Colloids and Surfaces B: Biointerfaces 170 (2018) 373–381

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Reduction/temperature/pH multi-stimuli responsive core cross-linked polypeptide hybrid micelles for triggered and intracellular drug release

Jing Qu[a], Qiu-yue Wang[a], Kang-long Chen[a], Jian-bin Luo[a], Qing-han Zhou[a,*], Juan Lin[b,*]

[a] College of Chemical and Environment Protection, Southwest Minzu University, First Ring Road, 4th Section No.16, Chengdu, Sichuan 610041, China
[b] School of Biomedical Sciences and Technology, Chengdu Medical College, 783 Xindu Road, Chengdu, Sichuan 610500, China

ARTICLE INFO

Keywords:
Core cross-linked micelles
Polypeptide
Multiple-stimuli responsive
Drug delivery

ABSTRACT

The high toxicity, poor stability, premature drug release, and lack of intracellular stimuli responsibility of current polymeric micelles still hinder them for potential clinical applications. To address these challenges, a novel type of multi-stimuli responsive, core cross-linked polypeptide hybrid micelles (CCMs) was developed for triggered anticancer drug delivery in tumor microenvironment. The CCMs was prepared via free radical copolymerization by using N,N'-methylene-bis-acylamide (BACy) as the cross-linking agent, 2,2-azobisisobutyronitrile (AIBN) as the initiator, where poly (γ-benzyl-L-glutamate) (PBLG) and N-isopropylacrylamide (NIPPAM) as comonomers. The doxorubicin (DOX) was then introduced into the CCMs by hydrazone bond to prepare the drug-incorporated core cross-linked micelles (CCMs-DOX). By the experimental results, the CCMs showed reduction responsibility due to the degradable disulfide bond in the polymer network. The hydrazone bond can be broken under acidic condition causing a controllable drug release for CCMs-DOX. Compared to only 7.7% DOX release under pH 7.4 at 37°C, a much higher DOX release rate up to 85.3% was observed under 10 mM GSH (pH 5.0, 42°C). *In vitro* cell assays showed that the blank CCMs showed almost no toxicity against HUVEC cells while the CCMS-DOX exhibited significant cancer cell killing effect. These experimental results suggested that the prepared multi-stimuli responsive polymeric micelles could serve as a smart and promising drug delivery candidate for anti-cancer therapy.

1. Introduction

Recently, stimuli-responsive polymeric micelles serving as functional drug vehicles have emerged as the most promising technology platform for various drug delivery system (DDS) due to their small size, core-shell structure, drug-loading capacity, and triggered drug release in response to biological stimuli such as differences in redox potential, pH, and temperature between normal cells and tumor cells [1–5]. The stimuli-responsive polymeric micelles consist of a hydrophobic core and a hydrophilic shell, and possess the functional group for controlled drug release [6]. Therefore, various anti-cancer drugs can be incorporated in the micellar core such as paclitaxel or doxorubicin (DOX), and the hydrophilic shell can protect them from degradation by enzymes, prevent micelle aggregation, improve the bioavailability of drugs in water, and decrease side effects on healthy cells [7–10]. Therefore, the stimuli-responsibility can realize the triggered release of anti-cancer drugs in cancer tissue for targeted delivery [11–14].

However, for the conventional polymeric micelles from linear amphiphilic block copolymers, the micellar instability caused by plasma proteins, high electrolyte concentration, variation of pH and temperature, massive dilution, and mechanical shear forces in the blood circulation still remains challenges for DDS. The premature drug release caused by micellar instability may cause serious toxicity problems and hinder the effect of drug delivery at aimed sites, which would extremely limit their application if used in vivo. Therefore, it is of great importance for drug delivery vectors to endure the complex biological environment. Covalent cross-links in micelles can remarkably enhance the structural stability rather than the weak noncovalent intermolecular interactions in linear polymer micelles. Therefore, the high stability of polymer micelles with cross-linked structure have promising applications in DDS [15,16]. However, the permanently cross-linked micelles cannot be disintegrated and may accumulate in the host cells or tissues causing a long-term toxicity. In order to address the dilemma, in the last decade various strategies have been developed for the fabrication of degradable crosslinked micelles, which can maintain their nano-structures in the complicated extracellular environment, but undergo the biodegradation process in response to intracellular microenvironment [17].

* Corresponding authors.
E-mail addresses: zhqinghan@swun.edu.cn (Q.-h. Zhou), linjuan@cmc.edu.cn (J. Lin).

https://doi.org/10.1016/j.colsurfb.2018.06.015
Received 18 April 2018; Received in revised form 8 June 2018; Accepted 11 June 2018
Available online 18 June 2018
0927-7765/

On the other hand, although synthesis of tailored biopolymers with controllable architectures and compositions has been facilitated by new polymerization process, comparing to the proteins or peptides, synthetic biopolymers have not yet match the natural structures and biofunctional diversity so far. Due to the good biodegradability, biocompatibility, biofunctionality, and structural analogous to natural biomolecules, synthetic polypeptides have gained great interest for DDS [18]. Recently, various kinds of polypeptides with stimuli responsibility have been developed, such as reduction, temperature, pH, and enzyme as well [19–25]. Owing to good colloidal stability, the polypeptide based cross-linked polymeric micelle has been intensively studied for drug, protein, and gene delivery. For example, by using a ring-opening polymerization, Zhang et al. reported a reduction-sensitive shell-crosslinked polyglutamate-*b*-polysaccharide micelles for DOX intracellular delivery [26]. Ren et al. reported the preparation of a pH/sugar-sensitive, core-cross-linked, polyion complex micelles based on poly glutamic acid via a phenylboronic acid–catechol interaction for protein intracellular delivery [27]. Gao et al. developed a reduction- and temperature-sensitive core-cross-linked polyglutamate hybrid micelle with pendant diethylene glycol with lower critical aggregation concentration for DOX delivery against HeLa cells [28]. Via a reduction-responsive disulfide cross-linked stearyl-peptide-based micelle system, Yao et al. achieved co-delivery of DOX and microRNA-34a for prostate cancer therapy [29]. Recently, our group reported a reduction-responsive core cross-linked polyethylene glycol-polypeptide hybrid micelle with high loading capacity and efficient intracellular drug release [30]. However, due to the difficulty in fabrication schemes that involve complex and multi-step synthesis procedures, it is still challenging to integrate reduction, temperature, and pH stimuli-responsibility into one cross-linked micelle based on polypeptide for intracellular and efficient drug delivery [31].

Herein, we developed a simple approach to fabricate the core cross-linked polypeptide hybrid micelles (CCMs) via free radical copolymerization, and the anticancer drug, DOX, was then introduced to the CCMs through an acid-sensitive hydrazone bond to prepare a novel type of reduction, temperature, and pH multi-stimuli responsive core cross-linked micelles (CCMs-DOX). Their chemical structure, size, and morphology were fully characterized, and the release experiments demonstrated that the CCMs-DOX exhibited multi-stimuli responsive drug release. The blank CCMs showed almost no toxicity against HUVEC cells (normal cell) while the CCMs-DOX showed great promise of anti-tumor efficacy and intracellular drug delivery against HeLa cells (cancer cell) by the CCK8 assay and CLSM analysis. Scheme 1 illustrates the preparation of the CCMs, CCMs-DOX, and the overall mechanism of the controlled drug release in response to the simulated tumor microenvironment.

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPPAM) (98%) and Acryl amide (99%) were obtained from Best Reagent Ltd. 2,2-azobisisobutyronitrile (AIBN) (99%) purchased from Kemio Chemical Reagent Ltd. Hydrazino (80%) were obtained from Zhiyuan Chemical Reagent Ltd. Glutathione (GSH) (98%) and DOX (98%) purchased from Hua Feng Chemical Materials Ltd. N,*N*-dimethylformamide (DMF), toluene, dichloromethane, tetrahydrofuran (THF), and ethyl acetate were used after distilled.

2.1.1. Synthesis of N,N'-methylene-bis-acylamide (BACy)

Cysteamine hydrochloride (2.30 g, 10.2 mmol) was dissolved in 18 mL water and cooled in ice-water bath for 30 min. Acryloyl chloride (1.8 mL, 21.5 mmol) was dissolved in 3 mL of THF. Both above solutions were added slowly into three-necked flask within 5 min; at the same time, NaOH solution (1.60 g, 40 mM) was also drop-wisely added. The mixture was stirred for 3 h in ice-water bath, and then at room temperature for another 12 h. The organic phase was extracted with dichloromethane, and then dried over anhydrous $MgSO_4$. The BACy was finally purified by recrystallization from ethyl acetate/hexane mixture (1:1, v/v). The ^{1}H spectrum of BACy in $CDCl_3$ was shown in Fig. 1a. 1.60 g (61 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 2.89 (t, 4H, –CONHCH$_2$C***H$_2$***S–), 3.68 (t, 4H, –CONHC***H$_2$***CH$_2$S–), 5.66–6.31 (m, 6H, C***H$_2$***=C***H***CO–), and 6.61 (brs, 2H, –CON***H***–).

2.1.2. Synthesis of vinyl poly (γ-benzyl-L-glutamate) (PBLG)

γ-Benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) was prepared according to a reported literature procedure [32]. Allylamine (5 μL, 66.2 μmol), BLG-NCA (0.70 g, 2.7 mmol), and 3 mL of DMF were added to a Schlenk flask. After degassed by three freeze-thaw cycles, the mixture solution was reacted for 24 h at room temperature by gently stirring under N_2 atmosphere. The product was precipitated in ice-cooled ether and dried under vacuum. The ^{1}H spectrum of PBLG in $CDCl_3$ was show in Fig. 1b. Yield: 0.90 g (79 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 1.80–2.16 (–COOCH$_2$C***H$_2$***–), 2.31–2.52 (–COOC***H$_2$***CH$_2$–), 2.54 (CH$_2$=CHC***H$_2$***–), 4.48–4.67 (–CONHC***H***–), 4.99–5.19 (C$_6$H$_5$C***H$_2$***–), 5.46 ~ 6.67 (C***H$_2$***=C***H***CH$_2$–), 7.21–7.39 (C$_6$***H$_5$***–), and 7.72–7.92 (–CON***H***CH–).

2.1.3. Preparation of CCMs

In a typical experiment, NIPPAM (0.61 g, 5.3 mmol), PBLG (0.20 g, 0.1 mmol), BACy (0.04 g, 0.2 mmol), AIBN (0.04 g, 0.2 mmol), and 150 mL of toluene were added into a three-necked flask, and the mixture solution was stirred for 12 h at 85℃ under N_2 atmosphere. The product was precipitated in a mixture of ice-cooled ether/ tetrahydrofuran solution. The product was then filtrated and dried under vacuum. The ^{1}H spectrum of the cross-linked copolymer was shown in Fig. 1c. Yield: 1.50 g (70 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 0.99 ~ 1.30 (–C***H***$_3$, in NIPPAM unit), 1.77–2.15 (–COOCH$_2$C***H$_2$***–, in PBLG unit), 2.32–2.53 (–COOC***H$_2$***CH$_2$–, in PBLG unit), 3.66–3.78 (–CONHC***H$_2$***CH$_2$S–, in BACy unit), 3.89–4.02 (–C***H***–, in NIPPAM unit), 4.52–4.67 (–C***H***–, in PBLG polymer backbone), 4.96–5.20 (C$_6$H$_5$C***H$_2$***–, in PBLG unit), 7.18 ~ 7.37 (C$_6$***H$_5$***–, in PBLG unit), 7.70 ~ 7.98 (–CON***H***CH–, in PBLG unit and NIPPAM unit).

The CCMs were prepared by a dialysis method. 10 mg of cross-linked copolymer was dissolved in 4 mL of DMF. The solution was drop-wisely added to 5 mL of deionized water and stirred for 1 h. After that, dialysis method was used to remove the unbonded molecules and organic solvents with a dialysis tube with molecular weight cut-off (MWCO) of 12,000 for 3 days. Finally, the CCMs were freeze-dried into white powder.

2.1.4. Preparation of CCMs-DOX

Briefly, 0.50 g of CCMs was dissolved in 50 mL of DMF, and then 10 mL of anhydrous hydrazine and excessive DOX (0.10 g, 0.2 mmol) were added into the mixture solution under stirring for 24 h at room temperature with the exclusion of light and protection of N_2 atmosphere. The product solution was dialyzed against PBS buffer (pH 7.4, 10 mM) with a dialysis tube (MWCO 12,000), and the PBS buffer was refreshed every 5 h for 3 days to remove the excess DOX. Finally, the product solution was freeze-dried into a red powder.

The CCMs-DOX were prepared by a dialysis method. 10 mg of as prepared product was dissolved in 4 mL of DMF. The solution was drop-wisely added to 5 mL of deionized water and stirred for 1 h. Then the solution was transferred into a dialysis tube (MWCO 12,000) and dialyzed against PBS buffer (pH 7.4, 10 mM) for 3 days to prepare the CCMs-DOX. Finally, the product was freeze-dried into a brown powder.

2.2. Characterization

^{1}H-NMR spectra of BACy, PBLG, and CCMs were obtained using a Bruker 400-MHz spectrometer with deuterated chloroform ($CDCl_3$) as solvent using tetramethylsilane (TMS) as the internal standard. FTIR