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Thiol-responsive gemini poly(ethylene glycol)-poly(lactide) with a cystine disulfide spacer as an intracellular drug delivery nanocarrier

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ABSTRACT

Thiol-responsive gemini micelles consisting of hydrophilic poly(ethylene glycol) (PEG) blocks and hydrophobic polylactide (PLA) blocks with a cystine disulfide spacer were reported as effective intracellular nanocarriers of drugs. In the presence of cellular glutathione (GSH) as a reducing agent, gemini micelles gradually destabilize into monomeric micelles through cleavage of the cystine linkage. This destabilization of the gemini micelles changed their size distribution, with the appearance of small aggregates, and led to the enhanced release of encapsulated doxorubicin (DOX). The results obtained from cell culture via confocal laser scanning microscopy (CLSM) for cellular uptake, as well as cell viability measurements for anticancer efficacy suggest the potential of disulfide-based gemini polymeric micelles as controlled drug delivery carriers.

1. Introduction

Gemini or dimeric surfactants consisting of two hydrophobic chains and two polar headgroups that are covalently linked by a rigid or flexible spacer have attracted much attention [1–6]. Compared with monomeric surfactants, gemini surfactants exhibit low critical micelle concentrations (CMCs), higher efficiencies at reducing surface tension, and higher viscoelasticities [1–6]. These properties make gemini surfactants of special interest for biological and biomedical applications. Gemini surfactants appear to be adequate drug carriers due to their much lower CMC values and higher solubilization capacities compared with those of the corresponding monomeric surfactants [7–10]. These characteristics suggest that gemini surfactants with a degradable or stimuli-sensitive spacer can form micelles for controlled drug release. Among the various degradable linkages, disulfide-thiol exchange chemistry has been employed in the design of redox-responsive drug delivery systems for site-specific drug release. In biological systems, the concentration of glutathione (GSH), a cysteine-containing tripeptide produced in the cell cytoplasm, is approximately10 mM in the cytosol, whereas it is approximately 10 μM in the plasma [11,12]. Importantly, the concentration of GSH in some tumors has been known to be several times higher than that in normal cells [13]. Therefore, polymeric micelles containing disulfide bonds have been prepared for redox-responsive drug delivery [14–27]. These assemblies exhibit the controlled release of encapsulated drugs through the cleavage of disulfide bonds, causing destabilization or disintegration of the micellar aggregates. It has been reported that gemini surfactants with a disulfide spacer degrade into monomeric surfactants in a reductive environment, causing significant changes in the CMC, surface tension, and aggregation behavior [28–30]. Disulfide-based cationic gemini surfactants have been investigated as a gene delivery system for the modulated release of DNA in a reductive environment [31–33]. Although gemini surfactants containing disulfide bonds have been studied, there are no reports on gemini surfactants containing a disulfide spacer as carriers of hydrophobic drugs.

Recently, we reported on thiol-responsive micelles of nonionic gemini surfactants with cystine disulfide spacers consisting of hydrophilic PEG and hydrophobic stearoyl groups [34]. The nanosized assemblies of this surfactant showed the adjustable release of encapsulated indomethacin (IMC) as a model hydrophobic drug depending on the GSH concentration. In this study, we synthesized gemini $(PEG)_2$-Cys-$(PLA)_2$ with cystine disulfide as the spacer, as well as monomeric PEG-PLA, to investigate their solution properties and further evaluate the use of thiol-responsive gemini micelles as

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effective intracellular drug delivery carriers. The disintegration or destabilization of the gemini micelles upon cleavage of the cystine disulfide linkages in the presence of GSH was investigated using gel permeation chromatography (GPC) and dynamic light scattering (DLS). The destabilization of the gemini micelles led to enhanced release of encapsulated doxorubicin (DOX), a hydrophobic anticancer drug. Furthermore, the GSH-responsive destabilization of DOX-loaded gemini micelles was investigated in cells using confocal laser scanning microscopy (CLSM) to monitor cellular uptake and cell viability assays to evaluate anticancer efficacy.

2. Materials and experimental procedures

2.1. Materials

Methoxypoly(ethylene glycol) (mPEG) with a molecular weight of 2000 g/mol, lactide, N_α,N_α'-di-Boc-L-cystine (Boc-Cys-OH)$_2$, succinic anhydride, trifluoroacetic acid (TFA), triethylamine (TEA), and *N*,*N*′-dicyclohexylcarboiimide (DCC) were purchased from Aldrich and used as received. KB cells were obtained from American Tissue Culture Collection. Eagle's Minimum Essential Medium (EMEM) was purchased from Life Technologies. Trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were obtained from HyClone Laboratory. EGM-2 cell culture medium was purchased from Lonza. A Cell Counting Kit-8 (CCK-8) was obtained from Enzo Life Science.

2.2. Measurements

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker NMR spectrometer (AVANCE III 400). The molecular weight and molecular weight distribution of the polymer were determined using gel permeation chromatography (GPC) (1515, Waters) equipped with a Waters 2414 refractive index detector and Waters Styragel HR columns. The size and size distribution in hydrodynamic diameter by volume were measured using dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments). Transmission electron microscopy (TEM) was performed using a Tecnai G_2 microscope (FEI Company, USA) at an acceleration voltage of 120 kV. The samples were stained with uranyl acetate. UV–vis absorption spectra were obtained using a Cary 100 Conc UV-vis spectrometer (Varian).

2.3. Synthesis

Synthesis of PLA and PLA-COOH: Butyl alcohol (0.3 g, 4.16 mmol) and D,L-lactide (6.0 g, 41.7 mmol) were introduced into a polymerization tube, and stannous octoate corresponding to 0.5 mol% of lactide was added. The tube was sealed, and the polymerization was allowed to proceed at 130 °C for 24 h. Then, the polymer was dissolved in methylene chloride and precipitated in an excess amount of cold diethyl ether. The precipitate was filtered, washed several times with diethyl ether, and dried under a vacuum to yield 5.2 g of product. The molecular weight calculated by NMR was 1140 g/mol. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.29–5.13 (m, –C(O)*CH*CH$_3$–), 4.13 (t, –O*CH$_2$*CH$_2$CH$_2$CH$_3$), 1.49–1.44 (m, –*CH$_3$*CHO–), 1.36–1.19 (m, –OCH$_2$*CH$_2$CH$_2$*CH$_3$), 0.87 (t, –OCH$_2$CH$_2$CH$_2$*CH$_3$*).

PLA (3.0 g, 2.63 mmol) and succinic anhydride (0.32 g, 3.20 mmol) were dissolved in $CHCl_3$ (50 mL), and TEA (0.32 mL, 3.20 mmol) was added to the solution. The reaction was refluxed at 70 °C for 24 h. The reaction solvent was evaporated, and the resulting product was precipitated in an excess amount of cold diethyl ether. The precipitate was filtered, washed several times with diethyl ether, and dried under a vacuum to obtain 2.78 g of product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.29–5.13 (m, –C(O)*CH*CH$_3$–), 4.13 (t, –O*CH$_2$*CH$_2$CH$_2$CH$_3$), 2.76 (d, –C(O)*CH$_2$CH$_2$*C(O)–), 1.49–1.44 (m, –*CH$_3$*CHO–), 1.36–1.19 (m, –OCH$_2$*CH$_2$CH$_2$*CH$_3$), 0.87 (t, –OCH$_2$CH$_2$CH$_2$*CH$_3$*).

Synthesis of PEG-PLA: PEG (0.50 g, 0.25 mmol) and PLA-COOH (0.36 g, 0.68 mmol) were dissolved in anhydrous dichloromethane (20 mL), and DCC (0.08 g, 0.39 mmol) was added to the solution. The reaction was allowed to proceed at room temperature for 3 days. The insoluble dicyclohexylurea was removed by filtration. The product was precipitated in diethyl ether, filtered, and dried at room temperature under a vacuum. The crude product was dissolved in deionized (DI) water. The solution was filtered to remove the unreacted PLA-COOH, dialyzed (molecular weight cut-off [MWCO]: 2000 Da) against deionized water for 3 days to remove the residual mPEG, and then lyophilized to obtain 0.68 g of product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.29–5.13 (m, –C(O)*CH*CH$_3$–), 4.22 (t, –C(O)O*CH$_2$*CH$_2$O–), 4.12 (t, –C(O)O*CH$_2$*CH$_2$CH$_2$CH$_3$), 3.65–3.54 (m, –*CH$_2$CH$_2$*O), 3.39 (s, *CH$_3$*O–), 2.71 (d, –C(O)*CH$_2$CH$_2$*C(O)–), 1.26–1.19 (d, –OCH$_2$*CH$_2$CH$_2$*CH$_3$), 0.92 (t, –OCH$_2$CH$_2$CH$_2$*CH$_3$*).

Synthesis of (PEG)$_2$-Cys-(Boc)$_2$: (Boc-Cys-OH)$_2$ (0.3 g, 0.68 mmol) and PEG (3.26 g, 1.63 mmol) were dissolved in anhydrous dichloromethane (50 mL), and DCC (0.42 g, 2.05 mmol) was added to the solution. The reaction was allowed to proceed at room temperature for 48 h. Insoluble dicyclohexylurea was removed by filtration. The product was precipitated in diethyl ether, filtered, and dried at room temperature under a vacuum. The crude product was dissolved in water. The solution was thoroughly dialyzed (MWCO: 3500 Da) against deionized water for 3 days to remove the residual PEG and then lyophilized to obtain 2.60 g of product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.28 (t, –C(O)O*CH$_2$*CH$_2$O–), 3.69–3.54 (m, –*CH$_2$CH$_2$*O), 3.36 (s, *CH$_3$*O–), 3.15 (d, –CH*CH$_2$*SS–), 1.43 (m, –NH*(CH$_3$)$_3$*).

Synthesis of (PEG)$_2$-cystine: (PEG)$_2$-Cys-(Boc)$_2$ (2 g, 0.41 mmol) was dissolved in anhydrous dichloromethane (20 mL) and TFA (10 mL). The reaction was allowed to proceed at room temperature for 3 h. The reaction solvent was evaporated, and the resulting product was precipitated in an excess amount of cold diethyl ether. The precipitate was filtered, washed several times with diethyl ether, and dried under a vacuum to obtain 1.57 g of product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.25 (t, –C(O)O*CH$_2$*CH$_2$O–), 3.81 (t, –C(O)*CH*(NH$_2$)CH$_2$–), 3.69–3.54 (m, –*CH$_2$CH$_2$*O), 3.36 (s, *CH$_3$*O–), 3.10 (d, –CH*CH$_2$*SS–).

Synthesis of (PEG)$_2$-Cys-(PLA)$_2$: (PEG)$_2$-Cystine (1 g, 0.22 mmol) and PLA-COOH (0.65 g, 0.54 mmol) were dissolved in anhydrous dichloromethane (20 mL), and DCC (0.11 g, 0.54 mmol) was added to the solution. The reaction was allowed to proceed at room temperature for 3 days. Insoluble dicyclohexylurea was removed by filtration. The product was precipitated in diethyl ether, filtered, and dried at room temperature under a vacuum. The crude product was dissolved in DI water. The solution was filtered to remove the unreacted PLA-COOH and then lyophilized to obtain 1.10 g of the product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.15 (m, –C(O)*CH*CH$_3$–), 4.41–4.21 (m, –C(O)O*CH$_2$*CH$_2$O–, –C(O)O*CH$_2$*CH$_2$CH$_2$CH$_3$, –NHC(O)*CH$_2$*CH$_2$–), 3.67–3.54 (m, –*CH$_2$CH$_2$*O), 3.39 (s, *CH$_3$*O–), 3.29–3.17 (m, –CH*CH$_2$*SS–), 2.64 (d, –C(O)*CH$_2$CH$_2$*C(O)–), 1.26–1.24 (d, –OCH$_2$*CH$_2$CH$_2$*CH$_3$), 0.87 (t, –OCH$_2$CH$_2$CH$_2$*CH$_3$*).

2.4. Micellization and CMC measurement of polymers

For the micellization of the monomeric PEG-PLA and gemini (PEG)$_2$-Cys-(PLA)$_2$, the polymer (5 mg) was dissolved in DI water (5 mL). The solution was stirred for 6 h at room temperature, yielding micelles with a hydrophobic core in an aqueous solution at 1 mg/mL. The CMC was determined using pyrene as a fluorescent probe. The polymer concentrations varied from 1×10^{-4} to 1 mg/mL, and the pyrene concentration was fixed at 0.6 μM.