Formation of Reversible Shell Cross-Linked Micelles from the Biodegradable Amphiphilic Diblock Copolymer Poly(t-cysteine)-block-Poly(t-lactide)

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A novel biodegradable diblock copolymer, poly(t-cysteine)-b-poly(t-lactide) (PLC\text{-}b\text{-}PLLA), was synthesized by ring-opening polymerization (ROP) of \(N\)-carboxyanhydride of \(\beta\)-benzoyloxy-carbonyl-t-cysteine (ZLC-NCA) with amino-terminated poly(t-lactide) (NH\text{-}2\text{-}PLLA) as a macroinitiator in a convenient way. The diblock copolymer and its precursor were characterized by \(^1\)H NMR, Fourier transform infrared (FT-IR), gel permeation chromatography (GPC), and X-ray photoelectron spectroscopy (XPS) measurements. The length of each block polymer could be tailored by molecular design and the ratios of feeding monomers. The cell adhesion and cell spread on the PZLC\text{-}b\text{-}PLLA and PLC\text{-}b\text{-}PLLA films were enhanced compared to those on pure PLA film. PLC\text{-}b\text{-}PLLA can self-assemble to form micelles in aqueous media. A pyrene probe is used to demonstrate the micelle formation of PLC\text{-}b\text{-}PLLA in aqueous solution. Due to the ease of disulfide exchange with thiols, the obtained micelles are reversible shell cross-linked (SCL) micelles. The morphology and size of the micelles are studied by dynamic light scattering (DLS) and environmental scanning electron microscopy (ESEM).

Introduction

Over the past decade, increasing academic interest has been given to the supramolecular structures formed by the spontaneous self-assembly of block copolymers in selective solvents, because of their potential applications in nanoscience and nanotechnology, such as carriers for drug and gene delivery, diagnostic imaging, and nanoreactors.1–4 Compared to low molecular weight surfactants, polymer micelles are more stabilized because of their macromolecular nature. However, the development of their applications has been hindered by their poor physical stability that arises from the weakness of the supramolecular interactions binding the aggregates together.5 In 1996, Wooley and co-workers published the first paper on shell cross-linked (SCL) micelles.6 Since then, several groups have demonstrated that cross-linking of micelles can dramatically improve the colloidal stability of these aggregates.7–8 Disulfide bonds are found in natural peptides and proteins and play an important role in keeping the structural stability and rigidity.9,10 Due to its stability under certain conditions and its reversibility under others, disulfide–thiol chemistry is becoming increasingly popular in conventional polymer syntheses and provides a facile route to the preparation of recyclable cross-linking of micelles.11,12 Moreover, disulfide can reversibly undergo reduction to thiols depending on the environmental thiol concentration. For example, the thiol concentration varies in our body, depending on the location as well as pathological conditions. It is about 10 \(\mu\)M in the plasma but about 10 mM in the cytosol. It is 7 times higher around some tumors.13–15 In addition, there exist other methods of reducing disulfide bonds to thiols, including dithiothreitol, zinc dust, and UV light.16–18 The thiol groups can be subsequently reacted with reactive groups such as activated disulfides, maleimides, iodoacetyl groups, and some thiol-containing biomolecules (e.g., antisense oligonucleotides).19,20 Poly(t-lactide) (PLLA) is a well-known synthetic biodegradable polymer used in surgical repair, as carriers in drug delivery, and as temporary matrixes or scaffolds in tissue engineering due to its biodegradability, biocompatibility, high mechanical prop-

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polypeptides is an increasingly utilized route to the synthesis of new nanostructured materials. However, only a few biodegradable copolymers consisting of aliphatic polyesters and polypeptide chains have been studied so far.23–27 Most of them are copolymers based on poly(lactic acid) (PLA) as the hydrophobic segment and neutral, anionic, or cationic polypeptides as the other segment. Up to now, few papers on the synthesis of polypeptides consisting of thiol groups have been published.28,29 Herein, we synthesized a new diblock copolymer, poly([beta]-cysteine)-b-poly(l-lactide) (PLC-PLA) by ring-opening polymerization (ROP) of N-carboxyanhydride of [beta]-benzoyloxycarbonyl-l-cysteine (ZLC-NCA) with amino-terminated poly(l-lactide) (NH2-PLLA) as a macrominitiator. NH2-PLLA was obtained through ROP of l-lactide in the presence of stannous octoate and NH2-protected aminoethanol. Ho¨cker et al.30 successfully synthesized MPEG-PLA-NH2 by endcapping PLA with N-tert-butoxycarbonyl (Boc) phenylalanine and then deprotecting the Boc group. Our group further used MPEG-PLA-NH2 as a macromolecular initiator to get triblock copolymers MPEG-b-PLLA-b-PBLG and MPEG-b-PLLA-b-PZLL.22,31 Another approach to amino-functionalized poly(l-lactide)30 was ring-opening polymerizing l-lactide with an NH2-bearing initiator, that is, zinc tert-butoxycarbonylamino-propanoxide, that was prepared by the reaction of diethylzinc and Boc-aminopropanol. Because of intrinsic instability, it must be used immediately after preparation. In the present study, stannous octoate (Sn(Oct)2) is used as the catalyst to prepare the PLA block at 110 °C. However, the Boc group is not stable enough at 110 °C.32 So, the benzoyloxycarbonyl (Z) group is chosen to be the amino protective group.

Furthermore, we investigate the self-assembly properties of this copolymer in aqueous media. Due to the ease of disulfide exchange with thiols, reversible shell cross-linked spherical micelles are obtained. In comparison with the former biodegradable copolymers consisting of aliphatic polyesters and polypeptide chains, poly([beta]-cysteine)-b-poly(l-lactide) owns more special properties because of the presence of poly([beta]-cysteine) and the exchange between thiol and disulfide groups. It can be widely used in drug release and protein delivery.

**Experimental Section**

Materials. L-Lactide (LLA) was purchased from Purac Biochem bv Gorinchem and recrystallized from ethyl acetate three times. ZLC-NCA was prepared according to the method of Daly and

![Scheme 1. Synthesis of Diblock Copolymers PZLC-b-PLLA and PLC-b-PLLA](image)

<table>
<thead>
<tr>
<th>Table 1. Molecular Weight Data of PLLA before and after Deprotection</th>
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<tbody>
<tr>
<td>entry</td>
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<tr>
<td>---</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
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</tbody>
</table>

$^a$ Calculated from feed composition. $^b$ Determined by $^1$H NMR in DMSO-$d_6$ solution. $^c$ Determined by GPC in THF.
Sanda, F.; Kamatani, J.; Endo, T.; Noguchi, J.; Katchalski, E.

**Aminoethanol.** A total of 6.7 g of aminoethanol was dissolved in a 10% water solution of NaCO3. The solution (150 mL) was added, and then the mixture was cooled to 0 °C. Benzyloxy carbonyl chloride (15 mL) was added dropwise in 3 h. The mixture was allowed to react at room temperature for 12 h. The product was precipitated with an excess of cold methanol to give a white product, benzyloxy carbonyl (Z) group terminated poly(l-lactide) (Z-NH-PLLA). Yield: 96%.

**Synthesis of the Benzyloxy carbonylaminoethanol.** A total of 33% with respect to the amino group in PLLA (Mg Kα X-rays at 1253.6 eV) and a 150 mm hemispherical electron energy analyzer. The samples were first dissolved in DMSO-d6, and y are solvent peaks.

![Figure 1](image_url)  
*Figure 1.* 1H NMR spectra and their assignments of PLLA (A, see entry 1 in Table 1), PLLA-NH2 (B, see entry 2 in Table 1), PZLC-b-PLLA (C, see entry 1 in Table 2), and PLC-b-PLLA (D, deprotection from C) in DMSO-d6.

**Table 2. Molecular Weight Data of Diblock Copolymer PZLC-b-PLLA**

<table>
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<th>entry</th>
<th>polymers</th>
<th>macroinitiator</th>
<th>M_n[a]</th>
<th>DP[a]</th>
<th>DP[b]</th>
<th>M_n[a]</th>
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<tbody>
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<td>NH2-PLLA-1</td>
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<td>10</td>
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<tr>
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<td>NH2-PLLA-1</td>
<td>3800</td>
<td>20</td>
<td>13</td>
<td>3100</td>
</tr>
<tr>
<td>3</td>
<td>PZLC-b-PLLA3</td>
<td>NH2-PLLA-2</td>
<td>5800</td>
<td>10</td>
<td>8</td>
<td>1900</td>
</tr>
</tbody>
</table>

[a] Calculated from 1H NMR. [b] Calculated from feed composition.

Aminoethanol was distilled under vacuum before use. A 33 wt% solution of HBr in HAc was supplied by Acros.

Benzyloxy carbonyl chloride and trifluoroacetic acid were purchased from GL Biochem (Shanghai) Ltd. Tetrahydrofuran (THF) was dried and distilled in the presence of sodium before use. Dimethylfor- mamide (DMF) was dried over CaH2 and distilled under vacuum before use. Ellman’s reagent (DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid) was obtained from Sigma-Aldrich.

**Synthesis of the Benzyloxy carbonyl (Z) Group Terminated PLLA.** L-Lactide was ring-opening polymerized in the presence of the initiator 2-benzyloxy carbonylaminoethanol and stannous octoate (Sn(Oct)2). First, given amounts of initiator, l-lactide, toluene (25 wt%), and Sn(Oct)2 (1% mol) were added into a dried glass reactor already flame-dried and nitrogen-purged three times, and then the sealed reactor was maintained at 110 °C for 12 h. The product was precipitated with an excess of cold methanol to give a white product, benzyloxy carbonyl (Z) group terminated poly(l-lactide) (Z-NH-PLLA). Yield: 91.2%.

**Synthesis of Amino-Terminated PLLA.** The benzyloxy carbonyl (Z) group in Z-NH-PLLA was removed by reacting with 4 equiv of HBr (in HAc, C = 33%) with respect to the amino group in PLLA in CF3COOH (0.1 g/mL) at 0 °C for 1.5 h. The product was precipitated with an excess of diethyl ether and redissolved in chloroform. It was then washed with saturated aqueous NaHCO3 and H2O. The polymer was precipitated with an excess of diethyl ether to get a white solid (NH2-PLLA) and was dried in vacuum at room temperature for 48 h. Yield: 92.0%.

**Synthesis of Diblock Copolymer PZLC-b-PLLA.** In a dried flask, given amounts of NH2-PLLA and ZLC-NCA were dissolved in dried DMF (10 wt%) and the solution was stirred for 72 h at 30 °C. The product mixture was precipitated with an excess of a mixture of acetic acid and methanol (1:3, v/v) under vigorous stirring to give a white solid (PZLC-b-PLLA) while the unreacted PLLA remained in the solution. The purified PZLC-b-PLLA was gained under vacuum at 40 °C for 24 h. Yield: 88.7%.

**Deprotection of PZLC-b-PLLA.** The diblock copolymer PZLC-b-PLLA was dissolved in CF3COOH (5 wt%), 4 equiv of a 33% solution of HBr in HAc with respect to the benzyloxy carbonyl (Z) groups was added, and then the solution was stirred under argon for 2 h at 0 °C. After that, the reaction mixture was precipitated with an excess of diethyl ether to give a white product (PLC-b-PLLA). The precipitate was dried in vacuo at 40 °C for 24 h.

**Measurements of the Block Copolymers.** 1H NMR spectra were measured in DMSO-d6, at room temperature (20 ± 1 °C) by using an AV-300 NMR spectrometer from Bruker. Fourier transform infrared (FT-IR) spectra were recorded on a Bio-Rad Win-IR instrument. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC apparatus with tetrahydrofuran (THF) as eluent (flow rate: 1 mL/min) at 35 °C. The molecular weights were calibrated against polystyrene (PS) standards. Surface elemental compositions of the samples were analyzed on an Escalab MKII photoelectron spectrometer (VG Scientific). The X-ray photoelectron spectroscopy (XPS) experiments were performed in the spectroscopy chamber using a standard Mg anode X-ray source (Mg Kα X-rays at 1253.6 eV) and a 150 mm hemispherical electron energy analyzer. The spectra were obtained for each sample using a 90° takeoff angle. The samples were first dissolved in N,N-dimethylformamide (DMF), and then the solution was deposited on a silicon wafer to form a very thin layer, followed by drying in vacuo at 40 °C for 2 days.

**Cell Experiments.** Test Cells. ECV-304 cells were chosen as test cells. The cells were purchased from Shanghai Institute of Cell
Biology, Chinese Academy of Sciences, and cultured with Dulbecco’s modified Eagle’s medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin, and 100 mg/L streptomycin (Sigma), and the culture medium was replaced once everyday.

Cell Adhesion and Spreading. The copolymers PZLC-b-PLLA and PLC-b-PLLA were dissolved in DMF and cast on cover slides. The pure PLA films were prepared in a similar way and were used as controls. The slides were kept under vacuum for 48 h to remove the last traces of DMF and then exposed to UV light for 2 h for sterilization. The cover slides coated with the polymer films were placed in the culture wells, and then the culture medium was added into each well. The cells were seeded at a cell density of 1 × 10^5 cells/well and were incubated at 37 °C in 5% CO₂. The growth medium was replaced with fresh medium once every 4 h. The pictures of each cover slide were taken with a digital camera (DXM1200F, Nikon) after 3, 24, and 48 h.

Preparation of Polymeric Micelles. The diblock copolymer was first dissolved in N,N-dimethylformamide (DMF), which is a common solvent for the two blocks, with an initial concentration of 2.0 wt %. A total of 50 equiv of dithiothreitol (DTT) with respect to the thiol groups was added, and the mixture was stirred for 2 h at room temperature. DTT was used as a reducing agent. A given amount of deionized water was then added to the copolymer solution under gentle stirring. To reach equilibrium, the mixture was stirred overnight. After that, the mixture was further diluted with a large amount of water. It was then dialyzed against deionized water to remove DMF from the solution and against phosphate buffer (pH 6.8) to form shell cross-linked micelles.

Measurements of the Thiol Group Concentration. There were two kinds of sulfur-containing groups in the diblock copolymers prepared, thiol groups and disulfide bonds. The amount of free thiol groups was determined photometrically with Ellman’s reagent. First, 0.5 mL of micellar solution was mixed with 5 mL of deionized water and 2 mL of phosphate buffer (pH 8.0), and then 20 μL of Ellman’s reagent (39.6 mg dissolved in 10 mL of phosphate buffer, pH 7.0) was added into 3 mL of the above mixture. The samples were incubated for 2 h at room temperature in the dark before measuring at 412 nm using a UV–vis spectrophotometer (Shimadzu 2401PC). In order to determine the content of disulfide bonds, the sample was reduced with NaBH₄ to convert disulfide bonds into thiol groups and Ellman’s assay was performed to obtain the total content of thiol groups.

Table 3. Relative Surface Elemental Composition in mol % Determined by XPS

<table>
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<tr>
<th>entry</th>
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<td>Z-NH-PLLA*</td>
<td>38.09</td>
<td>61.91</td>
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</tr>
<tr>
<td>2</td>
<td>PLC-b-PLLAb</td>
<td>39.90</td>
<td>50.81</td>
<td>3.76</td>
<td>5.52</td>
</tr>
</tbody>
</table>

* See entry 3 in Table 1. * Deprotection from polymer PZLC-b-PLLA3 in Table 2. * Approximately equal to zero.

Figure 2. Microscopic images of adhered and spread ECV-304 cells. Polymer films: PLLA (A, D, G; see entry 3 in Table 1), PZLC-b-PLLA (B, E, H; see entry 3 in Table 2), and PLC-b-PLLA (C, F, I; see entry 2 in Table 3). Incubation time: 3 h (A–C), 24 h (D–F), and 48 h (G–I).

Scheme 2. Formation of a Reversible Shell Cross-Linked Micelle

thiols. The difference between the two measurements (“total thiol” — “free thiol”) was considered as the content of disulfide bonds.

**Fluorescence Measurements.** A pyrene probe was used to probe the formation of micelles. Steady state fluorescence spectra were obtained by using a Perkin-Elmer LS50B luminescence spectrometer. The copolymer solutions without dialyzing against phosphate buffer (pH 6.8) and distilled water were added consecutively into the volumetric flasks containing pyrene, and the copolymer concentration (pH 6.8) and distilled water were added consecutively into the sample surface before measurement. The pyrene concentration in each final solution was from 10^-4 to 0.36 g/L. This course should be quick to avoid cross-linking. The pyrene concentration in each final solution was 6 × 10^-4 mol/L (the saturation solubility of pyrene in water at 22 °C). The emission wavelength was set at 391 nm for fluorescence excitation spectra. The spectra were recorded at a scan rate of 240 nm/min.

**Dynamic Light Scattering (DLS) Measurements.** DLS measurements were carried out with a DAMN EOS instrument equipped with a He–Ne laser at a scattering angle of 90°. The micelle solution of about 0.4 mg/mL was passed through a 0.45 μm filter before measurement.

**Environmental Scanning Electron Microscopy (ESEM) Measurements.** The ESEM images were recorded with a model XL 30 ESEM FEG microscope from Micro FEI Philips. The dilute micelle solution was deposited on a silicon wafer to form a very thin layer and dried at room temperature. A thin layer of Au was coated on the sample surface before measurement.

**Results and Discussion**

**Synthesis and Characterization of the Copolymer.** For the synthesis of a block copolymer composed of a polyester and a poly(α-amino acid), an amino end-group on the polyester chain is the key point, because primary amine is an efficient initiator of the polymerization of amino acid N-carboxyanhydrides. However, it is difficult to introduce an amino group into a PLLA chain by converting its end OH group directly into NH2, because the aliphatic ester bonds are often broken down under strong reaction conditions such as strong acid or strong alkali during this end-group conversion. Our synthetic route is outlined in Scheme 1. More detailed synthetic steps are discussed as follows:

**Synthesis of the Benzyloxy carbonyl (Z) Group Terminated PLLA.** The benzyloxy carbonyl-terminated PLLA (Z-NH-PLLA) is synthesized with high conversion directly from the ROP of \( \text{L-lactide} \) (LLA) in the presence of 2-benzyloxy carbonylamino-ethanol (\( \text{C}_2\text{H}_5\text{CH}_2\text{OC}(=\text{O})\text{NHCH}_2\text{CH}_2\text{OH} \)) and Sn-(Oct)\(_2\)) in toluene solution. The characteristics of the synthesized polymers are shown in Table 1. The block lengths of PLLA can be designed and adjusted by the molar ratio of the LLA to the initiator. The molecular weights and the polydispersity of Z-NH-PLLA are characterized by \(^1\text{H} \) NMR and GPC. Typical signals of both PLLA and the benzyloxy carbonyl unit are detected by \(^1\text{H} \) NMR as shown in Figure 1A. The peaks marked with the letters (a) and (b) are assigned to the protons in PLLA repeat units, such as a at 5.2 ppm (quadruplet, \(-\text{C(O)}\text{CH(\text{CH}_3)O}^-\)), b at 1.4 ppm (doublet, \(-\text{C(O)}\text{CH(\text{CH}_3)O}^-\)). The peaks c, d (at 4.0 ppm—4.2 ppm), e (at 5.0 ppm), and f (at 7.4 ppm) can be assigned to the protons of the benzyloxy carbonyl group. Degree of polymerization, \( \text{DP}_{\text{PLLA}} \), can be obtained from the integral ratio of \( \text{C}_2\text{H}_5^- \) (f at 7.4 ppm) to \(-\text{C(O)}\text{CH(\text{CH}_3)O}^-\text{C(O)}\text{CH(\text{CH}_3)O}^- \) (a at 5.2 ppm) in the \(^1\text{H} \) NMR spectrum of Z-NH-PLLA, as shown in the following formula: \( \text{DP}_{\text{PLLA}} = 5a/2f \).

**Synthesis of Amino-Terminated PLLA.** It is well-known that the benzyloxy carbonyl protective group can be removed by acidolysis with a 33% solution of HBr in HAc. Thus, the reduction of Z-NH-PLLA into NH2-PLLA is carried out by this method. The deprotection of the Z group is confirmed by \(^1\text{H} \) NMR shown in Figure 1B. The benzyl peaks at 5.0 and 7.3 ppm disappear completely, demonstrating the elimination of the benzyloxy carbonyl group. Peak d at 4.0 ppm shifts to 3.0 ppm due to the deprotection of the benzyloxy carbonyl group. Moreover, the GPC trace of NH2-PLLA in Figure S2B in the Supporting Information shows a unimodal shape. This further indicates that Z-NH-PLLA is successfully obtained.

**Synthesis of Diblock Copolymer PZLC-b-PLLA.** Primary amines can be used as initiators for the ROP of NCA to prepare poly(α-amino acids), undergoing a nucleophilic addition to the

**Figure 3.** Fluorescence excitation spectra of pyrene in aqueous PLLA-PLC (see entry 2 in Table 3) solution (concentration in g/L) at room temperature. The emission wavelength was set to 391 nm.

**Figure 4.** Plot of \( I_{335}/I_{333} \) of pyrene versus log C of the polymer in deionized water.
The 1H NMR spectrum of the diblock copolymer, as shown obtained from the integral ratio of a unimodal shape and a small shift to the higher molecular weight copolymer (Figure S2C in the Supporting Information) shows it also adopts this kind of structure in bulk. The GPC trace of A.; Bryson, N.; Meyrueix, R. Macromolecules A.; Bryson, N.; Meyrueix, R. Macromolecules at 697 and 749 cm−1 indicating formation of the polypeptide block. The absorptions attributed to the amide I and amide II modes, respectively, PZLC-b-PLLA according to Scheme 1.

The 1H NMR spectrum of the PZLC-b-PLLA block copolymer is shown in Figure 1C. Peak d at 3.0 ppm shifts to 4.0 ppm. Peak h at 7.4 ppm is attributed to the benzene ring of the protecting group. Peaks g at 4.6, e at 4.0, and f at 3.1 ppm are assigned to protons of the PZLC block. Peaks at 5.2 and 1.4 ppm are assigned to protons of the PLLA block. As it is known, the data from the FT-IR spectrum is the magnification of the aggregate. (C) DLS graphs of micelle size distribution: (C-a) micelles directly prepared in H2O, (C-b) micelles after adding DTT, and (C-c) micelles after dialyzing against deionized water.

The 1H NMR spectrum of the PZLC-b-PLLA block copolymer is also confirmed by IR spectra (Figure S1 in the Supporting Information). Disappearance of the benzyl protective groups can be removed by acidolysis with a 33% solution of HBr in HAc.28,37 The deprotection of PZLC-b-PLLA is confirmed by 1H NMR (Figure 1D) and FT-IR (Figure S1C in the Supporting Information). Disappearance of the benzyl peaks at 4.6 and 7.4 ppm and at 749 and 697 cm−1 indicates complete removal of the protective groups. The GPC data of the deprotected polymer (Figure S2D in the Supporting Information) suggested that main chain cleavage in the polymers did not occur during the deprotection reaction. However, Figure 1D provides us some unexpected information. Along with the disappearance of characteristic NMR peaks of the benzyl protective groups, other peaks associated with the PLC segments also disappear, such as those at 4.0 and 3.1 ppm (Figure 1C versus D), as if the cysteine residues or even poly(L-cysteine) chains were lost during deprotection. To make it clear, the elemental compositions on the polymer surfaces determined by XPS are listed in Table 3. According to the molecular formulas shown in Scheme 1, sulfur is only present in the PZLC and PLC segments. Therefore, the sulfur content can provide evidence for the presence of the polypeptide. Although the XPS data are qualitative and there may be some unexpected information. Along with the disappearance of characteristic NMR peaks of the benzyl protective groups, other peaks associated with the PLC segments also disappear, such as those at 4.0 and 3.1 ppm (Figure 1C versus D), as if the cysteine residues or even poly(L-cysteine) chains were lost during deprotection. To make it clear, the elemental compositions on the polymer surfaces determined by XPS are listed in Table 3. According to the molecular formulas shown in Scheme 1, sulfur is only present in the PZLC and PLC segments. Therefore, the sulfur content can provide evidence for the presence of the polypeptide. As indicated in Table 3, the sulfur content increases from 0.0% for PLLA to 5.52% for PLC-b-PLLA, which can definitively prove the existence of PLC-b-PLLA on the film surface. Although the XPS data are qualitative and there may be experimental error, there is no doubt that there exist poly(L-cysteine) segments in the sample. Moreover, Ellman’s assay after reduction with NaBH4, confirmed that 8.8 thiol groups per copolymer chain are present, in agreement with DP_{PZLC} = 7 in the copolymer calculated from the 1H NMR spectrum. Therefore, most probably, the “disappearance” of PLC segments observed by 1H NMR is due to the formation of some structures which

c-5 carbonyl group of NCA.38 Therefore, NH2-PLLA is used as a macromolecular initiator to synthesize the diblock copolymer PZLC-b-PLLA according to Scheme 1.

The absorption peak at 3292 cm−1 is assigned to the C=C group. Peaks at 4.6, e at 4.0, and f at 3.1 ppm are assigned to protons of the PZLC block. Peaks at 5.2 and 1.4 ppm are assigned to protons of the PLLA block. As it is known, the data from the FT-IR spectrum is the magnification of the aggregate. (C) DLS graphs of micelle size distribution: (C-a) micelles directly prepared in H2O, (C-b) micelles after adding DTT, and (C-c) micelles after dialyzing against deionized water.

The structure of the copolymer PZLC-b-PLLA is also confirmed by IR spectra (Figure S1 in the Supporting Information). The absorption peak at 3292 cm−1 is assigned to ν_{C=O} of PZLC, and the peaks at 1630 cm−1 (ν_{C=O}) and 1523 cm−1 (ν_{C-O-NH}) are attributed to the amide I and amide II modes, respectively, indicating formation of the polypeptide block. The absorptions at 697 and 749 cm−1 from the phenyl group are characteristic of the protective groups in the PZLC block. The peaks at 1755 cm−1 (ν_{C=O}) and 1087 cm−1 (ν_{C-O-C}) are corresponding to the Phenyl protective groups, respectively, indicating formation of the polypeptide block. The absorptions at 697 and 749 cm−1 from the phenyl group are characteristic of the protective groups in the PZLC block. The peaks at 1755 cm−1 (ν_{C=O}) and 1087 cm−1 (ν_{C-O-C}) are corresponding to the Phenyl protective groups, respectively, indicating formation of the polypeptide block. Therefore, the sulfur content can provide evidence for the presence of the polypeptide. As indicated in Table 3, the sulfur content increases from 0.0% for PLLA to 5.52% for PLC-b-PLLA, which can definitively prove the existence of PLC-b-PLLA on the film surface. Although the XPS data are qualitative and there may be experimental error, there is no doubt that there exist poly(L-cysteine) segments in the sample. Moreover, Ellman’s assay after reduction with NaBH4, confirmed that 8.8 thiol groups per copolymer chain are present, in agreement with DP_{PZLC} = 7 in the copolymer calculated from the 1H NMR spectrum. Therefore, most probably, the “disappearance” of PLC segments observed by 1H NMR is due to the formation of some structures which

C-5 carbonyl group of NCA.38 Therefore, NH₂⁻PLLAA is used as a macromolecular initiator to synthesize the diblock copolymer PZLC-b-PLLA according to Scheme 1.

The absorption peak at 3292 cm−1 is assigned to the benzene ring of the protecting group. Peaks at 4.6, e at 4.0, and f at 3.1 ppm are assigned to protons of the PZLC block. Peaks at 5.2 and 1.4 ppm are assigned to protons of the PLLA block. As it is known, the data from the FT-IR spectrum is the magnification of the aggregate. (C) DLS graphs of micelle size distribution: (C-a) micelles directly prepared in H₂O, (C-b) micelles after adding DTT, and (C-c) micelles after dialyzing against deionized water.

The structure of the copolymer PZLC-b-PLLA is also confirmed by IR spectra (Figure S1 in the Supporting Information). The absorption peak at 3292 cm−1 is assigned to ν_{O-H} of PZLC, and the peaks at 1630 cm−1 (ν_{C=O}) and 1523 cm−1 (ν_{C-O-NH}) are attributed to the amide I and amide II modes, respectively, indicating formation of the polypeptide block. The absorptions at 697 and 749 cm−1 from the phenyl group are characteristic of the protective groups in the PZLC block. The peaks at 1755 cm−1 (ν_{C=O}) and 1087 cm−1 (ν_{C-O-C}) are corresponding to the Phenyl protective groups, respectively, indicating formation of the polypeptide block. Therefore, the sulfur content can provide evidence for the presence of the polypeptide. As indicated in Table 3, the sulfur content increases from 0.0% for PLLA to 5.52% for PLC-b-PLLA, which can definitively prove the existence of PLC-b-PLLA on the film surface. Although the XPS data are qualitative and there may be experimental error, there is no doubt that there exist poly(L-cysteine) segments in the sample. Moreover, Ellman’s assay after reduction with NaBH₄, confirmed that 8.8 thiol groups per copolymer chain are present, in agreement with DP_{PZLC} = 7 in the copolymer calculated from the ¹H NMR spectrum. Therefore, most probably, the “disappearance” of PLC segments observed by ¹H NMR is due to the formation of some structures which

Figure 5. (A) ESEM micrograph of the spherical micelles prepared in H₂O. (B) ESEM micrograph of the aggregates after adding DTT. The inset is the magnification of the aggregate. (C) DLS graphs of micelle size distribution: (C-a) micelles directly prepared in H₂O, (C-b) micelles after adding DTT, and (C-c) micelles after dialyzing against deionized water.
are insoluble in the solvent, for example, β-sheets or disulfide bonds.

Cell Adhesion and Spreading. Cell adhesion of various films was evaluated by culturing ECV-304 cells in a culture medium of DMEM containing 10% FBS. The test samples were copolymers PZLC-b-PLLA (see entry 1 in Table 2) and PLC-b-PLLA (see entry 2 in Table 3); the control sample was pure PLLA. The cells underwent an adhesion—proliferation process. Cell morphology changes during incubation can be seen clearly in Figure 2. After incubation for 3 h, the cell densities on the copolymers PZLC-b-PLLA and PLC-b-PLLA were much greater than that on pure PLLA. After incubation for 24 h, almost all cells on the copolymers PZLC-b-PLLA and PLC-b-PLLA spread very well, and they were fatter and more dense than those on the PLLA film. After incubation for 2 days, the cells on the copolymers almost occupied the whole surface. In short, the cells adhered, spread better, and proliferated faster on the copolymer films than on the control films. It should be noted that there was no appreciable difference basically in the cell adhesion and spreading between PZLC-b-PLLA and PLC-b-PLLA, probably because PLC-b-PLLA underwent thiol-to-sulfide conversion and its cell affinity became similar to that of PZLC-b-PLLA. The above experimental results indicate that the copolymer is a promising biodegradable material for cell and tissue engineering.

Characterization of Shell Cross-Linked Micelles. The diblock copolymers in this study are composed of a hydrophobic PLLA block and a hydrophilic PLCL block. The sulfur element exists in two forms, thiols or disulfides. It is well-known that a disulfide bond can be cleaved specifically into two thiols under mild conditions by using reagents such as dithiothreitol (DTT).\(^\text{16}\) Therefore, the block copolymer PLC-b-PLLA was first treated with DTT and then was allowed to self-assemble into micelles in aqueous solution. The micellar solution obtained was dialyzed against phosphate buffer of pH 6.8 to realize shell cross-linking via the formation of disulfide bonds (Scheme 2).\(^\text{42}\) The sample PLC-b-PLLA (see entry 2 in Table 3) was employed for micelle preparation. A pyrene probe was used to prove micelle formation. Figure 3 shows the excitation spectra of pyrene in aqueous solution. With increasing concentration of polymer solution, the fluorescence intensity increases, accompanied by a red shift from 333 to 335 nm. This is ascribed to micelle formation at a certain concentration. The pyrene probe is preferentially partitioned into the hydrophobic core of the micelle. The critical micelle concentration (cmc) value is obtained when the intensity ratio \(I_{333}/I_{335}\) is plotted against the solution concentration (Figure 4). It is \(5.5 \times 10^{-7}\) g/L. The micelle morphology and size were studied by ESEM and DLS after dialyzing against phosphate buffer of pH 6.8. ESEM studies show that the micelles of the copolymer assume a regular sphere shape with a mean diameter of about 50 nm (Figure 5A). As it is seen, all particles are dispersed very well, and almost no cohesion happens during drying. This can be confirmed by DLS results (Figure 5C-a). The hydrodynamic radius \(R_h\) is calculated from the DLS data by using the Stokes—Einstein equation, assuming that the micelles are of sphere shape.\(^\text{43}\) The average \(R_h\) value measured by DLS is 47.1 nm. The particle diameter measured by ESEM is a little smaller than \(2R_h\) measured by DLS, probably because of the volume shrinkage during sample drying. Ellman’s assay performed on the micelle solution confirmed that less than 6% of free thiols remained in the sample. The rest had been converted into disulfides during dialysis against phosphate buffer of pH 6.8. That is to say shell cross-linking of the micelles had taken place.\(^\text{44}\) Moreover, to prove the reversibility of shell cross-linking (SCL), DTT is used to reduce the disulfide bonds. A total of 50 equiv of DTT with respect to the thiol groups was added into the above micellar solution, followed by stirring for 2 h. ESEM and DLS were then used to examine changes of the micelles. It is noticed that the particle \(R_h\) of the spheres measured by DLS after DTT addition becomes 55.1 nm (Figure 5C-b), slightly bigger than the spheres (47.1 nm) in the original aqueous media. This may be attributed to the removal of the shell cross-linking caused by DTT, because the shell of the micelles may expand without cross-linking. Moreover, the smaller micelle size in the absence of DTT further indicates that the cross-linking occurs within individual micelles and not between micelles. Otherwise, the measured \(R_h\) value should have been much larger. The above process is reversible. As shown in Figure 5C-c, when DTT is removed by dialyzing against deionized water, the shell cross-linked micelle forms again so that the determined \(R_h\) goes back to 47.1 nm, similar to that before adding DTT (Figure 5C-a). Figure 5A and B shows the micelle morphology before and after DTT addition, respectively. Before DTT addition, small spherical particles are observed (Figure 5A). After DTT addition and subsequent dialysis, the same morphology as in Figure 5A is observed (data not shown). These observations are in agreement with DLS results, indicating that the micelles are shell cross-linked via disulfide bond formation. However, in Figure 5B, many large spheres are observed in addition to a few small spherical ones. With careful examination, these large strawberry-like spheres of \(\sim 150-280\) nm in diameter are actually composed of many spherical micelles of \(\sim 50\) nm. That is to say many small spheres fuse into large strawberry-like aggregates during ESEM sample preparation, probably due to water evaporation and DTT crystallization. During drying, oxygen in the air can make contact with the free thiol groups. Under such an oxidative environment, both intra- and intermicellar disulfide bonds may form, resulting in the strawberry-like aggregates. Therefore, Figure 5B does not represent the real morphology of the micelles in solution. As revealed by DLS measurements, in the presence of DTT, disulfide bonds are converted to thiol groups, and correspondingly, the shell cross-linked micelles lose their cross-linking and become normal micelles with extended polycysteine segments in the shell layer.

Conclusions

In this study, a novel diblock copolymer poly(t-cysteine)-b-poly(t-lactide) (PLC-b-PLLA) is synthesized by ROP of N-carboxyanhydride of β-benzoxycarbonyl-l-cysteine (ZLNC) in the presence of amino-terminated PLLA. The chemical structure of the block copolymer is confirmed by NMR, FT-IR, XPS, and GPC. The adhesion and spreading of ECV-304 cells on the copolymer films are better than those on PLLA films, suggesting the possibility of utilizing this copolymer as a biomaterial for drug delivery and tissue engineering. The diblock copolymer can form shell cross-linked micelles in aqueous media. The cmc value obtained by a pyrene probe is \(5.5 \times 10^{-7}\) g/L. The morphology of the micelles is characterized with ESEM and DLS, and the reversibility of the shell cross-linking is demonstrated by these two techniques. The introduction of polycysteine into aliphatic polyesters opens a way to prepare micelles with both thiol and disulfide functions, both of which are attractive for drug delivery systems. Further investigation is being...
undertaken, and the application of these copolymers will be published elsewhere.

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Supporting Information Available: IR spectra of the polymers (Figure S1) and GPC chromatographs of the polymers (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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