A Facile Strategy to Prepare Redox-Responsive Amphiphilic PEGylated Prodrug with High Drug Loading Content and Low Critical Micelle Concentration

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A Facile Strategy to Prepare Redox-Responsive Amphiphilic PEGylated Prodrug with High Drug Loading Content and Low Critical Micelle Concentration†

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Redox-responsive amphiphilic polymeric prodrug was synthesized in a facile way by polycondensation of oligo(ethylene glycol) (OEG) with dicarboxylic acids including malic acid (MA) and 3,3'-dithiodipropionic acid (DTPA), followed by esterification with ibuprofen, which was used as a model drug. Taking advantage of amphiphilic nature and relatively high molecular weight, this polymeric prodrug can form stable micelles in aqueous media with low critical micellar concentration (CMC). Free ibuprofen molecules can be steadily incorporated into the core of these micelles with surprisingly high loading content (38.9 wt %), owing to the hydrophobic interaction and π-π stacking with the ibuprofen moieties in the copolymer. The *in vitro* release results indicate that conjugated ibuprofen moieties presented a relatively slow and sustained release, while encapsulated ibuprofen molecules showed a rapid release. Furthermore, both of conjugated ibuprofen and encapsulated ibuprofen showed an accelerated release in the presence of 10 mM DL-dithiothreitol (DTT) due to the cleavage of the disulfide bonds, which leads to the disassociation of the micelles. Notably, this prodrug was revealed to have excellent cell compatibilities via cell counting kit-8 (CCK-8) assay. Confocal laser scanning microscope (CLSM) observation indicated that the micelles based on the polymeric prodrug can be quickly uptake by cells and present a redox-responsive drug release in cytoplasm. This kind of polymeric nano-carrier with high drug loading contents, low CMC, excellent biocompatibility and rapid response to reductive environment may have tremendous scope in the area of controlled drug delivery.

Introduction

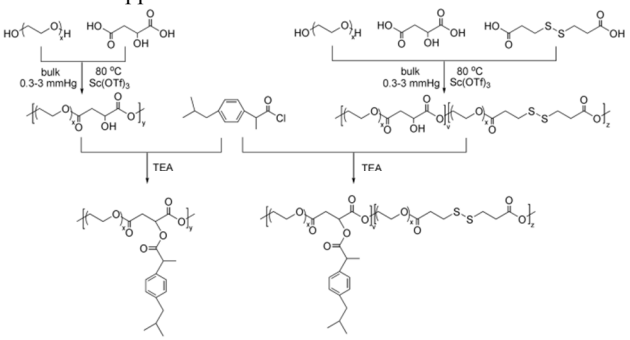
Most drugs are limited in clinical use for their poor water solubility, low selectivity, rapid blood clearance and severe side effects for healthy tissues with few can arrive at the desired site *in vivo*.[1, 2] Developing smart vehicles which selectively deliver drug molecules to target cells are urgent and attracted rapidly increasing attention.[3-5] Various polymeric drug delivery systems, such as polymeric micelles,[6-8] polymer-drug conjugates (polymeric prodrugs)[9-12] and polymeric gels[13, 14] have been developed to address these problems.

Polymeric micelle with their unique core-shell architecture, good stability under physiological condition and target cancerous tissues by passive accumulation via tumors' enhanced permeability and retention (EPR) effect has received tremendous interest in biomedical area.[15-18] Hydrophobic drugs can be encapsulated into the hydrophobic core of micelle carriers and protected by hydrophilic shell. However, many studies showed that the contents of encapsulated drug are generally not greater than 10 % to suppress premature burst release.[19-22]

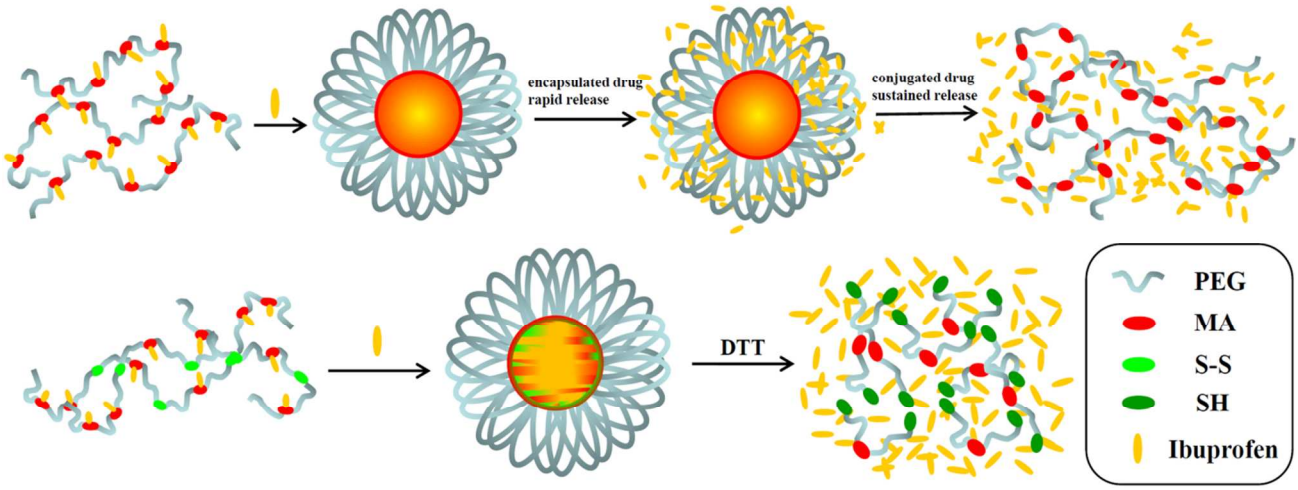
Recent attention has been focused on the conjugation of drug with polymer, termed polymeric prodrug, which can improve solubility, bioavailability, stability of the drug, and can prolong circulation time of drug to make it less likely to be prematurely released.[23-26] Notably, using drug as a part of nano-carrier can also minimize the use of other inert materials in drug carrier with unexpected side effects, and increase the drug loading content.[27] Among these, PEGylated prodrugs have been widely investigated as a biocompatible drug carrier for the reason that PEG can reduce nonspecific interactions with proteins and cells, as well as has been approved by U.S. Food and Drug Administration (FDA).[28-32] Recently, Shen and coworkers reported a PEGylated prodrug, synthesized through conjugating camptothecin (CPT) to oligo(ethylene glycol) (OEG) terminal via ester bond, realized rather high drug loading content.[33] But for this kind of method, short OEG chain is required for achieving high drug loading capacity and micelle formation. The obtained low molecular weight prodrugs, however, exhibit poor stability because of high critical micellar

concentration (CMC). To dissipate this defect, many papers reported on developing PEGylated prodrugs with high molecular weight, based on amphiphilic block copolymer with pendant functional groups for drug attachment,[34-36] which was synthesized by the ring-opening polymerization of functional cyclic monomer with PEG as macroinitiator. Nevertheless, the preparation is difficult and complicated with protection/deprotection, and comonomers without functional groups is required to reduce the steric hindrance, resulting in low drug loading content.[37]

Here, in order to overcome above mentioned shortcomings of PEGylated prodrugs, we develop a facile strategy to fabricate redox-responsive PEGylated prodrugs with high drug loading content and low CMC based on a multifunctional PEG derivative, poly[oligo(ethylene glycol) dithiodipropionicate-*co*-oligo(ethylene glycol) malicate] (POEGSSM), which was synthesized through direct chemoselective polycondensation of OEG diol with corresponding dicarboxylic acids under mild conditions using $Sc(OTf)_3$ as chemoselective catalyst.[38-41] Ibuprofen, a potent non-steroidal anti-inflammatory drug used in the treatment of rheumatism, arthritis, fever and other conditions, was selected as a model poorly water-soluble drug and conjugated onto the POEGSSM backbone through ester bonds. Owing to the much reduced steric hindrance from the neighboring pendent hydroxyl groups in POEGSSM, high drug loading PEG-ibuprofen polymeric prodrug was thus prepared, as shown in Scheme 1. As a control, a non-reducing cleavable polymeric prodrug based on poly[oligo(ethylene glycol) malicate] (POEGM) was also prepared in a similar manner. The prodrugs can form stable micelles in aqueous media with low CMC. Additionally, free ibuprofen molecules can be steadily encapsulated into the micelle. The *in vitro* release of conjugated ibuprofen from POEGSSIBu micelles was relatively slow and sustained, while physically encapsulated ibuprofen showed a rapid release. Both of them showed a DTT-triggered release as shown in Scheme 2. The biocompatibility and cell uptake of the polymeric prodrug micelles were also studied, and the results showed that this kind of biodegradable and biocompatiable polymeric nano-carriers with high drug loading content, low CMC and reductive response has great potential in biomedical applications.



Scheme 1. Synthetic routes of PEG-Ibuprofen polymeric prodrugs



Scheme 2. Illustrative preparation of Ibuprofen-loaded prodrug micelles and their redox-responsive Ibuprofen release

Experimental

Materials

Oligo(ethylene glycol) diols (OEG_{400}, M_n = 400 and OEG_{600}, M_n = 600, Aladdin, China) were dried by azeotropic distillation in the presence of dry toluene. Ibuprofen (IBu, Juhua Group Corporation; China), DL-dithiothreitol (DTT, Aladdin, China), DL-malic acid (MA, Aladdin, China), 3,3'-dithiodipropionic acid (DTPA, Aladdin, China), Nile red (NR, Sigma) and other reagents were used as received. $Sc(OTf)_3$ was synthesized according to our previous report.[42]

Preparation of POEGSSM and POEGM

28.0 g of OEG_{400} (70 mmol), 4.7 g of MA (35 mmol), 7.36 g of DPTA (35 mmol) and 0.35 g of $Sc(OTf)_3$ (0.7 mmol) were stirred in a 250 mL round-bottom reactor at 80 ºC under argon atmosphere for 4 h. Then the pressure was gradually decreased to 0.3-3 mmHg, stirred for 24 h. The crude product was purified by neutral alumina column chromatography with methylene choloride as eluent. After concentration, the solution was poured into ethyl ether to precipitate POEGSSM, which was dried in vacuum overnight to constant weight. Yield: 35.7 g (95 %). POEGM was synthesized from 27.0 g of OEG_{600} (45 mmol), 6.03 g of MA (45 mmol) and 0.22 g of $Sc(OTf)_3$ (0.45 mmol) in the same manner as described above.

Yield: 30.2 g (96 %). The ^{1}H NMR spectra of POEGSSM and POEGM were recorded using a Bruker Avance DMX500 spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. The molecular weight and molecular weight distribution of the obtained copolymers POEGSSM and POEGM were determined by gel permeation chromatography (GPC), which consisted of a Waters degasser, a Waters 1515 Isocratic HPLC pump, a Wyatt Optilab DSP interferometric refractometer, and columns: Styragel, HT 3, HT 4. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1.0 mL/min at 25 °C and polystyrenes with the molecular weight ranging from 580 to 600,000 were used as calibration standards.

Preparation of POEGSSMIBu and POEGMIBu via acylation reaction

IBu (10 g, 49 mmol) was dissolved into excess thionyl chloride and a drop of DMF was added as catalyst. The mixture was stirred at 80 ºC for 10 h, and then excess thionyl chloride was removed under vacuum to give IBu-acyl chloride (IBu-COCl) (9.8 g, 90 %). POEGSSM (2.0 g, 1.8 mmol) and TEA (0.77 mL, 5.4 mmol) were dissolved in 25 mL anhydrous CH_2Cl_2, after stirred at 0 °C for 30 min, IBu-COCl (1.23 g, 5.4 mmol) was added dropwise within 25 min. The solution was stirred overnight at room temperature and then washed successively with aqueous solutions of $NaHCO_3$ and NaCl. The organic phase was dried over anhydrous $MgSO_4$, filtered, evaporated to dryness and recrystallized from CH_2Cl_2/ethyl ether. The resulting solid POEGSSMIBu was dried in vacuum overnight to constant weight. Yield: 2.16 g (92 %). POEGMIBu was prepared from POEGM (1.7 g, 2.4 mmol), TEA (1.02 mL, 7.2 mmol) and IBu-COCl (1.64 g, 7.2 mmol) in the same manner. Yield: 2.1 g (93 %).

Preparation of POEGSSMIBu and POEGMIBu micelles

POEGSSMIBu (50 mg) was dissolved in 10 mL THF, and then 10 mL of PBS (0.01 M, pH 7.4) was added dropwise to the solution under stirring. The resulting solution was stirred for 2 h and dialyzed (MWCO 3500, Fisher Scientific) against PBS (0.01 M, pH 7.4) over 24 h to completely remove THF, forming micelle structure. The final concentration of the micellar solution was adjusted to 1.0 mg/mL. Meanwhile, micelles of POEGMIBu were prepared in the same manner. The size and size distribution of micelles were determined by dynamic light scattering (DLS) at 25 °C using Zetasizer Nano-ZS from Malvern Instruments with a He-Ne laser (633 nm). All micellar solutions had a final polymer concentration of 1.0 mg/mL and were filtered through a 0.45 μm filter. The morphology of the micelles was observed by using JEM-1230 transmission electron microscope (TEM) operating at an acceleration voltage of 60 kV. A drop of 1.0 mg/mL micellar solution was put onto the surface of Formvar-carbon film-coated copper grids. Excess solvent was quickly removed away with a filter paper and then stained by 2 wt% phosphotungstic acid aqueous solution.

Reductive degradation of POEGSSMIBu micelles

It is well known that disulfide linkages are stable under normal physiological conditions while respond to glutathione (GSH) or reductases via reversible cleavage into free thiols.[43, 44] In order to reduce the disufide bonds into free thiols entirely, 75 mg of DTT (0.5 mmol, 10 mM) was dissolved in POEGSSMIBu micellar solution (50 mL, 1.0 mg/mL) and stirred at 37 ºC overnight (10 h). Then the solution was added into a dialysis membrane bag (MWCO 3500, Fisher Scientific) and dialysized against double distilled water for another 24 h. The degraded product POEGSHMIBu was finally retrieved by lyophilization.

CMC of POEGSSMIBu, POEGMIBu and POEGSHMIBu

The CMC of POEGSSMIBu, POEGMIBu and POEGSHMIBu was estimated by fluorescence measurement using pyrene as a fluorescent probe. The pyrene concentration in the solution was fixed at 6.0×10^{-7} M and the concentration of block copolymer POEGSSMIBu, POEGMIBu and POEGSHMIBu was varied from 1.0×10^{-6} to 1.0 mg/mL. When polymeric micelles formed, pyrene will preferentially incorporate into hydrophobic micelle core instead of in polar environment (aqueous solution). And the sharp rise in intensity ratio (I_{338}/I_{333}) of pyrene in the excitation spectra indicates the onset of micellization (CMC) for amphiphilic copolymer. Fluorescence excitation spectra (300-360 nm) of the solutions were recorded using Hitachi F-4500 fluorescence spectrometer at 390 nm emission wavelength, with the excitation and emission bandwidths set at 5 nm slit width.

Encapsulation of free IBu into POEGSSMIBu and POEGMIBu micelles

POEGSSMIBu (50 mg) and IBu (15 mg) were dissolved in 10 mL THF, and then 5 mL of PBS (0.01 M, pH 7.4) was added dropwise to the solution under stirring. The resulting solution was stirred for 2 h and dialyzed (MWCO 3500, Fisher Scientific) against PBS (0.01 M, pH 7.4) over 24 h to remove THF, forming IBu-loaded micelles. The solution was filtered to remove unincorporated IBu. The final concentration of the micellar solution was adjusted to 1.0 mg/mL. The encapsulation of IBu to POEGMIBu micelle was carried out by a similar way. The solid state of drug-loaded micelles was retrieved by lyophilization and the amount of IBu trapped in the micelles was determined by ^{1}H NMR results (Fig. S1, S2).[45] Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations:

$$\mathrm{DLC(wt\,\%)} = W_{DM}/W_P \times 100\%$$

$$\mathrm{DLE(wt\,\%)} = (W_{DM} - W_{DC})/W_{Dt} \times 100\%$$

W_{DM} is the weight of IBu measured by ^{1}H NMR spectra, W_{DC} is the weight of conjugated IBu, W_P is the weight of POEGSSMIBu or POEGMIBu copolymer, W_{Dt} is the total weight of free IBu used in drug-encapsulated experiment.

***In vitro* IBu release from POEGSSMIBu and POEGMIBu micelles before and after loading with free IBu**

5 mL of POEGSSMIBu and IBu-loaded POEGSSMIBu micellar solutions (1.0 mg/mL) in phosphate buffer (PBS, 0.01 M, pH 7.4) were transferred into a dialysis membrane bag (MWCO 3500, Fisher Scientific) respectively, which were then immersed in 50 mL of PBS (0.01 M, pH 7.4) with or without DTT (10 mM), and held at a constant temperature of 37 °C in a water bath with horizontal shaking. At a predetermined time interval, 5 mL of incubated solution was taken out and replenished with an equal volume of corresponding PBS. IBu release from POEGMIBu and IBu-loaded POEGMIBu micelles were incubated in pure PBS (0.01 M, pH 7.4) and conducted in the same manner as describe above. IBu release profiles were determined by measuring the absorbance at a wavelength of 222 nm using a Shimadzu UV2550 UV-vis spectrophotometer. Calibration curves were established from known concentrations of IBu in PBS (0.01M, pH = 7.4) and PBS (0.01M, 10 mM DTT, pH = 7.4), respectively (Fig. S3). After the IBu moieties completely releasing from polymeric prodrug POEGMIBu, the remained polymer, named Re-POEGM, was retrieved by lyophilization and measured by GPC.

***In vitro* cytotoxicity of POEGSSMIBu and POEGMIBu**

The cytotoxicity of polymeric prodrugs POEGMSSIBu and POEGMIBu were evaluated by CCK-8 (Dojindo, Japan) assay. All tests were run five times. POEGSSMIBu and POEGMIBu were prepared in PBS (0.01 M, pH 7.4) and then sterilized by filtration (0.22 μm). MC3T3-E1 cells (Cell bank of the Chinese Academy of Science, China) were pre-incubated in a 96-well plate (5×10^3 cells/well) with culture medium 10% FBS/α-MEM (Invitrogen Co., Carlsbad, CA) in a humidified 5% CO_2-containing atmosphere at 37 °C for 24 h. Then cells were further incubated with POEGSSMIBu and POEGMIBu with four concentrations, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L for 24 h. Subsequently, media was aspirated and replenished with 100 μL of fresh culture medium. 10 μL CCK-8 reagents were added into each well, and the cells were incubated at dark for another 1 h. The absorbance at a wavelength of 450 nm of each well was measured using a microplate reader (Sunrise™ Basic; TECAN, Zurich, Switzerland). Non-treated cells were used as a negative control, wells without cell but culture medium was used as blank. The relative cell viability was calculated as follows:

$$\text{Cell viability (\%)} = [(OD_{450\,sample} - OD_{450\,blank})/(OD_{450\,control} - OD_{450\,blank})] \times 100\%$$

Data are presented as average ± SD (n = 5).

Cellular uptake and intracellular release of POEGSSMIBu and POEGMIBu micelles

The cellular uptake experiments were performed for POEGSSMIBu and POEGMIBu micelles by loading NR (red fluorescence) as a hydrophobic fluorescence probe in these micelles. NR (0.1 mg) with POEGSSMIBu (20 mg) or POEGMIBu (20 mg) were dissolved in 5 mL of tetrahydrofuran (THF) and stirred for 30 min at room temperature. Then 5 mL of PBS (0.01 M, pH 7.4) was added dropwise to the solution under stirring. The resulting solution was dialyzed against PBS (0.01 M, pH 7.4) for 24 h to remove THF and form NR-loaded POEGSSMIBu and POEGMIBu micelles. After that, the solution was filtered to remove unincorporated NR and the concentration of micelles was adjusted to 1.0 mg/mL.

Cellular uptakes intracellular NR release of NR-loaded POEGSSMIBu and POEGMIBu micelles were observed on CLSM (BX61W1-FV1000, OLYMPUS, Japan). Cellular uptake of free NR was performed as a control. Autoclave sterilized coverslips were placed in 6-well plate. The MC3T3-E1 cells were seeded with a concentration of 2×10^4 cells/well in 1.8 mL of complete α-MEM and cultured for 24 h. Then NR-loaded micelles or free NR were added, and cultured for 2 h, 4 h. Then the culture media was removed, followed by rinsing the cells three times with PBS (0.01 M, pH 7.4) and fixing with 4 % paraformaldehyde at room temperature for 15 min. Cell cytoskeletons and nucleus were identified following double staining of actin (green fluorescence) and nuclear (blue fluorescence), respectively, using fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich) and Hoechst 33342 (Sigma-Aldrich). After being mounted with neutral balsam, samples were observed with 60× magnification microscope.

The amount of NR in cells was analyzed by Spectramax M5 plate reader (Molecular Devices, LLC, USA). 180 μL suspension of the MC3T3-E1 cells were seeded into each well of 96-well plate (5 × 103 cells) and equal culture mediate was added into wells without cells as blank. After pre-incubated under the same condition for 24 h, NR-loaded micelles or free NR in 20 μL PBS (0.01 M, pH 7.4) were added. Equivalent amount of PBS were added into the negative control and blank. After 2 or 4 h's incubation, cells were washed with PBS twice, and finally 100 μL of PBS was added. The content of NR inside cells was determined by fluorescence intensity (Spectramax M5, ex: 550 nm, em: 605 nm). Each group was tested in quintuplicate.

Results and discussion

Synthesis and characterization of POEGSSM and POEGM

POEGSSM was prepared via the direct polycondensation of OEG diol with malic acid and 3,3'-dithiodipropionic acid, which was carried out under reduced pressure in bulk using $Sc(OTf)_3$ as chemoselective catalyst. The ^{1}H NMR spectrum of POEGSSM was shown in Fig. 1A with all the relevant signals well labeled, especially for the signals ascribed to DTPA, which clearly implied the introduction of disulfide bond into the backbone of the polyester. Notably, the appearance of the signal centered at 4.55 ppm (H^d), attributing to the protons of methylidyne group in malicate unit, indicated the acquisition of the polyesters with pendent secondary hydroxyl groups by chemoselective esterification of primary hydroxyl groups with carboxyl groups. Meanwhile, the molar ratio of OEG/MA/DPTA in the copolymer is in agreement with the feeding molar ratio (2: 1: 1) according to the integrals of corresponding protons. As a control group, another PEG derivative without disulfide bonds (POEGM) was also synthesized in the same manner by adding OEG diol with an equimolar amount of malic acid. The ^{1}H NMR spectrum of POEGM, as well as the signal assignments, was presented in Fig. 2A, which also clearly verified the acquisition of PEG derivative with pendant hydroxyl groups. The molecular weights of the copolymers were determined by GPC (Fig.

3), which were unimodal, further confirming the successful synthesis of POEGSSM and POEGM via polycondensation.

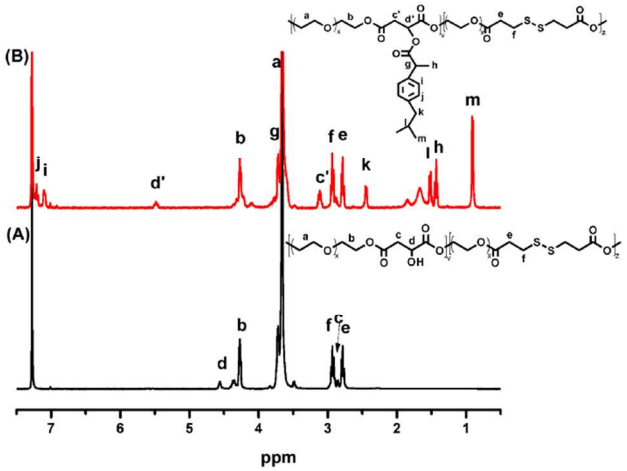


Fig. 1 [1]H NMR spectra of POEGSSM (A) and POEGSSMIBu (B).

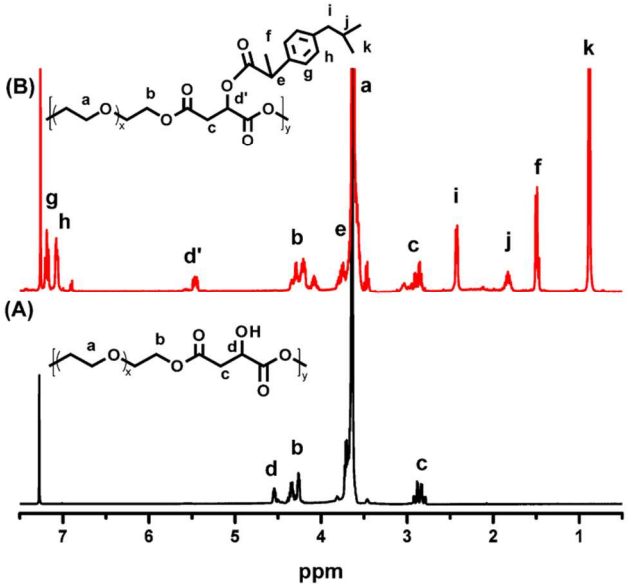


Fig. 2 [1]H NMR spectra of POEGM (A) and POEGMIBu (B).

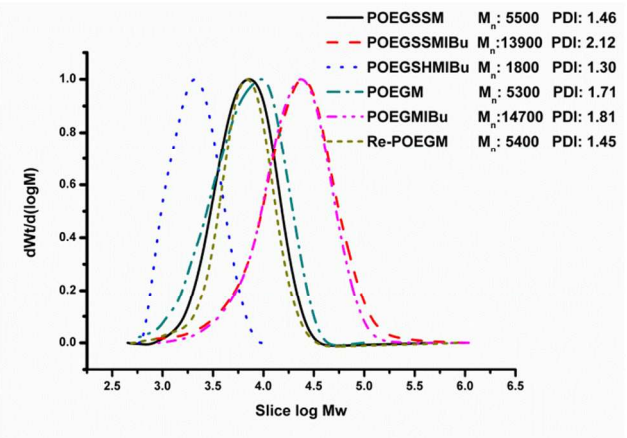


Fig. 3 GPC curves of POEGSSM, POEGSSMIBu, POEGSHMIBu, POEGM, POEGMIBu, Re-POEGM.

Synthesis and characterization of POEGSSMIBu and POEGMIBu

The acquisition of reductive-sensitive polymeric prodrug via the acylation reaction of IBu-COCl to POEGSSM was performed at 0 ºC in the presence of TEA. A representative [1]H NMR spectrum of POEGSSMIBu was shown in Fig. 1B, clearly exhibiting the variation of chemical structure compared with its precursor (POEGSSM). The shift of the signal from δ 4.55 ppm (H^d) to δ 5.46 ppm ($H^{d'}$), as well as the presence of the signals assigned to IBU moieties, intelligibly implied the successful conjugation of IBu to POEGSSM by ester bond, resulting in polymeric prodrug with disulfide bond in the backbone. Meanwhile, the non-reducing cleavable prodrug POEGMIBu was prepared in a similar way and the [1]H NMR spectrum was presented in Fig. 2B. Moreover, the POEGSSMIBu and POEGMIBu were further characterized by GPC measurements. As shown in Fig. 3, compared with their corresponding precursors, the curves of polymeric prodrugs significantly shifted to high molecular weight region, but still maintained unimodal, signifying the successful synthesis of polymeric prodrugs.

The disulfide bonds of POEGSSMIBu were completely cloven by treating with DTT (10 mM) at 37 ºC for 10 h, resulting in a reductively degraded product (POEGSHMIBu), which was measured by GPC. Compared with POEGSSMIBu, the GPC curve of POEGSHMIBu shifted to low molecular weight region, which clearly implies the reductive degradation of POEGSSMIBu owing to the cleavage of disulfide bonds in the backbone.

Preparation of micelles from POEGSSMIBu and POEGMIBu

Amphiphilic polymeric prodrugs can self-assemble into micelles in an aqueous solution by dialysis method, which were characterized by measuring the CMC using pyrene as a fluorescent probe.[46] The intensity ratio of bands at 338 and 333 nm (I_{338}/I_{333}) was calculated and plotted versus the polymer concentrations, presenting a sigmoid curve as shown in Fig. 4. The sharp rise in intensity ratio of peaks at 338 and 333 nm of pyrene in the excitation spectra points the on-set of micellization (CMC) for amphiphilic copolymer. The CMC values of POEGSSMIBu and PEOGMIBu were about 5.09 mg/L (Fig. 4A) and 5.39 mg/L (Fig. 4B), respectively. The rather low CMC intelligibly indicate that the amphiphilic polymeric prodrugs can form stable micelles under such a low micellar concentration. The CMC of POEGSHMIBu was also evaluated by fluorescence spectroscopy. After treating with 10 mM DTT at 37 ºC for 10 h, the CMC value of the polymeric prodrug showed a tremendous increase from 5.09 mg/L to 38.0 mg/L (Fig. 4C), which indicates that the micelles formed by amphiphilic polymeric prodrug containing disulfide bonds become unstable in reductive environment due to the cleavage of disulfide bonds.

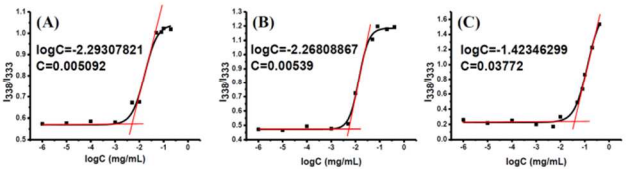


Fig. 4 Plots of fluorescence intensity ratio I_{338}/I_{333} from pyrene excitation spectra vs. log C for POEGSSMIBu (A), POEGMIBu (B) and POEGSHMIBu (C).

Preparation of IBu-loaded micelles based on polymeric prodrugs POEGSSMIBu and POEGMIBu

The polymeric prodrug micelles can steadily encapsulate IBu, which was prepared in an aqueous solution by dialysis method. The initial weight ratio of IBu to polymer was 3/10. The drug loading efficiency was surprisingly high, that is 75.2 % for POEGSSMIBu micelles and 77.2 % for POEGMIBu micelles. The total drug loading content can reached as high as 38.9 wt % for POEGSSMIBu micelles and 46.6 wt % for POEGMIBu micelles, respectively. These high drug loading capacities of the POEGSSMIBu and POEGMIBu micelles might be attributed to the π-π aromatic stacking force between covalently bonded IBu moieties and free IBu molecules, which makes more free IBu molecules incorporating into the micellar core.[47]

Size and morphology of micelles

The size and morphology of POEGSSMIBu micelles were determined by DLS and TEM, as shown in Fig. 5. The diameter of micelles measured by DLS was approximately 22 nm, which is in good accordance with that detected by TEM. Interestingly, after loading free IBu into the micelles, the diameter of the micelles significantly enlarged to 52 nm, but the morphology of the micelles still maintained spherical shape according to TEM image. The increase of the micelle size after drug loading probably assigned to the entrapment of the drug in the core of the micelles. The size change of POEGSSMIBu micelles in response to 10 mM DTT was also determined by DLS. After treating with DTT for 10 h, large stable aggregates with broad size distribution were observed. This result indicates the rapid response of POEGSSMIBu micelles upon reductive environment, which is in good accordance with GPC and CMC data.

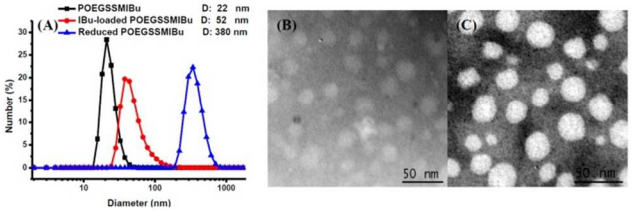


Fig. 5 Particle size, distribution of POEGSSMIBu micelles, IBu-loaded POEGSSMIBu micelles and Reduced POEGSSMIBu micelles measured by DLS (A); TEM images of POEGSSMIBu micelles (B) and IBu-loaded POEGSSMIBu micelles (C).

Similarly, the size and morphology of POEGMIBu micelles were also determined by DLS and TEM as shown in Fig. 6. The typical TEM images show spherical morphology of the micelles with uniform size distribution. Compared to the blank micelles, the size of IBu-loaded micelles also showed a significant increase.

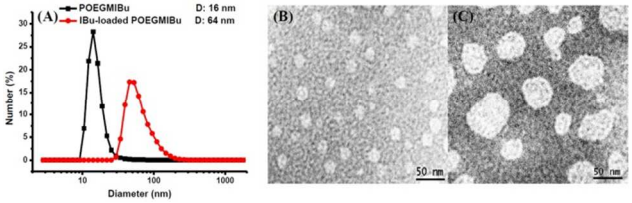


Fig. 6 Particle size, distribution of POEGMIBu micelles and IBu-loaded POEGMIBu micelles measured by DLS (A); TEM images of POEGMIBu micelles (B) and IBu-loaded POEGMIBu micelles (C).

***In vitro* IBu release from micelles**

The intracellular concentration of GSH (0.5–10 mM) is significantly higher than that of outside cells (2–20 μM).[48, 49] The huge difference may selectively trigger the cleavage of the disulfide bonds of the polymeric prodrug, resulting in a controlled release of the loaded drugs into cells. Here, 10 mM DTT was chosen to mimic the reductive environment in cytoplasmic environment.[50-52] DTT-induced IBu release from POEGSSMIBu micelles was investigated by dialysis. The solutions taken out at predetermined intervals were characterized by UV-vis spectrometry at 222 nm depending on the calibration curves, which were established from known concentrations of IBu in corresponding medium. For POEGSSMIBu micelles, almost 35 % of conjugated IBu was released within 30 h in the presence of DTT (10 mM), whereas little release of IBu was observed from the POEGSSMIBu micelles in pure PBS even after 100 h (Fig. 7A), which intelligibly reveals that the release rates of conjugated IBu had a great dependence on the reducing reagent. Meanwhile, for IBu-loaded POEGSSMIBu micelles, as shown in Fig. 7B, a burst release of IBu was observed in the presence of DTT within first 10 h, and a relatively delayed drug release was observed during the same period without DTT. After 10 h, the release behaviors of IBu-loaded POEGSSMIBu micelles are similar to those of POEGSSMIBu micelles. It could be concluded that the release of conjugated IBu was slow and sustained owing to the gradual hydrolysis of the ester bonds, while the release rate of the encapsulated IBu from the micelles was much faster, but both of them clearly showed reducing-dependent release behaviors. The reductive cleavage of disulfide bonds can not only accelerate the release of encapsulated free IBu with the disassociation of the micelles, but also induce the fast hydrolysis of ester bonds to release conjugated ibuprofen by reducing the steric hindrance that water molecules encountering in the hydrolysis of the ester bonds. The IBu release behaviors of POEGMIBu micelles (Fig. 7C) and IBu-loaded POEGMIBu micelles (Fig. 7D) are similar to those of POEGSSMIBu micelles and IBu-loaded POEGSSMIBu micelles in pure PBS without DTT. The release of conjugated IBu could sustain for more than 40 days. After all of IBu moieties were hydrolyzed and released from POEGMIBu micelles, the remained polymer (Re-

POEGM) was recovered by lyophilization and measured by GPC. As shown in Fig. 3, the GPC cure and molecular weight of Re-POEGM is similar to those of POEGM, which intelligibly indicates that the hydrolysis of ester bonds formed from the pendent secondary hydroxyl groups was relatively faster than those in the polymer backbone based on primary hydroxyl groups, and the polymer backbone hardly hydrolyzed even after 40 days.

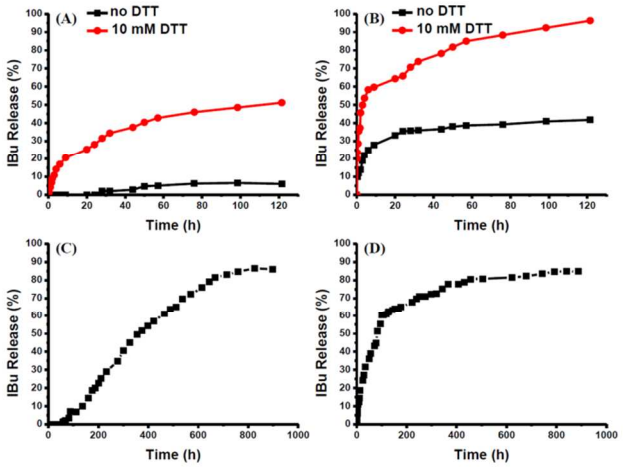


Fig. 7 *In vitro* IBu release profiles from POEGSSMIBu micelles (A); IBu-loaded POEGSSMIBu micelles (B); POEGMIBu micelles (C) and IBu-loaded POEGMIBu micelles (D).

In vitro cytotoxicities of POEGSSMIBu and POEGMIBu

The cytotoxicities of POEGSSMIBu and POEGMIBu were both determined by CCK-8 assays against MC3T3-E1 cells. A series of polymeric prodrug solutions with various concentrations were used to investigate the biocompatibility of PEG-IBu copolymer. The results indicate that both POEGSSMIBu and POEGMIBu showed great biocompatibility to MC3T3-E1 cells (Fig. 8). IBu itself might be toxic,[53, 54] however, the great biocompatibility of the PEG-IBu copolymer shown in Fig. 8 intelligibly indicated that the toxicity of IBu could be greatly reduced by conjugating with PEG derivatives.

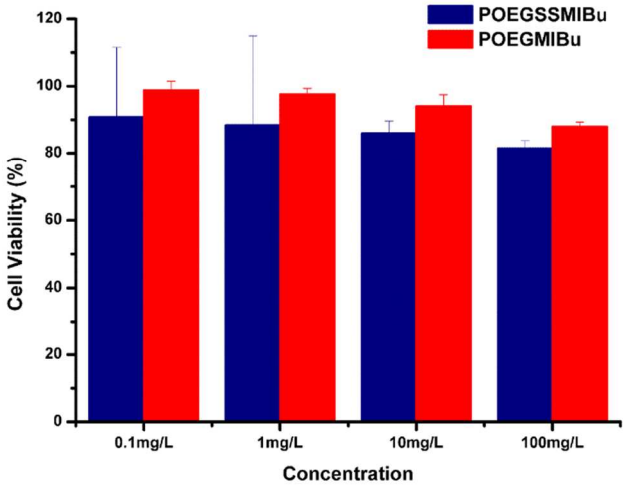


Fig. 8 Relative cell viability of POEGSSMIBu and POESMIBu against normal cultivated MC3T3-E1 cells for 24 h.

Cellular uptake and intracellular drug release of POEGSSMIBu and POEGMIBu micelles

The cellular uptake and intracellular release of NR-loaded micelles in response to cellular GSH against MC3T3-E1 cells were investigated by CLSM and Spectramax M5 plate reader with NR (red fluorescence) as a hydrophobic fluorescence probe. The MC3T3-E1 cells were incubated with NR-loaded micelles for 2 or 4 h. Meanwhile, the MC3T3-E1 cells incubated with equivalent amount of free NR for 2 or 4 h were used as control. As shown in Fig. 9A and B, little fluorescence of NR can be detected in the cytoplasm of the cells when the cells are treated with free NR for 2 h and 4 h, indicating that free hydrophobic molecules could hardly be taken up by the cells. In contrast, the fluorescence of NR could be clearly observed in the cytoplasm of the cells after treating with NR-loaded POEGMIBu and POEGSSMIBu micelles for both 2 h and 4 h (Fig. 9C, D, E and F), which implied that POEGMIBu and POEGSSMIBu micelles can be internalized into the cells efficiently. Moreover, the fluorescence intensity increased with the incubation time, owing to the continuously internalization of the NR loaded micelles. To sum up, the hydrophobic agents could be well transported into the cells by encapsulated them into the hydrophobic core of the micelles, which is very important and potential for their use as ideal drug carriers. More importantly, cells incubated with NR-loaded POEGSSMIBu micelles emitted obviously stronger fluorescence of NR than those treated with NR-loaded POEGMIBu micelles for the same time. This phenomenon clearly confirms the efficient cellular uptake and successive destabilization of POEGSSMIBu micelles by high intracellular GSH concentrations in cytoplasm of the cells, which accelerates the release of encapsulated NR with the cleavage of disulfide bonds in response to the reductive environment (Scheme 3). These results are consistent with the results of *in vitro* release of encapsulated IBu from POEGSSMIBu and POEGMIBu micelles in the presence of DTT.

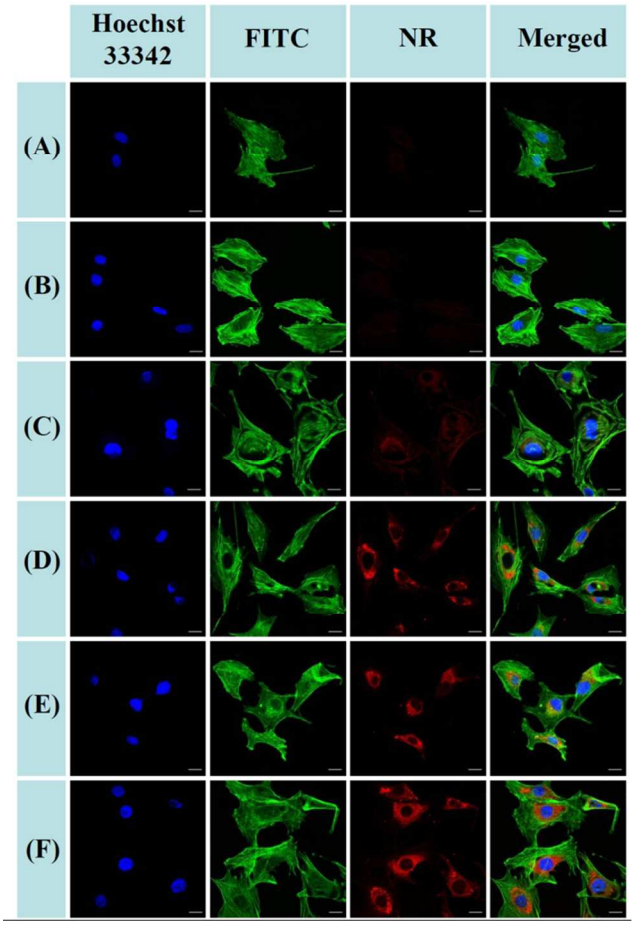
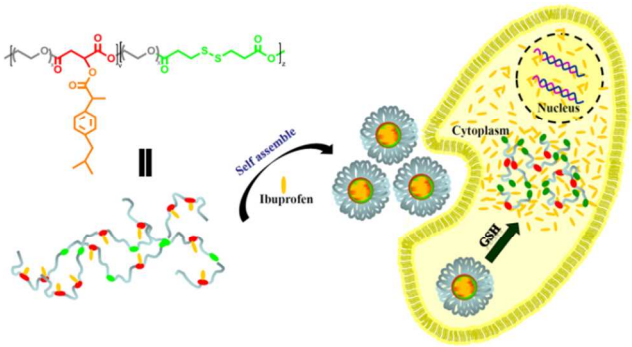


Fig. 9 CLSM images of MC3T3-E1 cells after 2 or 4 h incubation with NR-loaded micelles and free NR. For each panel, the images from left to right showed cell nuclei stained by Hoechst 33342 (blue), cytoskeletons stained by fluorescein isothiocyanate (FITC)-labeled phalloidin (green), NR fluorescence in cells (red), overlays of three left images. The scale bars correspond to 20 μm in all the images. (A) free NR, 2 h incubation; (B) free NR, 4 h incubation; (C) NR-loaded POEGMIbu micelles, 2 h incubation; (D) NR-loaded POEGMIBu micelles, 4 h incubation; (E) NR-loaded POEGSSMIBu micelles, 2 h incubation; (F) NR-loaded POEGSSMIBu micelles, 4 h incubation.



Scheme 3. Schematic illustration of the fabrication of Ibuprofen-loaded prodrug micelles for their efficient cellular uptake and intracellular Ibuprofen release triggered by GSH

The quantitative fluorescence intensities in the MC3T3-E1 cells were determined by Spectramax M5. The MC3T3-E1 cells were incubated similar to CLSM measurements. As showed in Fig. 10, analogous to the control group (cells treated with pure PBS), almost no fluorescence signals were detected in the cells after treating with free NR, clearly indicated that free hydrophobic NR could hardly be taken up by the cells. Nevertheless the MC3T3-E1 cells cultured in NR-loaded POEGMIBu and POEGSSMIBu micellar solution all showed fairly high fluorescence intensity of NR. Furthermore, the MC3T3-E1 cells treated with POEGSSMIBu micelles showed enhanced intracellular fluorescence of NR than those incubated with POEGMIBu micelles under the same conditions, which clearly implied rapid and complete intracellular NR release from POEGSSMIBu micelles under reductive environment. Additionally, of course, the fluorescence intensity also increased with the prolongation of incubation time. These results quantify the CLSM measurements quite well.

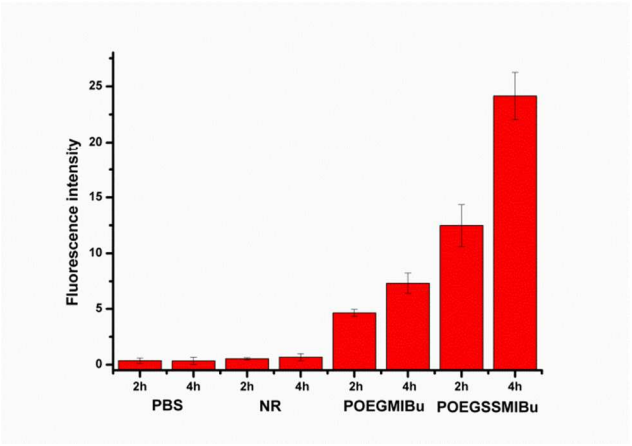


Fig. 10 Fluorescence intensity in MC3T3-E1 cells after incubation with PBS, free NR, NR-loaded POEGMIBu and POEGSSMIBu micelles for 2 h and 4 h.

Conclusions

In this study, an amphiphilic polymeric prodrug with pendent hydrophobic IBu moieties and hydrophilic backbone containing multiple disulfide bonds was facilely synthesized by a combination of polycondensation and esterification, which can form stable nano-sized micelles in aqueous solution with low CMC, and encapsulate free IBu molecules with the total IBu loading content as high as 38.9 wt%. The conjugated IBu could release from the nanocarriers by the hydrolysis of the ester bonds, presenting a slow and sustained release. While the encapsulated IBu molecules could release by the diffusion, showing a relatively fast release. Importantly, this kind of nanocarriers owns a stimuli release under reductive environment due to the cleavage of disulfide bonds in the polymeric backbone, which could accelerate the release of encapsulated and conjugated IBu simultaneously. The micelles based on this amphiphilic polymeric prodrug shows excellent biocompatibility, and could be efficiently taken up by cells, realizing a redox-responsive release because of the high intracellular GSH concentration. This study provides a facile and universal strategy to prepare efficient and smart drug delivery

system for controlled drug release. Meanwhile, amphiphilic polymeric prodrugs for cancer therapeutics could also be synthesized based on the PEG derivative with pendant hydroxyl groups and anticancer drug within one or two synthetic step. Even more, other stimuli-responsive bonds, such as pH-responsive bond, thermal-responsive bond and light-responsive bond, could be facilely introduced between the polymeric backbone and anticancer drug moiety. The strategy developed in our study provides an efficient and smart drug delivery system with multi-response could be fabricated facilely and low costly in a large scale for cancer chemotherapy.

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Notes and references

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† Electronic Supplementary Information (ESI) available: ESI includes the determination of the loading content of POEGSSMIBu and POEGMIBu micelles and the calibration curves of IBu in PBS with and without DTT. See DOI: 10.1039/b000000x/

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Graphic abstract

A Facile Strategy to Prepare Redox-Responsive Amphiphilic PEGylated Prodrug with High Drug Loading Content and Low Critical Micelle Concentration

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We prepared redox-responsive amphiphilic PEGylated prodrug with high drug loading content and low critical micelle concentration by simple polycondensation and esterification.