



Preparation of pH/redox dual responsive polymeric micelles with enhanced stability and drug controlled release

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ABSTRACT

Stimuli-responsive polymeric micelles were prepared through self-assembly of amphiphilic copolymers poly (ethylene glycol)-poly(γ -benzyl L-glutamate), followed by a core-crosslinking reaction using cystamine as the crosslinking agent. The crosslinked micelles with spherical morphologies in nanometer size showed enhanced stability against dilution and concentrated salt solutions compared to the micelles before crosslinking. Doxorubicin (DOX) as a model drug was encapsulated into the core of micelles through electrostatic interactions between carboxylic acid and DOX. In vitro drug release under pH and redox conditions was investigated. Furthermore, the cytotoxicity of micelles was evaluated before and after drug loading. The endocytosis of DOX-loaded micelles and the intracellular drug release were studied. DOX-loaded micelles exhibited accelerated drug release behaviors in an acidic and reductive environment, and showed an inhibited premature release behavior as compared to the noncrosslinked micelles. Considering their enhanced stability, pH and redox dual triggered responsive characteristics, the polymeric micelles can serve as potential systems for controlled drug delivery.

1. Introduction

Various nanoparticles, such as liposomes [1], inorganic nanoparticles [2, 3], and polymeric nanoparticles [4–7], have been widely applied as drug carriers [8]. Among them, polymeric micelles as promising platforms for drug delivery have attracted much attention due to their excellent biocompatibility, improved drug bioavailability, reduced off-target cytotoxicity, the versatility of tailoring for molecular weight and function groups, and preferential accumulation at tumor sites by the enhanced permeability and retention effect [9, 10]. However, polymeric micelles represent a minor contribution in the general nanomedicine field when compared to other polymeric and lipidic nanocarriers, on account of the relatively low physical stability upon dilution in body fluids to final concentrations below the critical micelle concentration (CMC) [11–15]. Another practical challenge with polymeric micelles is their low stability in vivo related to the interactions with cells and biomolecules present in the blood, which often lead to premature drug release and a diminished ability of the drug to reach its target [16]. Therefore, the improvement of polymeric micelle physical stabilities under extreme dilution conditions and a high salt concentration are of importance for the design of a promise drug delivery system.

Cross-linking of the micelle can effectively prevent the shell-core

structure from decomposing prematurely and is frequently used to stabilize drug encapsulations. In the past few years, cross-linking of the hydrophilic shell, the hydrophobic core or the core-shell interface have been adopted to overcome the instability problem [17]. Core cross-linking is one of the most effectively practiced strategies to stabilize drug encapsulations [18–20]. The resultant smart nanocarriers with crosslinked structures not only improve their structural stability but also retain their stimuli responsiveness. Environmental responsive technologies combined with covalent cross-linking have exhibited outstanding performance in the controlled release of encapsulated drugs. For example, micelles based on disulfide or ketal crosslinked polymers have been developed as promising biomaterials for controlled drug delivery due to their unique redox responsiveness [21–24]. Therefore, it is of great interest to develop disulfide crosslinked micelles and investigate their triggered release behavior under redox condition.

Various block co-polymeric micelles based on polyethylene glycol-poly(amino acids) (PEG-PAA) have been utilized for micelle-preparation to carry chemotherapeutic drugs in cancer. PEG-PAA utilizes polymerized amino acids as hydrophobic core. The most exploited amino acids to prepare micelles for drug delivery applications include lysine, aspartic acid, glutamic acid, and histidine [10]. Poly(L-glutamic acid) (PLGA) and its derivatives are one of the most widely investigated biomedical materials due to their good in vivo biocompatibility and

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biodegradability, and modifiable carboxyl side groups that can easily be modified to confer stimuli-responsive capabilities. Yan et al. fabricated pH-responsive hollow poly(L-glutamic acid)/chitosan nanogels with rapid antineoplastic agent mitoxantrone release behavior under acidic conditions [25]. Yang et al. designed a unique nano-rod-shaped microstructure via self-assembly in phosphate buffer and then co-assemble with poly(L-glutamic acid) at a proper polymer-drug ratio [26]. Wang et al. synthesized novel reduction-sensitive therapeutic micelles consisting of poly(ethylene glycol)-poly(L-glutamic acid) with significant antitumor activity and reduced side effects [27]. However, few reports showed that polymeric micelles based on crosslinked PLGA derivatives with enhanced stability served as dual-responsive to pH and reduction nanocarriers in drug delivery systems.

In this work, we designed a novel core cross-linked copolymer with hydrophobic poly(γ -benzyl-L-glutamates) (PBLG) core and hydrophilic PEG shell for dual responsive drug delivery. Cystamine was used as a crosslinking agent and the resultant micelle had a more compact spherical morphology which was much more stable against dilution and concentrated salt solutions. Disulfide bonds could be broken under reduction condition, leading to complete destruction of micelles. Doxorubicin (DOX) as a model drug was encapsulated into the core of micelles through electrostatic interactions. The physicochemical properties, drug loading and release performances of the polymeric micelles have been investigated. Furthermore, cell cytotoxicity and internalization behavior of the drug loaded copolymer have also been explored to evaluate the potential for in vivo drug delivery.

2. Experimental

2.1. Materials

γ -benzyl-L-glutamates (BLG) and γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) were synthesized as described in previous literatures [1, 2]. doxorubicin hydrochloride (DOX-HCl) was purchased from Shanghai Hao Cloud Chemical Technology Co., Ltd.; Cystamine dihydrochloride (Cys.HCl) and Poly(ethylene glycol) methacrylate (MAPEG, average M_n 475 g·mol⁻¹) were bought from Sigma-Aldrich; Glutathione (GSH), 2-aminoethanethiol (AET), azodiisobutyronitrile (AIBN), trifluoroacetic acid (TFA), phenol, n-butyl acetate, acetic acid (HAc), and hydrogen bromide 33% w/w (45% w/v) solution in acetic acid (HBr/HAc) were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2. Preparation of BLG-NCA

L-glutamate-benzyl ester (3.05 g) and THF (30 mL) were added into a 100 mL dried glass reactor previously flamed and nitrogen purged several times. Triphosgene (4.2 g) was added, and the mixture was reacted at 45 °C for 5 h. The reaction solution was slowly added dropwise to a large quantity of dry petroleum ether, and was placed in an ice bath for precipitation. The white needle crystals were precipitated and filtrated under reduced pressure. After purification in tetrahydrofuran/petroleum ether twice and drying in vacuum for 12 h, the final product (BLG-NCA) was obtained (yield: 89%).

2.3. Synthesis of core-crosslinked micelles of poly (ethylene glycol)-poly(γ -benzyl L-glutamate)

The synthetic procedure was shown in Fig. 1. Mercapto-terminated Poly(γ -benzyl L-glutamate) (PLT) was synthesized through the reaction of AET and BLG-NCA with different molar ratio in dichloromethane. Poly (ethylene glycol)-poly(γ -benzyl L-glutamate) (PLE) was obtained after the free radical polymerization of poly(ethylene glycol)methacrylate (MAPEG) in the presence of PLT served as a chain transfer agent. Finally, PLE was crosslinked by cystamine, producing the cross-linking nano micelles (PLS) after dialysis. Briefly, cystamine and PLE-2

were placed in a three neck flask according to the molar ratio of 1:1, 2:1 or 4:1 respectively, then 10 g of DMF as the solvent was added. The reaction was carried out at 30 °C under magnetic stirring for 5 h. The cross-linking nano micelles (PLS) were obtained after dialysis for 72 h.

2.4. Preparation of the drug loaded micelles

Doxorubicin(DOX) was encapsulated into the core of micelles through electrostatic interactions between carboxylic acid and DOX as showed in the following reaction (Scheme 1):

Briefly, 10 g of PLS-2 micelles suspension was mixed with TFA (5 wt %) and HBr/HAc (the molar ratio between HBr and benzyl is > 20) at 0 °C under stirring for 1 h. Then DOX-HCl was added to the mixture and the concentration was 5 mg·g⁻¹. After stirring for 1 h, ultrapure water was added dropwise until the solution color turned red and an obvious Tyndall effect can be observed. To stabilize the DOX-loaded micelles, the DOX-loaded micelle suspension was kept stirring for 12 h in darkness. 2 g stable DOX-loaded micelle solution was then transferred to a dialysis bag for dialyzing against pure water for 24 h in dark place. In the process of dialysis, the dialysis solution was analyzed for testing the acid residues of TFA, HBr and HOAc using a pH measuring meter. After removing the residues completely, the dialysis solution was transferred to a volumetric flask and diluted the solution to volume. The rest of DOX-loaded micelle solution was measure and dialyzed against pure water for 72 h. the mass of micelles and concentration of DOX-loaded micelles were determined after the micelle suspension was lyophilized. The DOX-loaded crosslinked micelle was denoted as PLS-DOX while the non-crosslinked micelle was PLE-DOX.

For measuring encapsulation efficiency (EE) and DOX loading content (LC), the DOX-loaded micelles were dissolved in dimethyl sulfoxide (DMSO) solution for completely destroying the micelle structure and releasing all the DOX. The total content of DOX in DMSO was determined using UV-Vis spectroscopy (TU-1901 type, Beijing Purkinje General Corporation) monitored at 483 nm using a calibration curve obtained from DOX/DMSO solutions with different DOX concentrations. The encapsulation efficiency (EE) and DOX loading content (LC) were obtained using the following formula:

$$EE\% = \frac{W_L}{W_S} \times 100 \quad (1)$$

$$LC\% = \frac{W_L}{W_S} \times 100 \quad (2)$$

where W_L , W_S , and W_F are weights of drug loaded, the micelle samples and drug in feed respectively.

The other detailed information for the preparations can be seen in the supporting information.

2.5. In vitro drug release under pH and GSH conditions

Drug release was performed in a shaking water bath (200 rap) at 37 °C shielded from light. The micellar solution (5.0 mL) with a concentration of 1 mg mL⁻¹ was dialyzed against 50 mL of phosphate-buffered saline (PBS) (pH 7.4 and 5.0) with or without 10 mM GSH. At each time interval, 4 mL of the medium was withdrawn and replaced with 4 mL of fresh medium. The concentration of DOX was determined by UV-Vis spectroscopy. Accumulative release of DOX from the DOX-loaded micelles was expressed as a percentage of the released DOX (formula (3)).

$$C_r(\%) = \frac{V_0 C_n + V_e \sum_{i=1}^{n-1} C_i}{W_0} \quad (3)$$

C_i : the concentration of DOX in dialysate at time i (g mL⁻¹); V_e : volume of the medium withdrawn a time (mL); V_0 : the volume of PBS (mL); W_0 : initial weight of DOX in the micelles (g); n : the number of

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