] with a molecular weight of 4786.9 g/mol (determined by ESI MS, m/z = 1595.5 [$(M + 3H)^{3+}$, calcd: 1595.8]) after purification by RP-HPLC. The deprotected collagen-like peptide forms thermally stable triple helices ($T_m \sim 45$ °C) in aqueous solution as indicated by CD spectroscopy and differential scanning calorimetry,[33,34,39] indicating its promise as an assembling domain in bioactive materials; its sequence is shown in Scheme 1. The thermal stability of the peptide relative to the LCST of the PDEGMEMA (LCST ∼ 26 °C)[35,36] is relevant to the potential thermal modulation of self-assembled structures from these building blocks and is expected to facilitate further studies on the mutual effect of these two temperature-dependent phenomena on each other.

The two building blocks, synthetic polymer and peptide, were conjugated to form the polymer-*b*-collagen-*b*-polymer triblock copolymer (PCP) by mixing 1.5 equiv of PDEGMEMA per primary amine group of the peptide (Scheme 1). The reaction was carried out in DMF at 35 °C for 2 days, and 2 μL of triethylamine was added. The resulting hybrid polymer was isolated by 3-fold precipitation into cold diethyl ether and dried in vacuum. A GPC in DMF showed one product signal that was clearly shifted toward higher molecular weight compared to either of the building blocks (Figure 1a). The GPC trace of the peptide was

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plotted only for comparison, although GPC is not the ideal method to measure the monodispersity of peptides, as they may interact with the column material and thus cause asymmetric elugrams.[40] According to the evaluation of the GPC data via calibration with PMMA standards, the molecular weight of the triblock system was M_n = 13 700 g/mol with a M_w/M_n = 1.32. The absence of a lower molecular weight shoulder indicated the absence of any unconverted peptide or homopolymer, confirming successful reaction of the peptide and complete separation of the homopolymer from the product by precipitation into cold diethyl ether.

After the successful conjugation with the polymer, the peptide was deprotected in a mixture of trifluoroacetic acid (TFA), deionized water, 1,2-ethanedithiol, and triisopropylsilane (94.5:2.5:2.5:1) at room temperature (2 h) and afterward precipitated into a cold mixture of diethyl ether and hexane (50:50). The ester linkages in the polymer side groups were found to be stable under these conditions (see Supporting Information). Retention of the PCP triblock structure after deprotection was verified via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 1b). The gel clearly showed that the smeared band for the deprotected PCP (lane 2) is distinct from a simple blend of polymer and peptide (lane 4; multiple bands arise from the presence of both unfolded monomer and folded triple helix in the peptide sample (as illustrated in lane 5)); the data also illustrate that the product did not contain any nonconjugated peptide and that PCP exhibits a higher molecular weight than the peptide itself.[41] The triblock structure was also indicated by ^{1}H NMR. The spectrum of the deprotected hybrid polymer (Figure 2a) shows clearly the strong signals of the two polymer blocks as well as signals characteristic of the peptide block; i.e., some of the signals representing the protons at the α-carbon atoms between 5 and 4 ppm and the signals of the protons in the peptide bond (9.00−6.37 ppm) (for comparison with the respective building blocks, see Figure 2b,c). The sum of the integrals of the latter (36 protons per molecule) were compared to the integral of the signal for the methyl group in the polymer backbone (~164 protons in the triblock copolymer) between 1.04 and 0.52 ppm, and this comparison showed that the product contained at least 86% of the triblock copolymer and not more than 14% of the diblock copolymer was formed as byproduct. Thus, the SDS-PAGE and ^{1}H NMR data confirm the formation of the PCP as suggested by GPC. Of functional interest, the deprotected PCP exhibits an LCST of ~38 °C (onset) in water, which is higher than that of the pure homopolymer, as expected.

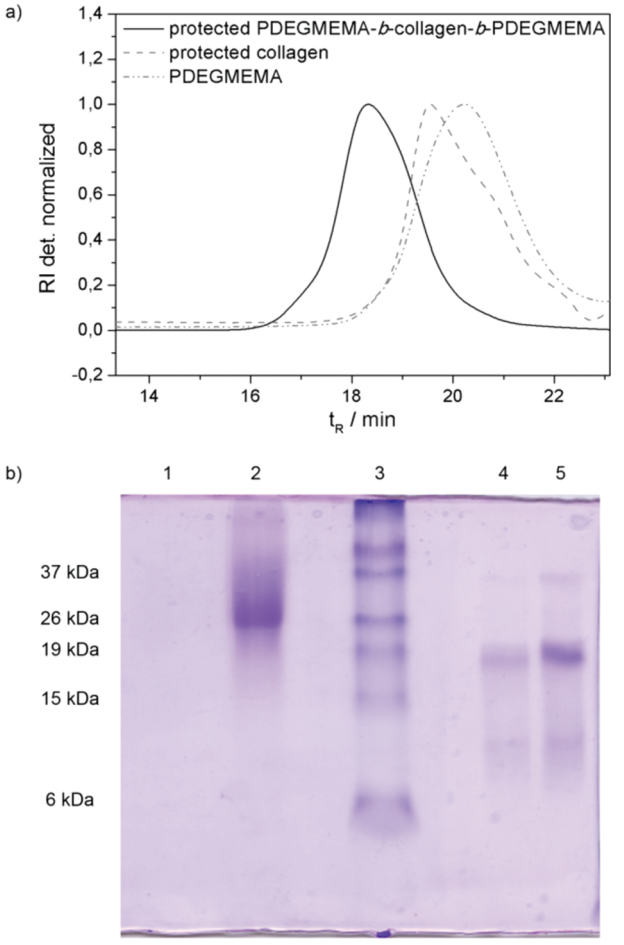
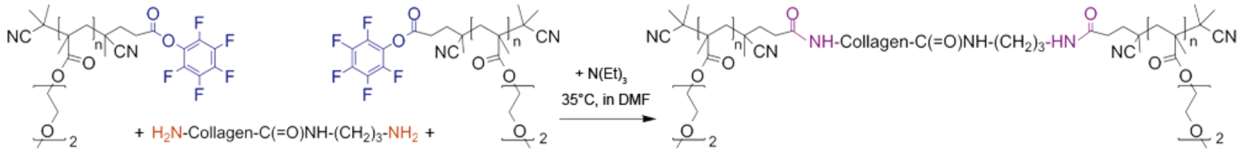


Figure 1. (a) GPC elugram of the protected triblock copolymer in DMF (+ 0.01 M LiCl) in comparison with the elugrams of the homopolymer and the collagen-like peptide. (b) SDS PAGE: lane 1 contains the homopolymer (not stained), lane 2 the deprotected triblock copolymer PDEGMEMA-*b*-collagen-*b*-PDEGMEMA, lane 3 a protein ladder, lane 4 a blend of homopolymer and peptide (only the peptide band is visible), and lane 5 only the collagen-like peptide. The samples were run on a 14% gel, and the bands were visualized via treatment with Coomassie Blue.

A 137 μM solution (concentration determined by amino acid analysis) of the deprotected PCP in phosphate buffered saline (PBS, pH = 7.4) was incubated at 4 °C overnight in order to allow the peptide to form the collagen triple-helical structure. This solution was analyzed via CD spectroscopy to evaluate triple-helix formation of the peptide block under these conditions (Figure 3a). The CD spectrum at 5 °C featured the typical maximum of a collagen triple-helix centered at 225 nm and a minimum at 202 nm.[39,42] The CD spectrum of pure PDEGMEMA was also measured and, as expected, did not show any CD activity. Hence, these results suggest that the collagen-like peptide sequence successfully promoted the self-organization of PCP into assemblies containing triple helices. Further, the thermal denaturation of PCP was monitored via change in mean residue ellipticity ($[\theta]_{MRE}$) at 202 and 225 nm (Figure 3b). While the nonfunctionalized collagen-like peptide showed a standard sigmoidal unfolding curve at 225 nm,[43] in the case of PCP, the changes in the $[\theta]_{MRE}$ values at 202 and 225 nm indicated a more gradual and complicated unfolding with two potential transitions, suggesting multistate noncooperative transition behavior and a dual responsiveness caused by the convolution of the thermally responsive behavior of the polymer blocks and the unfolding of the collagen block. This self-assembly behavior, the delayed thermal denaturation of the triple helix in the presence of

Scheme 1. Synthesis of a Hybrid Triblock Copolymer via Activated Ester Chemistry from PDEGMEMA and a Collagen-like Peptide with Protected Residual Groups (Cysteine: Trt; Glutamic Acid: *t*-Bu; Lysine: Boc; Arginine: Pbf)[a]



[a] Definition: $H_2N-Collagen-C(=O)NH-(CH_2)_3-NH_2$ = $H_2N-GGPPGPPGPPGPRGEKGERGPRGPPGPPGPPGPCCG-C(=O)NH-(CH_2)_3-NH_2$.

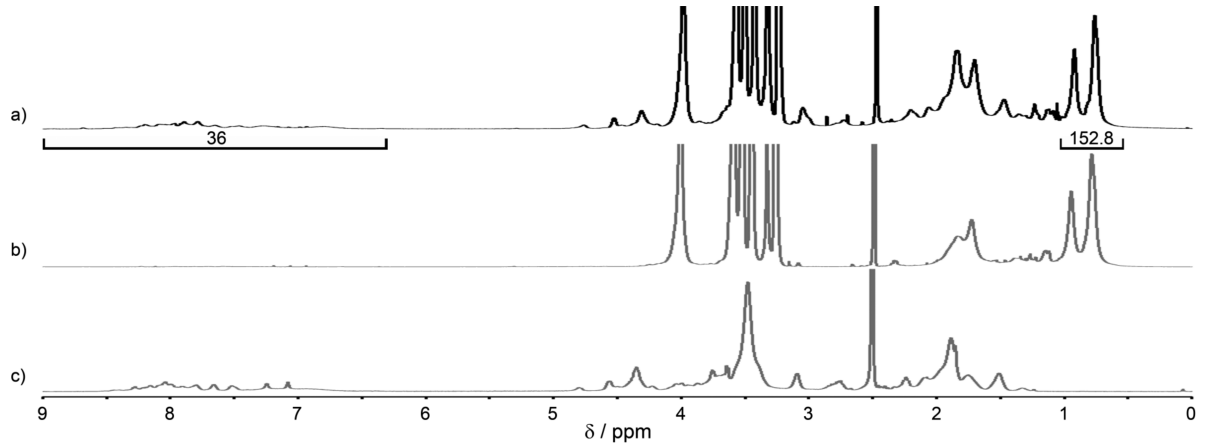


Figure 2. ^{1}H NMR spectra of (a) deprotected PDEGMEMA-*b*-collagen-*b*-PDEGMEMA (600 MHz), (b) PDEGMEMA (400 MHz), and (c) the deprotected peptide (400 MHz) in d_6-DMSO.

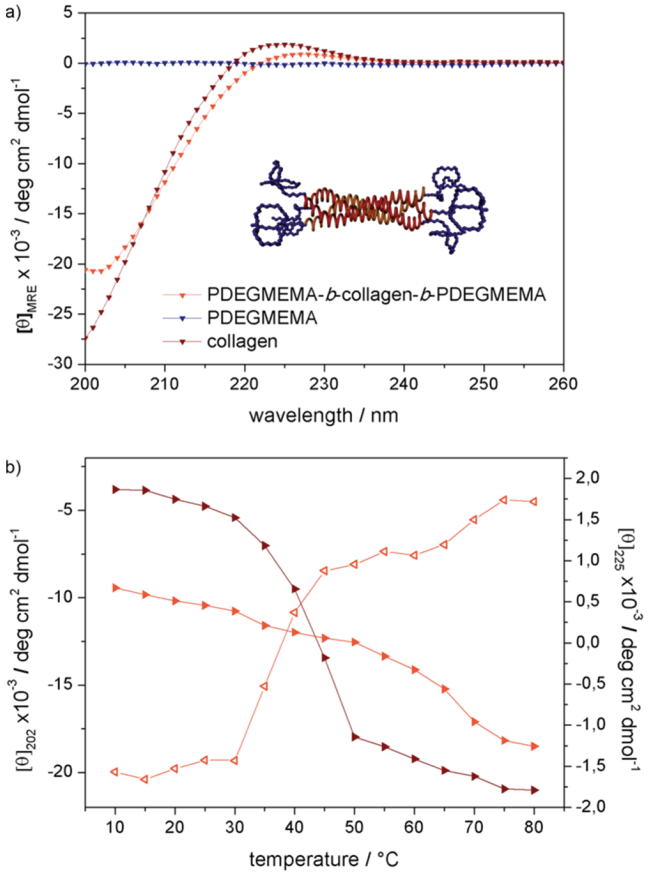


Figure 3. (a) CD spectra of the deprotected triblock copolymer PDEGMEMA-*b*-collagen-*b*-PDEGMEMA, the homopolymer, and the collagen-like peptide in PBS at 5 °C. (b) Thermal denaturation curves of the deprotected triblock copolymer (orange hollow triangles at 202 nm, orange filled triangles at 225 nm) and the collagen-like peptide (red triangles at 225 nm) in PBS measured via CD spectroscopy.

the polymer, and the potential of this hybrid material to form nanometer-scale structures are extremely promising given the organization of the isolated peptide domain into nano- and microscale structures as suggested by electron microscopy.[34] Further, the stimuli-responsive character of the involved polymer blocks offers intriguing possibilities to modulate the behavior of the biohybrid polymer[44] and is currently under investigation.

In summary, a versatile synthetic approach for the successful bioconjugation of RAFT polymers to peptides, without post-polymerization functionalization of either of the two building blocks, was established. This approach could be applied to various peptides with addressable amine groups and to a variety of synthetic polymers amenable to synthesis by RAFT polymerization using the described functional CTA. As an example, the site-selective conjugation of a stimuli-responsive poly(methacrylate) and a collagen-like peptide containing a Boc-protected lysine was demonstrated. The resulting PDEGMEMA-*b*-collagen-*b*-PDEGMEMA triblock copolymer exhibited the expected collagen triple-helical structure, suggesting opportunities to sequentially drive self-assembly behavior of the triblock via simple changes in temperature. Further studies of the self-organization of this and similar hybrid materials will follow. This synthetic approach is broadly applicable and could also be employed in the synthesis of comparable diblock copolymers or multiblock copolymers.

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Supporting Information Available: Experimental information, ^{19}F NMR of PDEGMEMA before and after treatment with TFA (deprotection conditions for the peptide), and GPC elugrams of PDEGMEMA before and after treatment with TFA. This material is available free of charge via the Internet at http://pubs.acs.org.

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