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Highly hemocompatible zwitterionic micelles stabilized by reversible cross-linkage for anti-cancer drug delivery

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ABSTRACT

Both blood stability and intelligent-responsiveness after reaching the drug-targeting site are very important features to make desirable nano-drug vehicles (NDVs). Here, a highly nonfouling cross-linked micelle based on a copolymer composed of carboxybetaine methacrylate (CBMA) as hydrophilic segment and 2-(methacryloyloxy)ethyl lipoate (MAEL) as hydrophobic and cross-linked segment is reported. Furthermore, a simple method to evaluate the hemocompatibility of NDVs through examining the activation of a blood-clotting protein (fibrinogen) was introduced. The micelles can encapsulate anticancer drug doxorubicin (DOX) conveniently and release DOX quickly in response to an intracellular reductive environment. With the advantages of excellent stability in fibrinogen (1 mg/mL) PBS solution and 50% fetal bovine serum (FBS), and accelerated intracellular drug release, the biocompatible zwitterionic micelles stabilized by reversible cross-linkage might be a promising drug carrier for cancer chemotherapy.

1. Introduction

Micelles formed by self-assembly of amphiphilic copolymer have attracted significant interest as drug delivery systems because of their distinct advantages including improving the solubility of hydrophobic drugs, long circulation *via* evading recognition by reticuloendothelial system (RES) and passive targeting ability of tumor tissues by the enhanced permeability and retention (EPR) effect [1–4]. However, one of the significant disadvantages of normal self-assembled polymeric micelles is that micelles are not stable and they may dissociate upon dilution. Cross-linking approaches have been shown to be an effective way to improve the stability of micelles. In the past decade, cross-linking approaches have been developed in the core, in the shell, or at the interface of the hydrophobic and hydrophilic layer [5–8]. However, most techniques resulted in overly stable micelles, which are not desirable due to the extremely slow drug release after the micelles arrive at the target sites. In this sense, the application of degradable linkages for cross-linking would facilitate the drug release. Stimuli-responsive polymeric micelles are very promising because they can achieve sudden drug release with environmental stimulus such as temperature [9], pH, [10] light [11], redox [12–14], etc.

Reduction-sensitive micelles are particularly interesting, because of the presence of high glutathione tripeptide (γ-glutamyl-cysteinyl-glycine, GSH) concentration inside cells. In blood and extracellular matrices, as well as on the cell surface, the disulfide cross-linked micelles are rather stable as a result of a relatively high redox potential due to a low concentration of GSH. On the other hand, the micelles with disulfide cross-linkage could rapidly release the loaded drugs intracellularly through the cleavage of disulfide bonds by GSH [15–17]. Recently, Zhong and coworkers [18] reported reversibly stabilized dextran nanoparticles for the triggered intracellular delivery. The reported dextran nanoparticles were stable under extracellular conditions but were rapidly destabilized under reductive environments and triggered the release of DOX. Thayumanavan and coworkers [19] reported biocompatible nanogels that provided the ability to encapsulate hydrophobic guest molecules using intra/intermolecular disulfide formation of polymers containing pyridyl disulfide (PDS). The release of guest molecules could be induced by GSH. Additionally, the surface of this nanogel could be efficiently functionalized under mild conditions.

Another necessary property of self-assembled polymeric micelles is the stability in blood stream. Polyethylene glycol (PEG) and oligo(ethylene glycol) (OEG) are the most commonly used materials to form the shells of micelles to make them stable [20,21]. However, it is now recognized that PEG or OEG decomposes in the presence of oxygen and transition metal ions found in most biochemically relevant solutions [22]. And also a possible

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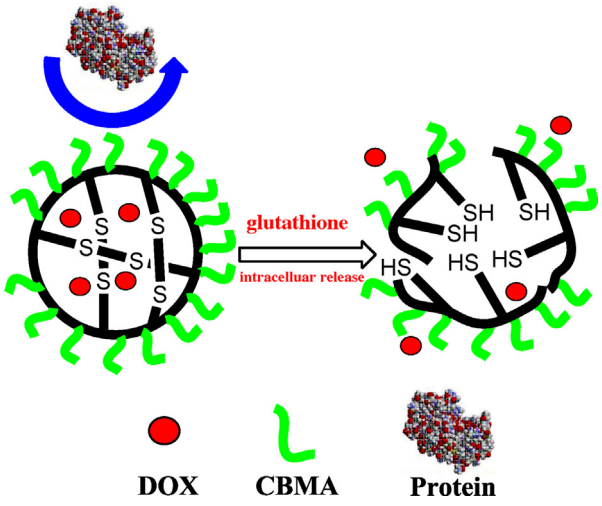

Scheme 1. Schematic representation of hemocompatible reversible cross-linked micelles for intracellular release of DOX.

immunoreaction to PEGylated protein drug has been observed [23]. The complex formation between albumin and PEG further suggests that a possible unknown mechanism of PEG protection might be involved [24]. Thus, all these potential negative effects limit their long-term applications. With the close investigation on the mechanism of nonfouling materials in recent years, polyzwitterionic materials, such as poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC) [25–27], poly(sulfobetaine methacrylate) (pSBMA) [28–30], poly(carboxybetaine methacrylate) (pCBMA) [31,32] and simply mixed-charge materials [33–36] have been recognized as effective nonfouling materials which can maintain the stability of micelles in complex media such as serum. Therefore, polyzwitterionic materials might be good alternatives of PEGs for excellent stability in blood.

However, because of the extremely strong hydrophilicity of zwitterionic materials, it is rather difficult to prepare a copolymer with both highly hydrophilic and hydrophobic segments for efficient and stable polymeric drug carrier. Thus, we propose to use short cross-linkable moiety to stabilize and entrap drugs. Here, a reversible cross-linked micelle as a hemocompatible, robust and smart nanocarrier for controlled release of anticancer drug Doxorubicin (DOX) was prepared and investigated. The micelles can entrap DOX easily and release it quickly in response to an intracellular reductive environment. Moreover, this nanocarrier exhibited excellent stability in fibrinogen (1 mg/mL) PBS solution and 50% fetal bovine serum (FBS), and accelerated intracellular drug release (Scheme 1). These hemocompatible zwitterionic micelles stabilized by reversible cross-linkage might be a promising drug carrier for cancer chemotherapy.

2. Experimental methods

2.1. Materials

N,*N*-Dicyclohexylcarbodiimide (DCC, 99%) was purchased from J&K China Chemical Ltd. Doxorubicin hydrochloride (DOX·HCl, 99%) was purchased from Taizhou XinFangXiang Chemical Co., Ltd. DL-Dithiothreitol (DTT, 99%), lipoic acid (99%), 2-hydroxyethyl methacrylate (HEMA, 98%), 4-(dimethylamino)pyridine (DMAP, 99%), 2,2′-azobisisobutyronitrile (AIBN, 99%) and dimethylaminoethyl methacrylate (DMAEMA, 99%) were purchased from Aladdin-reagent (Shanghai). Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, β-propiolactone and glutathione monoethyl ester (GSH-OEt) were purchased from TCI (Shanghai). Neutralized TCEP was prepared following the procedures reported previously [8]. AIBN was purified by recrystallization from ethanol.

2.2. Characterization

All ^{1}H NMR spectra were recorded in CD_3OD, $CDCl_3$, DMSO-d_6 or D_2O using a Bruker ADVANCE2B/400MHZ instrument at room temperature. The critical micelle concentration (CMC) determination used pyrene as a hydrophobic fluorescent probe and carried out by FLS920 spectrofluorometer at room temperature. For fluorescence measurement, the emission fluorescence of pyrene was monitored at 394 nm when excited at 339 and 334 nm respectively. The concentration of the copolymer were varied from 1.0×10^{-4} to 0.6 mg/mL and the concentration of pyrene was fixed at 6.02×10^{-7} mol/L. Transmission electron microscopy (TEM) samples were prepared by drying a drop of aqueous solution of copolymer micelles (1 mg/mL) onto a carbon-coated copper grid. TEM analysis was performed by JEM-1200EX TEM operating at an accelerating voltage of 80 kV. The average diameter and size distribution of the micelles were measured by Zetasizer Nano-ZS (Malvern Instruments) with the scattering angle at 173°.

2.3. Synthesis of carboxybetaine methacrylate (CBMA)

Carboxybetaine methacrylate (CBMA) was prepared as the procedures reported previously [37]. Briefly, β-propiolactone (3.53 mL, 55 mmol) in 5 mL anhydrous acetone was added dropwise to a solution of DMAEM (8.43 mL, 50 mmol) dissolved in 45 mL anhydrous acetone. The reaction was stirred under nitrogen protection at 15 °C for 6 h. The reaction mixture was filtrated and a white solid was collected after washed by 50 mL anhydrous acetone. The white solid was dried in vacuum drying oven to obtain the final product with a yield of 77%. ^{1}H NMR (400 MHz, D_2O): 1.83(3H, s), 2.62(2H, t), 3.08 (6H, s), 3.56 (2H, t), 3.68 (2H, t), 4.54 (2H, t), 5.67(1H, s), 6.05 (1H, s).

2.4. Synthesis of 2-(methacryloyloxy) ethyl lipoate (MAEL)

Typically, DCC (2.47 g, 12 mmol) was added into 20 mL anhydrous CH_2Cl_2 solution comprising lipoic acid (2.06 g, 10 mmol), HEMA (1.30 g, 10 mmol) and DMAP (145 mg, 1.2 mmol). The mixture was stirred at 0 °C under nitrogen. The reaction mixture was allowed to warm up to ambient temperature and stirred overnight. After filtration the solution was evaporated and the residue was purified by column chromatography (silica gel 100–200 mesh) using $CHCl_3$ as eluent solvent. MAEL was obtained as a yellow liquid, with the yield of 85%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.45 (2H, m), 1.67 (m, 4H), 1.87 (m,1H), 1.94 (s, 3H), 2.35 (t, 2H), 2.45 (m, 1H), 3.14 (m, 2H), 3.55 (m, 1H),4.33(s, 4H), 5.58 (s, 1H), 6.13 (s, 1H).

2.5. Synthesis of poly(CBMA-co-MAEL)

A typical polymerization procedure of poly(CBMA-*co*-MAEL) was as carried out as follows (Scheme 2): CBMA (0.311 g, 1.35 mmol), MAEL (0.286 g, 0.9 mmol) and AIBN (0.018 g, 0.11 mmol) were first dissolved in methanol (10 mL). Then, the mixture was added into a 25 mL flask with a magnetic stirrer bar and degassed by nitrogen for 30 min. The polymerization tube was then placed into an oil bath thermostat at 60 °C for 16 h. The reaction mixture was concentrated with a rotary evaporator, then repeatedly precipitated in cold isopropanol for three times and dried under vacuum to obtain copolymer 2 (CP 2) as a yellow solid. The yield of CP 1, CP 2 and CP 3 was 68.7%, 78.8% and 76.5%, respectively.