Communication

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Water-Soluble Poly(L-serine)s with Elongated and Charged Side-Chains: Synthesis, Conformations, and Cell-Penetrating Properties

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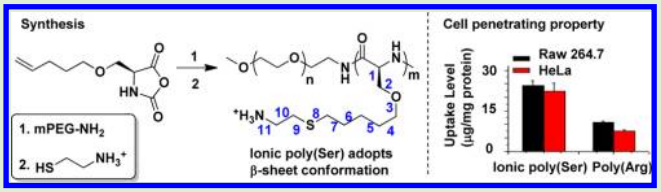
Supporting Information

ABSTRACT: Water-soluble poly(L-serine)s bearing long side-chain with terminal charge groups were synthesized via ring-opening polymerization of *O*-pentenyl-L-serine *N*-carboxyanhydride followed by thiol−ene reactions. These side-chain modified poly(L-serine)s adopt β-sheet conformation in aqueous solution with excellent stability against changes in pH and temperature. These water-soluble poly(L-serine) derivatives with charged side-chain functional groups and stable β-sheet conformations showed membrane-penetrating capabilities in different cell lines with low cytotoxicity.



INTRODUCTION

The secondary structures of proteins facilitate the folding of proteins into unique three-dimensional structures and regulate their biological activities.[1] To better understand protein folding/unfolding and eventually mimic their biological functions, there has been growing interest in designing and synthesizing water-soluble, synthetic polypeptides that adopt stable secondary conformations.[2−7] A variety of water-soluble polypeptides bearing neutral hydrophilic functional moieties[8−14] or groups[15−21] have been prepared, which can adopt stable α-helical or β-sheet conformations against various denaturing conditions. However, these polypeptides lack electrostatic property, which dictates critical interactions regulating the folding/unfolding and controlling the biological activities of these polypeptides.[22]

Water-soluble polypeptides electrolytes, such as negatively charged poly-L-glutamate and positively charged poly-L-lysine, have been extensively used in various applications. They have been used as the building blocks of self-assembly,[23] drug delivery vehicles[24] and coating materials.[25] However, they adopt random coil conformations in water solution at physiological conditions due to the electrostatic repulsion of their charged side chains, which tends to destabilize the helical conformation.[26] Much effort has been devoted to achieve water-soluble helical peptides with side-chain charged amino acid residues. The α-helical conformation of such peptides can be stabilized by side-chain hydrophobic interactions,[27] salt bridges,[28] or tethering of the side-chain functional groups situated on the same helical surface.[29] However, these strategies require the design of peptides with specific sequences that involve time-consuming, stepwise synthesis and tedious chemistry to control the orientation of peptide side chains.[30]

Recently, we demonstrated that the helical structure of cationic polypeptides can be stabilized by increasing the hydrophobic interaction of the side chain and minimizing the effect of side chain charge repulsion.[31,32] These ionic polypeptides with unusual helical stability were realized by maintaining a minimum separation distance of 11 σ-bonds between the peptide backbone and the side chain charge. Further enhanced helicity and helical stability were achieved when the side chain charges are situated even further away from the peptide backbone.[33] Nevertheless, all these designs of polypeptides are based on glutamate, an amino acid with high helical propensity. Ionic polypeptide derivatives from other amino acids, especially those atypical helix-favoring amino acids (e.g., L-serine), have never been investigated. Doruker and Bahar have observed that the peptide with serine residue has higher unwinding rate of helical states than the peptides with alanine or valine residues.[34] However, in a separate study, Hwang and Deming designed and synthesized poly(L-serine) with pendant oligoethylene glycol moieties.[35] They observed a random coil to β-sheet transition with the addition of methanol to the aqueous polypeptide solution but did not observe the formation of helical conformation. Here, we report the design and synthesis of a poly(L-serine) bearing terminally charged long hydrocarbon side chains and evaluation of their physical and biological properties, aiming to expand our understanding of polypeptide electrolytes. These polypeptides adopt stable β-sheet conformation against external environmental changes (e.g., pH and temperature) and showed interesting cell membrane permeability in several cell-lines with low cytotoxicity. To our knowledge, this is the first demonstration of cell membrane penetration with a peptide containing a large portion of β-sheet instead of α-helical conformation.

EXPERIMENTAL SECTION

Materials. Anhydrous dimethylformamide (DMF) was dried by columns packed with 4 Å molecular sieves and stored in a glovebox.

Received: June 21, 2012
Revised: July 28, 2012
Published: August 1, 2012

Tetrahydrofuran (THF), ethyl acetate (EtOAc) and hexane were dried by columns packed with alumina and stored in a glovebox. PEG_{112}-NH_2 (MW = 5000) was purchased from Laysan Bio Inc. (Arab, AL, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Raw264.7 (mouse monocyte macrophage) and HeLa (human cervix adenocarcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS).

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Varian U500 MHz or a UI500NB MHz spectrometer. Chemical shifts were reported in ppm and referenced to the solvent proton and ^{13}C resonances. Size-exclusion chromatography (SEC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multiangle laser light scattering (MALLS) detector, and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 10^3 Å, and 10^4 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 M LiBr as the mobile phase. The MALLS detector is calibrated using pure toluene with no need for calibration using polymer standards and can be used for the determination of the absolute molecular weights (MWs). Infrared spectra were recorded on a Perkin-Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film. Circular dichroism (CD) measurements were carried out on a JASCO J-700 CD spectrometer. The polymer samples for CD analysis were prepared at concentrations of 0.1−2.5 mg/mL in general unless otherwise specified. The solution was placed in a quartz cell with a path length of 0.2 cm. The mean residue molar ellipticity of each polymer was calculated based on the measured apparent ellipticity by following the literature reported formula: Ellipticity ($[\theta]$ in deg·cm^2·dmol^{-1}) = (millidegrees × mean residue weight)/(path length in millimeters × concentration of polypeptide in mg/mL). Lyophilization was performed on a FreeZone lyophilizer (Labconco, Kansas City, MO, USA). UV light was generated by an OmiCure S1000 UV lamp (EXFO, Mississauga, Canada).

Synthesis of *O*-Pentenyl-L-serine (3). In an ice−water bath, Boc-Ser-OH (6.7 g, 33.6 mmol) was dissolved in anhydrous DMF (100 mL) followed by the slow addition of sodium hydride (1.7 g, 95%, 67.2 mmol). Thirty minutes later, 5-bromo-1-pentene (10.0 g, 67.2 mmol) was added in one portion. The reaction mixture was stirred at room temperature for 16 h. DMF was distilled at 60 °C under vacuum. The residue was stirred in water (75 mL) for 16 h, followed by extracting with diethyl ether (30 mL × 2). The aqueous layer was acidified to pH 3 with 1N HCl and then extracted with EtOAc (50 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, collected by filtration and removed under vacuum to yield the product as a yellow oil (**2**, 7.0 g, 76% yield). The deprotection of **2** (7.0 g, 25.6 mmol) was performed in HCl/dioxane (4 M, 150 mL) by stirring the reaction solution at room temperature for 4 h. The solid precipitate was collected by centrifuge and purified by washing with diethyl ether (100 mL). The resulting product, *O*-pentenyl-L-serine (**3**, 4.0 g, 90% yield), was dried at room temperature under vacuum for 8 h before use. ^{1}H NMR (500 MHz, D_2O): δ 5.74 (m, 1H, CH_2=C*H*CH_2−), 4.88 (m, 2H, CH_2=$CHCH_2$−), 4.01 (m, 1H, −C*H*NH_2), 3.90 (m, 2H, −O$CH_2$$CHNH_2$), 3.44 (m, 2H, CH_2=$CHCH_2CH_2CH_2$−), 1.97 (m, 2H, CH_2=$CHCH_2CH_2CH_2$−) and 1.54 (m, 2H, CH_2=$CHCH_2CH_2CH_2$−).

Synthesis of *O*-pentenyl-L-serine-based *N*-carboxylanhydride (PE-L-Ser NCA, 4). *O*-pentenyl-L-serine **3** (1.0 g, 4.8 mmol) and triphosgene (1.1 g, 3.7 mmol) were mixed in anhydrous THF (15 mL) in a round-bottomed flask. The reaction was stirred at room temperature for 16 h. The solvent was distilled at room temperature under vacuum to yield a brown liquid residue which was purified by silica gel chromatography (Note: the silica gel was dried at 120 °C under vacuum for 8 h before use) with hexane as the eluent containing EtOAc gradient (from 0% to 40%). A light yellow liquid product was obtained (0.8 g, 83% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.74 (s, 1H, -NH), 5.75 (m, 1H, CH_2=C*H*$CH_2CH_2CH_2$−), 5.22 (t, 2H, CH_2=$CHCH_2CH_2CH_2$−), 4.43 (t, 1H, −C*H*NH_2), 3.73 (d, 2H, -OCH_2CHNH), 3.46 (m, 2H, CH_2=$CHCH_2CH_2CH_2$-), 2.04 (m, 2H, CH_2=$CHCH_2CH_2CH_2$−) and 1.61 (m, 2H, CH_2=$CHCH_2CH_2CH_2$−). ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.0, 153.0, 138.0, 115.3, 71.4, 68.7, 58.8, 30.2, 28.6.

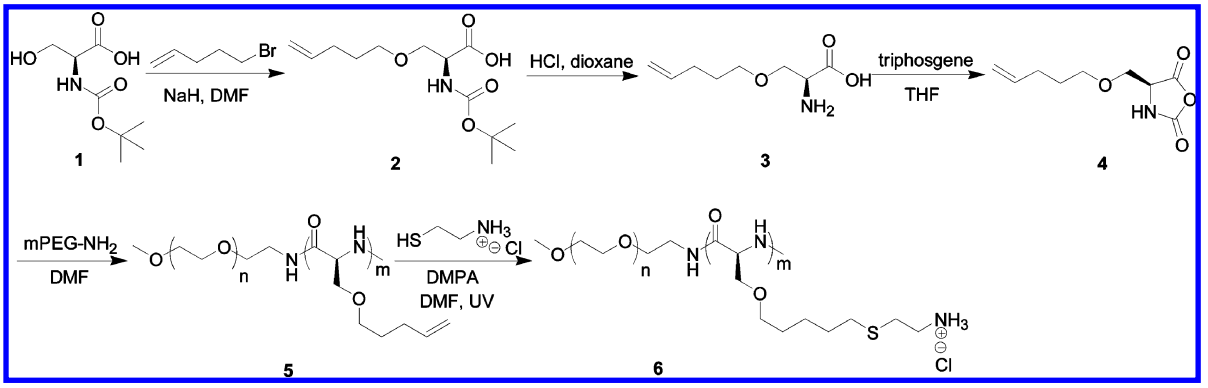
Synthesis of Polyethylene Glycol-*block*-Poly(*O*-pentenyl-L-serine) (PEG-*b*-PPELS, 5). In a glovebox, PEG_{114}-NH_2 (22.5 mg, 4.5 μmol of amino groups) and **4** (90 mg, 450 mmol) were dissolved in DMF (2.0 mL). The polymerization was carried out at room temperature for 48 h. Fourier transform infrared (FTIR) was used to monitor the polymerization until the conversion of **4** was above 99%. The product **5** in DMF solution was directly used for the next-step reaction. A small portion of **5** was isolated for characterization. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.76 (m, 1H, CH_2=C*H*$CH_2CH_2CH_2O$−), 4.95 (m, 2H, CH_2=$CHCH_2CH_2CH_2O$−), 4.43−4.24 (m, 3H, C*H*CH_2−), 3.62 (s, 4H, −CH_2CH_2O− of PEG), 3.55 (m, 2H, CH_2=$CHCH_2CH_2CH_2O$−), 2.05 and 1.62 (m, 4H, CH_2=$CHCH_2CH_2CH_2O$−). It should be noted that **5** was characterized by NMR immediately after the polymerization solution was concentrated under vacuum. If **5** is stored in solid form for an extended period of time, it is no longer soluble in common organic solvents (e.g., DMF and $CHCl_3$), presumably due to the interchain H-bonding of **5** that has a large portion of β-sheet conformation and side-chain hydrophobic interaction that makes it very difficult for solvent molecules to get back in once they are depleted (completely dried).

Synthesis of Poly(ethylene glycol)-*block*-Poly(*O*-pentenyl-L-serine-*graft*-cysteamine) (PEG-*b*-(PPELS-*g*-CA), 6). In a quartz flask, cysteamine hydrochloride (100 mg, 0.75 mmol, 1.7 equiv. of the allyl groups) and photoinitiator (2,2-dimethoxy-2-phenyl acetophenone, DMPA, 5 mg) were added to the polymerization solution (**5**, 0.45 mmol in 2 mL DMF), followed by purging with N_2 for 5 min. The quartz flask was sealed and irradiated by UV (365 nm, 16 mW) for 30 min. HCl (1N, 5 mL) was added to the DMF solution, and the resulting mixture was dialyzed against deionized (DI) water in a dialysis bag with a cutoff molecular weight of 1000 g/mol. After lyophilization, **6** in a white solid form was obtained (43 mg, 36% yield). ^{1}H NMR (500 MHz, D_2O): δ 3.66 (br, 2H, $CHCH_2O$−), 3.57 (br, 4H, −CH_2CH_2O− of PEG), 3.41 (br, 3H, C*H*CH_2O− and −O$CH_2CH_2CH_2CH_2CH_2S$−), 3.09 (br, 2H, −$SCH_2CH_2NH_3Cl$), 2.72 (br, 2H, −$SCH_2CH_2NH_3Cl$), 2.47 (br, 2H, −$OCH_2CH_2CH_2CH_2CH_2S$−), 1.48 (br, 4H, −$OCH_2CH_2CH_2CH_2CH_2S$−), 1.28 (br, 2H, −$OCH_2CH_2CH_2CH_2CH_2S$−).

Synthesis of Rhodamine−Polypeptide Conjugates. PEG-*b*-(PPELS-*g*-CA) (10 mg, 3×10^{-5} mol of primary amine) was dissolved in a $NaHCO_3$ solution (4 mL, 0.2 M). Rhodamine B isothiocyanate (RhB-NCS, 0.83 mg, 1.5 μmol) in dimethyl sulfoxide (DMSO; 83 μL) was added to the polymer solution. The reaction vial was wrapped with aluminum foil. The reaction solution was stirred at room temperature for 12 h followed by dialysis in DI H_2O to yield rhodamine-labeled polypeptide (8.0 mg, 80% yield).

Cell Uptake. Cells were seeded in a 96-well plate at a density of 10 000 cells/well. The medium was replaced with serum-free DMEM 24 h later, followed by the addition of the rhodamine-polypeptide. After incubation for a predefined period of time, the cells were washed with cold phosphate-buffered saline (PBS; 3 × 200 μL, containing 20 U/mL heparin) to completely remove surface-bound cationic proteins from the cells.[36] Cells were then lysed with 100 μL RIPA buffer; the rhodamine content in the lysate was quantified by spectrofluorimetry. A series of rhodamine−polypeptide solutions were prepared through the dilution of the stock solution (1 mg/mL) with RIPA lysis buffer to final concentrations of 100, 80, 60, 40, 20, 10, or 5 μg/mL. Regression curves were obtained by plotting the fluorescence intensity of the standard solution (E_x = 560 nm, E_m = 590 nm) against concentration (μg/mL). The amount of rhodamine−polypeptide in the cell lysate was calculated based on the regression curve. The protein level in the cell lysate was determined by the BCA kit. The uptake level was

Scheme 1. Synthesis of PEG-*b*-(PPELS-*g*-CA) (6)



expressed as micrograms of polypeptide relative to 1 mg of cellular protein.

Cell Uptake Mechanisms. To explore the mechanism of polypeptide internalization, cells were preincubated with one of an endocytosis inhibitor (chlorpromazine (10 μg/mL), genistein (100 μg/mL), methyl-β-cyclodextrin (mβCD, 5 mM), wortmannin (10 μg/mL), or dynasore (80 μM)) for 30 min prior to the addition of polypeptide, which was present in the cell culture medium throughout the 2-h uptake experiment at 37 °C. Similar cell uptake experiments were also performed at 4 °C. Results were expressed as percentage uptake level of the control cells, which were incubated with polypeptides at 37 °C for 2 h in the absence of endocytic inhibitors.

Cytotoxicity Measurement. Cells were seeded in a 96-well plate at a density of 10 000 cells/well and incubated for 24 h at 37 °C. The medium was replaced with serum-free DMEM. Polypeptides were then added at 10, 5, 2, 1, and 0.5 μg/well, respectively. Cells were incubated for 4 h, and then the medium was replaced with serum-containing DMEM. After another 20-h incubation at 37 °C, cell viability was assessed using the standard MTT assay.

RESULTS AND DISCUSSION

O-Pentene-L-serine *N*-carboxylanhydride (PE-L-Ser NCA, **4**) was synthesized in three steps from Boc-Ser-OH (**1**) with an overall yield of 50−60% (Scheme 1). Specifically, **4** was synthesized via the etherification of **1** with 5-bromo-1-pentene in the presence of sodium hydride to afford **2**, followed by the deprotection of *tert*-butyloxycarbonyl group of **2** in HCl/dioxane and the cyclization of *O*-pentenyl-L-serine (**3**) with triphosgene. **4** is readily soluble in THF, EtOAc, ethyl ether, dichloromethane, and chloroform, but insoluble in hexane. Purification of **4** via recrystallization was attempted but only resulted in a dark-colored oily residue. Therefore, a recently developed purification method by Deming and co-workers through the use of silica gel chromatography was used to purify **4**,[37] which yielded a colorless oil with satisfactory purity. The molecular structure of **4** was verified by NMR (Figure 1) and FTIR (Figure S1).

The NCAs can be polymerized using primary amines and hexamethyldisilazane (HMDS).[38,39] However, the resulting polymers showed poor solubility in common organic solvents, such as DMF, chloroform, dichloromethane, and THF, which made it difficult to assess the MWs of these polymers. PEG_{112}-NH_2 was then used to initiate the ring-opening polymerization of **4** in DMF, aiming to obtain polymers with improved solubility yet undistorted polypeptide conformation via terminal grafting of PEG that has excellent solubility in common organic solvents and water. The MWs of the PEG-poly(**4**) (denoted as PEG_n-*b*-$PPELS_m$) were determined by ^{1}H NMR and SEC. PEG_n-*b*-$PPELS_m$ with a PPELS segment up to

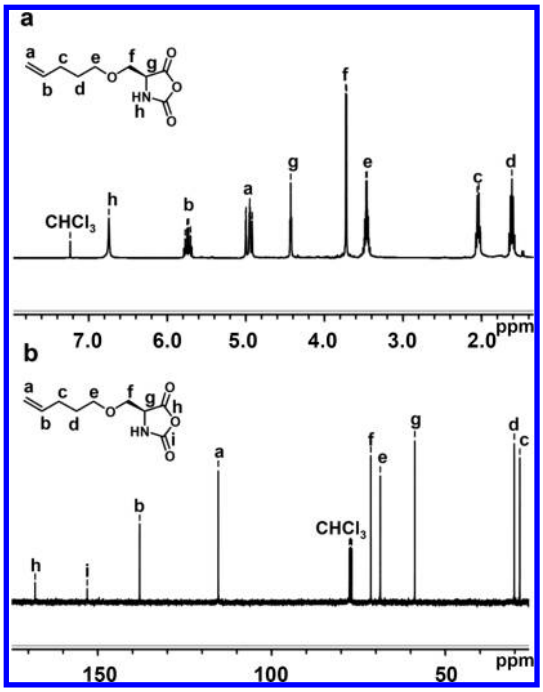


Figure 1. (a) ^{1}H and (b) ^{13}C NMR spectra of PE-L-Ser NCA (**4**) in $CDCl_3$.

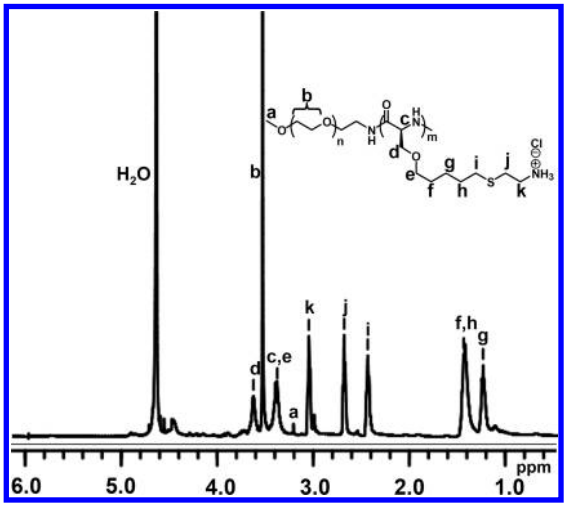


Figure 2. ^{1}H NMR spectrum of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) (**6**) in D_2O.

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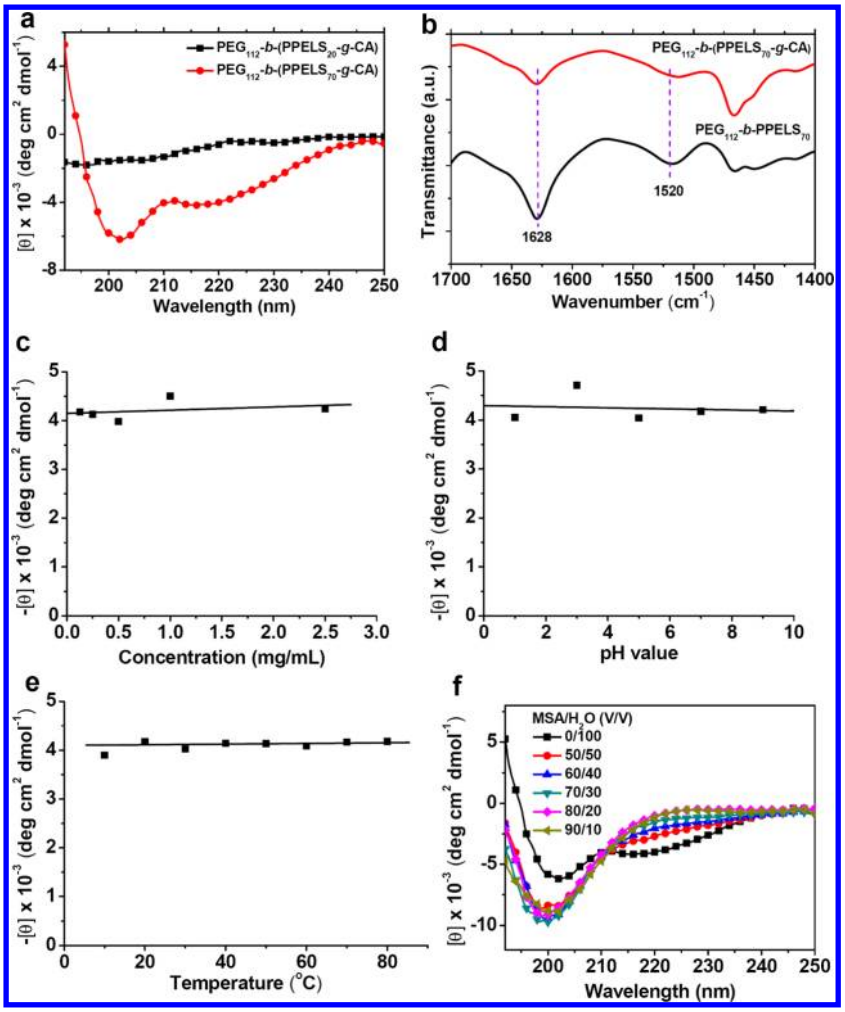

Figure 3. (a) CD spectra of PEG_{112}-*b*-($PPELS_{20}$-*g*-CA) and PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in water. (b) FTIR spectra of PEG_{112}-*b*-$PPELS_{70}$ and PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in the solid state. (c) The concentration dependence of the residue molar ellipticity at 216 nm for PEG_{112}-*b*-($PPELS_{70}$-*g*-CA). (d) The pH dependence of the residue molar ellipticity at 216 nm for PEG_{112}-*b*-($PPELS_{70}$-*g*-CA). (e) The temperature dependence of the molar ellipticity at 216 nm for PEG_{112}-*b*-($PPELS_{70}$-*g*-CA). (f) CD spectra of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in a mixed solvent of MSA and H_2O.

70 mer (m = degree of polymerization (DP) = 70) was prepared, which was verified by SEC (Figure S2) and ^{1}H NMR (Figure S3). A prominent peak shift was observed by comparing the SEC traces of PEG_{112}-NH_2 and PEG_{112}-*b*-$PPELS_{70}$ (Figure S2), indicating the successful synthesis of PEG_n-*b*-$PPELS_m$. The PPELS with a DP larger than 70 in the PEG_n-*b*-$PPELS_m$ was also attempted. However, precipitation was observed in the DMF polymerization solution. The precipitates, presumably aggregated PEG_n-*b*-$PPELS_m$ with a PPELS block larger than ca. 70 repeating units, was insoluble in most common organic solvents including THF and chloroform.

Even for a PEG_n-*b*-$PPELS_m$ prepared with a DP < 70 that is soluble in the DMF polymerization solution, it becomes insoluble in DMF after it is dried and stored for an extended period of time. Because PEG_n-*b*-$PPELS_m$ has very hydrophobic side chains, it should be very difficult for the polar organic solvent molecules, such as DMF, to return to the nonpolar, hydrophobic side chain areas and solvate (redissolve) the aggregated polypeptide side chains once the organic solvent molecules are depleted during the drying process. Therefore, the polymerization solutions of PEG_n-*b*-$PPELS_m$ were directly used for the thiol−ene "click" reaction, which was performed inside a quartz flask under N_2 with catalytic amount of DMPA, the photo initiator, and purified by dialysis. Highly water-soluble (solubility >5 mg/mL) ionic poly(L-serine) analogues (**6**) were obtained after freeze-drying. The molecular structure of **6** was verified by ^{1}H NMR (Figure 2). A complete disappearance of the characteristic peaks of vinyl groups (CH_2═CH−) (Figure 2 and Figure S3) indicates the quantitative grafting efficiency.

The solution conformations of PEG-*b*-(PPELS-*g*-CA)s were studied by CD spectroscopy. PEG_{112}-*b*-($PPELS_{20}$-*g*-CA) showed no Cotton effect in the scanning range between 190 and 250 nm, while PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) showed two negative dichroic peaks at 202 and 216 nm (Figure 3a). CDPro (CONTIN method) was used to determine the secondary structure of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA), which indicated that PEG_{112}-*b*-($PPELS_{70}$-g-CA) adopts 45% of β-sheet and significant portion of random coil. FTIR results revealed an amide I peak at 1628 $cm^{-1}$and an amide II peak at 1520 $cm^{-1}$for PEG_{112}-*b*-($PPELS_{70}$-*g*-CA), confirming that PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) adopts β-sheet conformation in the solid state (Figure 3b), which is consistent with the conformation determined by CD. While PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) has comprehensive β-sheet conformations in aqueous solution, it showed concentration independence of its $-[\theta]_{216}$ values (Figure 3c) and remarkable stability against pH changes (from pH 1 to pH 9, Figure 3d). These properties of PEG_{112}-*b*-

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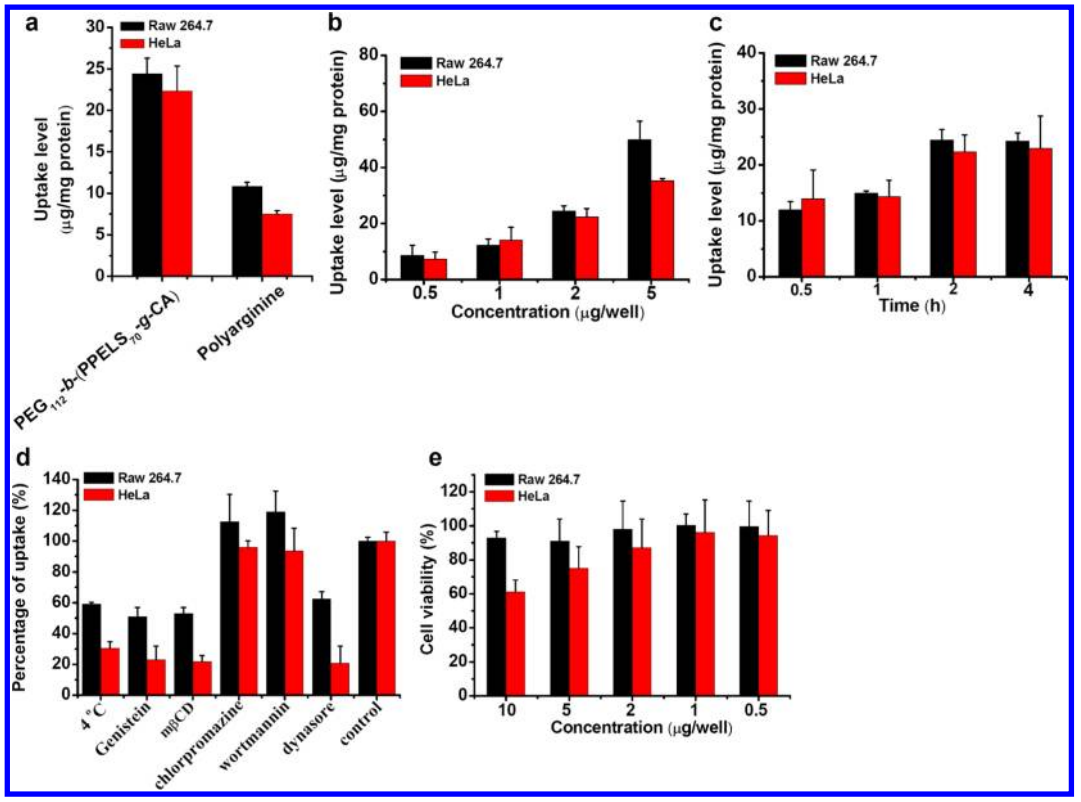

Figure 4. (a) Uptake level of rhodamine-labeled PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in Raw 264.7 and HeLa cells. Cells in 96-well plates were treated with polypeptides (2 μg/well) for 2 h, and the uptake level was expressed as μg polypeptide associated with 1 mg cellular protein (n = 3). (b) Concentration-dependent uptake level of rhodamine-labeled PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in Raw 264.7 and HeLa cells. Cells in 96-well plates were treated with polypeptides (0.5, 1, 2, and 5 μg/well) for 2 h (n = 3). (c) Time-dependent uptake level of rhodamine-labeled PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in Raw 264.7 and HeLa cells. Cells in 96-well plates were treated with polypeptides (2 μg/well) for 0.4, 1, 2, and 4 h, respectively (n = 3). (d) Elucidation of the mechanisms underlying cell uptake of polypeptides. Cells were treated with rhodamine-labeled PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) (2 μg/well) for 2 h in the presence of various endocytic inhibitors, and results were expressed as percentage uptake (%) of control cells which were incubated in the absence of endocytic inhibitors (n = 3). (e) Cytotoxicity of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) as determined by the MTT assay. Cells in 96-well plates were incubated with polypeptides at different concentrations for 4 h in serum-free media, and were further cultured in serum-containing media for another 20 h before the cell viability assessment. Results were expressed as percentage viability of control cells that did not receive polypeptide treatment (n = 3).

($PPELS_{70}$-*g*-CA) resemble the properties of ionic poly(L-glutamate)s bearing elongated and charged side-chains.[27] Trifluoroethanol (TFE) and methanol are well-known solvents to induce the formation and enhance the stability of secondary structures of peptides.[35,40] For example, Hwang and Deming have reported a random coil to β-sheet transition by adding methanol to the aqueous solution of PEGylated poly(L-serine)s.[35] We did similar experiments by using these solvents to trigger the conformation transitions of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in the aqueous solution, but observed an unusual stability of the β-sheet conformation of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) by varying the fraction of MeOH or TFE (Figure S4) in water. PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) also showed excellent stability against elevated temperatures (Figure 3e), with negligible change of $-[\theta]_{216}$ value at a temperature ranging from 10 to 80 °C. A typical secondary structure transition was observed when increasing the volume fraction of methanesulfonic acid (MSA), a very strong peptide denaturing agent (Figure 3f), presumably due to the destruction of the hydrogen bonding. By comparing our results with those reported by Deming,[35] it is apparent that the hydrophobic side chains play an important role in stabilizing the β-sheet conformation of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA). We also noticed that PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in aqueous solution showed some degree of aggregation based on the analysis of dynamic light scattering. It is unclear, however, how exactly the hydrophobicity of side chains improves the β-sheet stability, which is subjected to further study.

Cationic polypeptides with rigid molecular structures, mostly α-helical conformations, have been previously demonstrated to show membrane activities.[41−43] A report of using a β-sheet containing cationic polypeptide to mediate cell membrane permeation has not been reported in the literature to the best of our knowledge. We were motivated to explore the cell penetration potentials of the resulting ionic poly(L-serine)s with rich β-sheet conformation using rhodamine-labeled polypeptide. HeLa, a cervical cancer cell line, and Raw 264.7, a macrophage cell line, were selected for the cell penetration assessment. As shown in Figure 4a, PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) showed interesting cell penetrating properties and outperformed polyarginine (5000−15000 Da), a well-known membrane active polypeptide, with respect to the cell uptake.[23] The cell uptake level increased with incubation time and the feeding amount, and the maximal cell uptake level was noted 2 h post polypeptide treatment (Figure 4b,c). To further understand the mechanisms underlying the polypeptide-mediated cell penetration, we performed the cell uptake study at lower temperature (4 °C) or in the presence of various

dx.doi.org/10.1021/bm3009445 | *Biomacromolecules* 2012, 13, 2609−2615

endocytic inhibitors. The cell uptake level was inhibited by 40−70% at 4 °C when energy-dependent endocytosis was completely blocked (Figure 4d), suggesting that part of the polypeptide was internalized through endocytosis while the remaining entered the cells via energy-independent transduction. Genistein and mβCD inhibited the cell uptake process, while chlorpromazine showed unappreciable inhibitory effect, suggesting that the endocytosis was related to the caveolae- rather than clathrin-mediated pathway. Furthermore, the cell uptake level was decreased in the presence of dynasore, which inhibited both the clathrin- and caveolar-mediated pathways by inhibiting dynamins, further substantiating the above observed results. Unappreciable inhibitory effect was observed for wortmannin, indicating that macropinocytosis was not involved. Despite their desired cell penetrating properties, the resulting polypeptides showed good cell tolerability as determined by the MTT assay, as evidenced by slight toxicity at high concentrations (5 and 10 μg/well) and unappreciable toxicity at concentrations less than or equal to 2 μg/well (Figure 4e). Such results further supported that the cell penetration was achieved without compromising the cell viability and biological functions.

CONCLUSIONS

In summary, PEG_{112}-*b*-($PPELS_{70}$-*g*-CA), a water-soluble, poly(L-serine) analogue with elongated, positively charged side chains was synthesized through mPEG-NH_2-initiated ring-opening polymerization of *O*-pentenyl-L-serine *N*-carboxyanhydrides followed by thiol−ene reactions to introduce the charged groups. PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) adopted a mixed β-sheet and random coil conformation, and the β-sheet conformation was stable against changes in pH and temperature. The resulting polypeptides showed cell-penetrating properties. We are currently investigating whether these materials can be used as gene delivery vehicles.

ASSOCIATED CONTENT

Supporting Information

FTIR spectrum of PE-L-Ser NCA (**4**) (Figure S1), SEC traces of PEG_{112}-NH_2 and PEG_{112}-*b*-$PPELS_{70}$ (Figure S2), ^{1}H NMR spectrum of PEG_{112}-*b*-$PPELS_{70}$ (**5**) in $CDCl_3$ (Figure S3). The MW of PEG-*b*-PPELS can be calculated from the integrations of H_b and H_g (Figure S3). MeOH and TFE concentration dependence of the molar ellipticity at 216 nm for PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) (Figure S4). CD spectra of PEG_{112}-*b*-($PPELS_{70}$-*g*-CA) in aqueous solutions against various changes (Figrue S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by the NIH (R21 1R21EB013379 and Director's New Innovator Award 1DP2OD007246). We thank Professor Martin Gruebele for allowing us to use their CD spectrometer.

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