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Thermoresponsive Polypeptides from Pegylated Poly-L-glutamates

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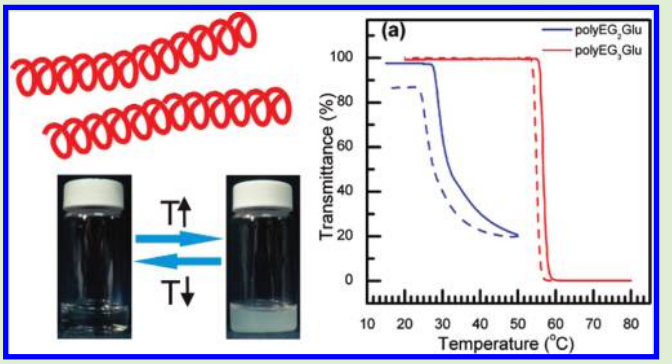
Supporting Information

ABSTRACT: The synthesis and characterization of new thermoresponsive pegylated poly-L-glutamate (poly-L-EG$_x$Glu) are described. The obtained polypeptides display low critical solution temperature (LCST) behaviors in water, and the LCST can be tuned via copolymerization of different amino acid monomers at varied molar ratio. This is the first example of thermoresponsive polypeptide made from ring-opening polymerization of α-amino acid *N*-carboxyanhydrides (NCAs). Circular dichroism characterizations reveal that the secondary structure of poly-L-EG$_x$Glu depended on the chain length of the side chain.



Thermoresponsive polymers are currently a center of active research because of their extensive applications in biotechnology.[1−3] Meanwhile, many well-studied thermoresponsive polymers had some limitations regarding their biocompatibility and biodegradability for in vivo applications. Hence, fully biodegradable responsive biopolymers are greatly desirable for biomedical applications. Although synthetic polypeptides have been studied for decades to construct functional biomaterials,[4−6] thermoresponsive polypeptides were still rare, except those with unique sequences such as elastin. There has been active research recently in developing synthetic routes to prepare (co)polypeptides with various functionalities.[7−14] However, studies of making completely biodegradable thermoresponsive polypeptides with transformable secondary structures have not been reported. Herein we report the first demonstration of synthetic thermoresponsive polypeptides, which is significant because of their addition of hydrogen bonding and ordered chain conformations that are not found in the well-studied (meth) acrylate analogues.

For natural polypeptides, most water-soluble polypeptides are polyelectrolytes, which have some issues regarding pH-dependent solubility and aggregation with oppositely charged biomolecules in vivo. Therefore, nonionic water-soluble polypeptide materials would be ideal examples to avoid the abovementioned problems. On the basis of natural amino acids, several side-chain-functionalized nonionic and water-soluble polypeptides were developed.[9−12,15−17]Although these polypeptides displayed promising properties with potential biomedical applications, they did not display thermoresponsive properties. Adding additional thermoresponsive moiety to polypeptides would be greatly desirable to make intelligent biomaterials.[7,18−20] Furthermore, postmodification of available polypeptides was also applied to prepare thermoresponsive polymers, however, with poor control over functionalities and architectures.[21−24]

PEG is a well-known stealth and biocompatible polymer, and oligo(ethylene glycol) (OEG)-grafted poly(meth)acrylate displayed tunable lower critical solution temperature (LCST) behaviors.[25,26] However, the nondegradable carbon−carbon backbone raised some issues regarding in vivo applications. In this work, we used OEG to functionalize glutamic acid via ester bond, so we can suppress side-chain hydrogen bonding formation while retaining the thermoresponsive characteristic of OEG units. It is also worth noting that the starting materials were commercially available, and the synthesis can be easily scaled up.

The pegylated-L-glutamic acids were prepared by direct coupling between methylated ethyleneglycols and L-glutamic acid via sulfuric-acid-catalyzed esterification (Scheme 1). After purification, these amino acids were then converted into corresponding α-amino acid *N*-carboxyanhydrides (NCAs) using triphosgene in THF. The obtained NCAs were viscous oils at room temperature and difficult to purify using recrystallization strategy. We thus applied recently developed flash column chromatography method to purify them and obtained NCAs with good purity allowing controlled living polymerization.[27] All monomers and NCAs were unambiguously characterized using NMR, HRMS, and elemental analysis shown in the Supporting Information.

All three NCAs were readily soluble in common solvents such as THF, ethyl acetate, and dichloromethane but insoluble in hexane and ether, and they underwent polymerization efficiently using Ni(COD)depe as initiator in DMF at RT.[28] We also found that these NCAs can also be polymerized using classical amine

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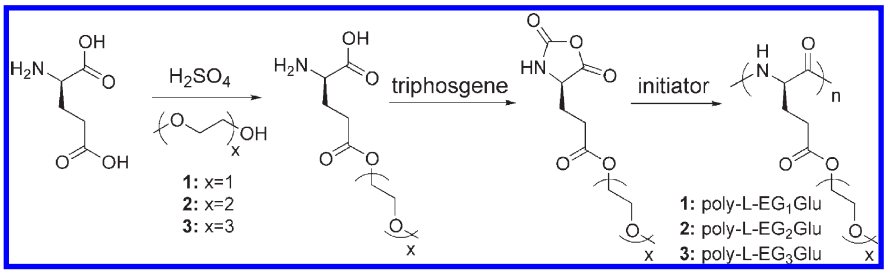
Scheme 1. Synthetic Routes to Poly-L-EG$_x$Glu Homopolypeptides



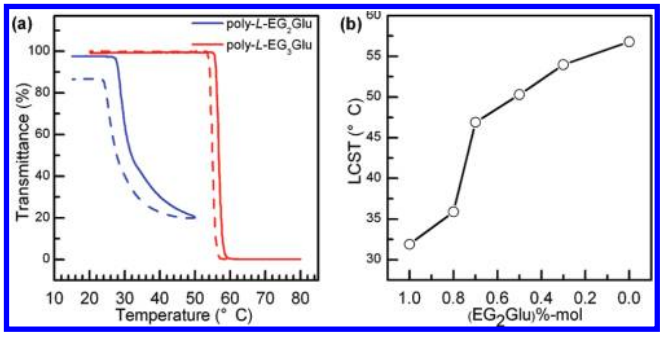


Figure 1. (a) Plots of transmittance as a function of temperature for aqueous solutions (2 mg/mL) of poly-L-EG$_2$Glu and poly-L-EG$_3$Glu. Solid line: heating; dashed line: cooling. (b) LCST of poly(EG$_2$Glu-EG$_3$Glu) copolypeptides as a function of sample composition.

initiator[29] or hexamethyldisilazane in DMF.[30] Here we used the transition-metal-mediated ring-opening polymerization, which allowed good control over product molecular weight and polydispersity (PDI < 1.2).[28] Using Ni(COD)depe as initiator and DMF as solvent, we prepared different homopolypeptides and random copolypeptides with narrow PDIs (Table S1 of the Supporting Information). The obtained polypeptides were generally purified via dialysis except noted, and white solid products were obtained after lyophilization with yield of ~90%. The products are designated as poly-L-EG$_x$Glu, where x (= 1, 2, and 3) represents the repeat unit oligoethylene glycol at the side chain. The solubility of poly-L-EG$_x$Glu homopolypeptides was found to depend strongly on the length of the OEG side chain (Table S2 of the Supporting Information). In particular, poly-L-EG$_1$Glu became insoluble in any common solvents after precipitation from DMF. Poly-L-EG$_2$Glu and poly-L-EG$_3$Glu were soluble in water and common polar solvents such as DMF, DMSO, and DCM but insoluble in THF.

Because poly-L-EG$_2$Glu and poly-L-EG$_3$Glu were soluble in water, we then focused on their aqueous solution properties. For both poly-L-EG$_2$Glu and poly-L-EG$_3$Glu, simple heating of their aqueous solution (2 mg/mL) caused a fast transition from clear solution to cloudy and cloudy to clear transition upon cooling, suggesting a reversible LCST behavior. We applied turbidimetry to determine corresponding LCSTs shown in Figure 1. Figure 1a showed the phase transition traces for poly-L-EG$_2$Glu and poly-L-EG$_3$Glu. For poly-L-EG$_2$Glu, the transmittance decreased from 100 to 20% when temperature increased from 27 to 50 °C, which gave an LCST of ~32 °C. In contrast, poly-L-EG$_3$Glu displayed a sharp LCST around 57 °C. The cooling traces of both poly-L-EG$_2$Glu and poly-L-EG$_3$Glu showed recovery of transmittances, indicating reversible LCST transition. For poly-L-EG$_3$Glu, we performed 10 heating−cooling cycles between 45 and 65 °C and observed complete recovery of transmittance (Figure S1 of the Supporting Information). In contrast, poly-L-EG$_2$Glu with a less ethylene glycol unit had only partially reversible LCST behavior (Figure 1a). Poly-L-EG$_2$Glu recovered only 90% transmittance and had 4 degrees of hysteresis in cooling ramp compared with 2 degrees for poly-L-EG$_3$Glu. Moreover, poly-L-EG$_2$Glu survived only two heating−cooling cycles prior to a phase separation, which was found to be due to secondary structure transformation from α-helix to β-strand, as discussed later. Note that both samples displayed hysteresis in phase transition during cooling ramp, which was probably due to redissolution of EG unit requiring slight overcooling to overcome the energy barriers.[31]

For biomedical applications, it is particularly important to have LCST around 37 °C. We have shown that the poly-L-EG$_2$Glu and poly-L-EG$_3$Glu had LCST about 32 and 57 °C, respectively. A question arose whether the LCST can be varied by copolymerization of L-EG$_2$Glu-NCA and L-EG$_3$Glu-NCA to avoid making new monomers. We made several random copolypeptides with different EG$_2$Glu/EG$_3$Glu ratio and found that the LCST can be varied from 36 °C with 80 mol % L-EG$_2$Glu to 54 °C with 30 mol % L-EG$_2$Glu shown in Figure 1b. It was worth noting that an advantage of using Ni(COD)depe as initiator was its high reactivity and efficiency and good copolymerization of the different comonomers, as evidenced by single LCSTs.[28] The yield was almost quantitative, and GPC characterization showed that all copolypeptides had narrow molecular weight distribution with PDI < 1.2 (Table S1 of the Supporting Information).

The secondary structures of poly-L-EG$_2$Glu and poly-L-EG$_3$Glu were characterized using circular dichroism (CD) and FTIR spectroscopy shown in Figure 2 and Figures S4−S6 of the Supporting Information. We found that the secondary structure of poly-L-EG$_2$Glu strongly depended on sample history. Poly-L-EG$_2$Glu purified by dialysis did not have a well-defined secondary structure, which was composed of 16% helix, 32% β-strand, 20% turns, and 32% random coil, respectively. Heating the same solution above its LCST did not cause obvious change in corresponding secondary structures (Figure 2a) within a CD measurement time range. Actually, precipitation occurred for long time stored poly-L-EG$_2$Glu aqueous solution, for example, 2 mg/mL, possibly due to an increase in β-strand percentage. If we precipitated poly-L-EG$_2$Glu from DMF solution into ether right after polymerization, then poly-L-EG$_2$Glu formed almost 100% α-helix in freshly prepared aqueous solution. Heating this solution above its LCST did not change its secondary structure after two cycles (Tables S3 and S4 of the Supporting Information), whereas long-time aging induced substantial transformation from α-helix to β-strand (Figure S5 of the Supporting Information). Eventually, poly-L-EG$_2$Glu lost its

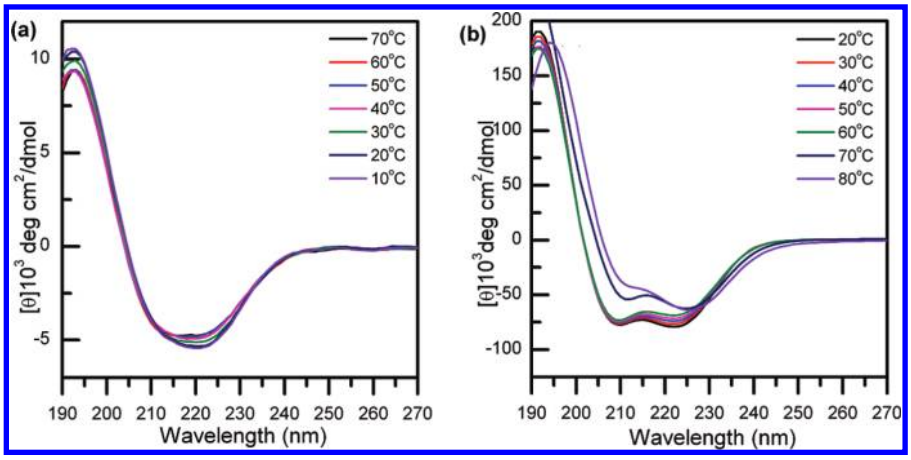


Figure 2. CD spectra of (a) poly-L-EG_2Glu and (b) poly-L-EG_3Glu as a function of temperature (heating scan).

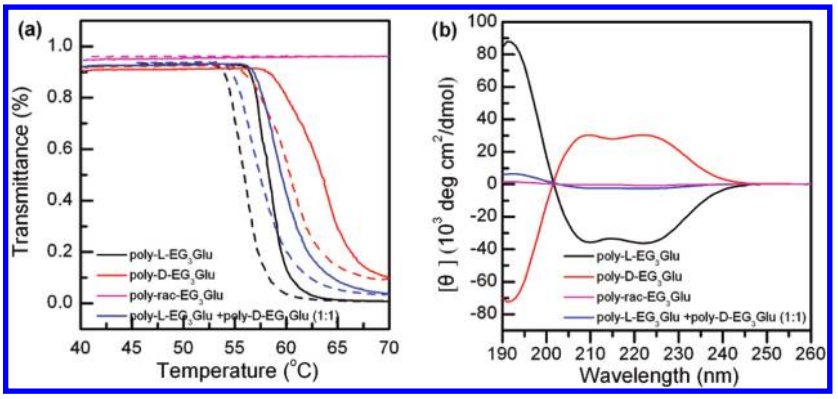


Figure 3. Plots of transmittance as a function of (a) temperature and (b) CD spectra for poly-L-EG_3Glu and poly-D-EG_3Glu and poly-*rac*-EG_3Glu homopolypeptide. Solid line: heating; dashed line: cooling.

thermoresponsive property due to increase in β-strand content. In contrast, the secondary structure of poly-L-EG_3Glu in water was more regular. CD measurements revealed that poly-L-EG_3Glu formed stable α-helix in water with 100% helicity, and its secondary structure was independent of temperature (Figure 2b). The change of CD signal in Figure 2b was most likely due to aggregation when the solution was heated above its LCST. Cooling scan of the same solution showed negligible change of its secondary structure (Figure S7 of the Supporting Information).

Because poly-L-EG_1Glu was insoluble in most solvents, we tried using FTIR to characterize its secondary structure in the bulk state and found that poly-L-EG_1Glu had strong absorption bands at 1631 (amide I band) and 1520 cm^{-1}(amide II band), indicating predominate β-strand conformation (Figure S4 of the Supporting Information). For poly-L-EG_2Glu, FTIR showed that it had mixed conformations containing both α-helix and β-strand (Figure S4 of the Supporting Information). FTIR characterization of poly-L-EG_3Glu displayed the strong carbonyl absorption at 1653 cm^{-1} (amide I) and the N−H amide II absorption at 1551 cm^{-1}, suggesting α-helical conformation.[32] These results were consistent with CD measurements discussed above, suggesting that the longer the length of side OEG chains, the more helical conformation.[33]

Regarding copolypeptides composed of L-EG_2Glu and L-EG_3Glu, their secondary structures depended on the molar ratio of each component (Table S3 of the Supporting Information). Copolypeptides containing 70, 50, and 30 mol % L-EG_2Glu have about 82, 71, and 100% helicity, respectively, from CD data analysis. These results indicated that increase in L-EG_3Glu content in copolypeptide helps to stabilize α-helix conformation. For all three samples, CD results also showed that heating solution above their LCST did not cause obvious change in their secondary structure accordingly. Also, note that all of these copolypeptides solutions were stable over time, in contrast with poly-L-EG_2Glu solution (Table S3 and S4 of the Supporting Information).

We also explored effects of polypeptide chirality toward their LCST behaviors. Using D-glutamic acid, we synthesized D-EG_3Glu, D-EG_3Glu-NCA, and poly-D-EG_3Glu, respectively. Similar to poly-L-EG_3Glu, poly-D-EG_3Glu displayed reversible LCST behaviors in water with LCST = 63 °C for polypeptide with DP = 247. CD measurement revealed that poly-D-EG_3Glu adopted α-helix conformation in water (Figure 3b). Note that poly-D-EG_3Glu has higher LCST than poly-L-EG_3Glu. We attributed this difference to the optical purity arising from synthetic D-glutamic acid because CD measurement found that it has only 85% helicity in contrast with 100% helicity for poly-L-EG_3Glu. Mixing equal amount of poly-L-EG_3Glu and poly-D-EG_3Glu did not change their LCST behaviors (Figure 3a), whereas their apparent CD signals canceled each other. The LCST of the above polypeptide blend ranged between corresponding

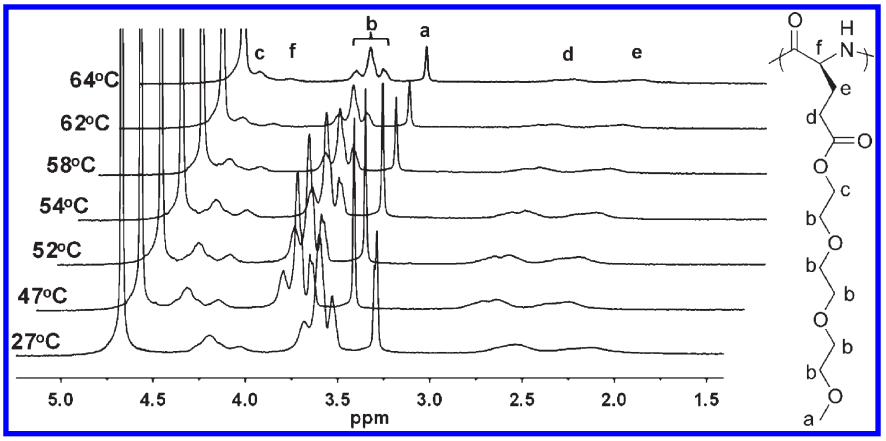


Figure 4. ^{1}H NMR spectra of poly-L-EG_3Glu as a function of temperature in D_2O.

homopolypeptides. Using equal molar L-EG_3Glu-NCA and D-EG_3Glu-NCA, we prepared racemic poly-*rac*-EG_3Glu, which adopted a random coil conformation suggested from CD spectrum (Figure 3b). Surprisingly, the racemic homopolypeptide did not show LCST behaviors up to 70 °C (Figure 3a). We believe that such transition arises from its conformation change. Both poly-L-EG_3Glu and poly-D-EG_3Glu formed stable α-helix in water. Presumably, their backbone amide bonds form intramolecular hydrogen bondings, which minimize their interaction with water molecules. Consequently, their water solubility arises from the grafted OEG units. The breaking symmetry in poly-*rac*-EG_3Glu suppressed forming intramolecular hydrogen bonding but promoted formation of intermolecular hydrogen bonding with water molecules, which accordingly improved polypeptide solubility. As a result, poly-*rac*-EG_3Glu did not display LCST behavior up to 100 °C.

We have demonstrated that poly-L-EG_2Glu and poly-L-EG_3Glu had reversible LCST behaviors. An important question is to understand the underlying mechanism regarding the driving force of LCST behaviors. For poly-L-EG_xGlu, there were several types of hydrogen bonding competition. The amide bonds in polypeptide backbone intended to form intramolecular or intermolecular hydrogen bonding, for which variation of temperature within small range might not disrupt their integrity in water. For example diethyleneglycol-functionalized poly-L-lysine displayed LCST around 100 °C.[17] In general, OEG units can form hydrogen bonding with water and will have reversible dehydration and hydration with variation of temperature.[31] We thus applied temperature-dependent ^{1}H NMR to explore the local chemical environmental variation of these characteristic protons versus temperature changes (Figure 4). At room temperature, the protons of methoxy (δ 3.3), methylene (δ 3.6), and β-methylene (δ 2.1) were easily identified for poly-L-EG_2Glu and poly-L-EG_3Glu (Figures S2 and S4 of the Supporting Information). With the increase in temperature, we found that protons of end methoxy and methylene groups became more and more broad, accompanying a substantial decrease in signal intensity. Further increase above their corresponding LCST caused almost disappearance of their resonances. These results suggested that temperature increase induced dehydration of ethylene glycol groups[34] without disrupting peptide secondary structures referred from CD characterization (Figure 2). Also, the normalized integration area of methoxy group as a function of temperature shown in Figure S3d of the Supporting Information displayed a similar transition to turbidity experiment. Taking the middle point of transition temperature, we obtained an LCST of ~55 °C, in good agreement with turbidity measurements described above. For poly-L-EG_2Glu and copolypeptides with different compositions, we observed similar results shown in Figures S2 and S3 of the Supporting Information. These results were also corroborated from solution properties of poly-*rac*-EG_3Glu, which lost its LCST behavior because of disruption of its secondary structure from α-helix to random coil.

In summary, we demonstrated a facile and economic strategy to prepare biodegradable thermoresponsive polypeptides and copolypeptides with narrow molecular weight distribution. All materials were commercially available, and monomers were synthesized by direct esterification. The LCST can be tuned via copolymerization of different monomer at varied ratio. CD characterization suggested that the secondary structures of poly-L-EG_xGlu and copolypeptides relied on chain length of OEG side chain. We believe that these thermoresponsive polypeptides with tunable LCST will have great promising to construct new intelligent biomaterials for biomedical applications.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and characterization and CD, FTIR, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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