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Nanomicelles based on a boronate ester-linked diblock copolymer as the carrier of doxorubicin with enhanced cellular uptake

Yan Xu[a], Yuanyuan Lu[a], Lei Wang[a], Wei Lu[a], Jin Huang[b], Ben Muir[c], Jiahui Yu[a,*]

[a] Shanghai Engineering Research Centre of Molecular Therapeutics and New Drug Development, School of Chemistry and Molecular Engineering, East China Normal University, Shanghai 200062, PR China
[b] College of Chemistry, Chemical Engineering and Life Science, Wuhan University of Technology, Wuhan, PR China
[c] The Commonwealth Scientific and Industrial Research Organisation, Bayview Ave, Bag 10, Clayton South, Melbourne, Victoria 3169, Australia

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ABSTRACT

This study sought to develop a new type nanomicelle based on boronate ester-linked poly(ethylene glycol)-*b*-poly(benzyl glutamate) (PEG-BC-PBLG) diblock copolymer as the carrier of doxorubicin (Dox) to achieve acid-induced detachment of PEG shells and subsequent boronic acid-mediated enhanced endocytosis. *In vitro* studies revealed that the PEG-BC-PBLG copolymer was stable in neutral solutions but tend to hydrolysed under acidic conditions, which was attributed to the acid-sensitive properties of boronate ester bonds. The formation of PEG-BC@PBLG micelles was confirmed based on critical micelle concentration (CMC), particle size, and morphology observations. It was observed that these micelles were spherical with an average particle size of approximately 80 nm, as measured by dynamic laser scattering (DLS), suggesting their passive targeting to tumour tissue and endocytosis potential. Dox-loaded PEG-BC@PBLG micelles (PEG-BC@PBLG·Dox) showed sustained drug release profiles over 9 h, and their cumulative drug release was dependent on the pH value of the environment. Remarkably, cellular uptake ability of PEG-BC@PBLG micelles was found to be higher than that of non-boronate ester-linked PEG@PBLG micelles due to boronic acid-mediated endocytosis, as revealed by confocal laser scanning microscopy (CLSM) imaging of fluorescein isothiocyanate (FITC) green-conjugated micelles, thereby providing higher cytotoxicity against HepG2 cells. The antitumour activity and toxicity of PEG-BC@PBLG·Dox micelles *in vivo* were evaluated in BLAB/c mice against HepG2 cell-derived tumours. Compared with Dox, PEG-BC@PBLG·Dox showed reduced toxicity, whereas its tumour growth inhibition rate was 17% higher than that of free Dox. These results indicate the great potential of PEG-BC@PBLG micelles as the carrier of various lipophilic anticancer drugs with improved anti-tumour efficacy.

1. Introduction

Conventional chemotherapy has achieved great success in cancer therapy but remains limited by a lack of tumour-selectivity, drug resistance, and other side effects [1,2]. To overcome these limitations, substantial efforts has been devoted to the development of anticancer drug delivery carriers because of their prolonged circulation time and high passive targeting ability to tumour sites *via* the enhanced permeability and retention (EPR) effect [3,4]. For cancer therapy, it is desirable to achieve a sufficient drug concentration at tumour tissues, which may enhance the therapeutic efficacy and reduce the probability of drug resistance in cells [5,6]. Therefore, various stimuli-sensitive drug carriers have been designed in recent years based on the physiological properties of the tumour microenvironment or the expression of tumour-associated receptors [7–9]. The difference in the pH value between tumour and normal tissue is often used as a stimulus factor [10]. The pH value of normal plasma is 7.35–7.45, whereas the pH in tumour microenvironment decreases to 6.6–6.8 depending on the distance from blood vessels because anaerobic glycolysis is facilitated by the limited oxygen supply [11]. Previously designed pH-sensitive carriers are relatively stable in normal blood circulation but can degrade when they accumulate at target tumours, mainly owing to the breakage pH-sensitive chemical bonds [12,13].

Boronate ester has been demonstrated as a pH-sensitive chemical bond, which can be easily hydrolysed under acid conditions but relatively stable in neutral and alkaline solutions [14,15]. Levkin et al. [16] reported the formation of a boronate dextran polymer (B-Dex) that realised four-fold higher release of incorporated anticancer drug at pH 5.0 in comparison to pH 7.4. For delivery to solid

* Corresponding author. Fax: +86 21 6223 7026.
E-mail address: jhyu@sist.ecnu.edu.cn (J. Yu).

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tumours, the surfaces of nanoparticles were modified with water soluble polymers, such as PEG, to afford a long circulation time in blood and passive targeting potential to the tumour mass [17,18]. However, PEGylation inhibits cellular uptake of nanoparticles [19]. Receptor-mediated cellular uptake of PEGylated nanoparticles is usually achieved by immobilization of antibodies or other ligands that recognize tumour-associated antigens or ligand receptors at the terminal end of PEG [20,21]. It has been reported that boronic acid shows high affinity with the polysaccharide called Sialyl Lewis X, which is abundant on the surface of HepG2 cells. Additionally, Zhong et al. revealed that the transfection of phenylboronic acid-modified PEIs was more efficient in HepG2 cells, which realised receptor-mediated endocytosis [22–24].

In this paper, a new strategy to prepare boronate ester-linked core-shell nanomicelles as the carrier of doxorubicin was developed to achieve acid-induced detachment of PEG shells and subsequent boronic acid-mediated enhanced endocytosis. Particularly, PEG-BC@PBLG nanomicelles were fabricated by self-assembly of boronate ester-linked PEG-BC-PBLG copolymer. Because boronate ester is stable in neutral environments, PEG-BC@PBLG micelles could maintain their structure and integrate into blood circulation and could reach tumour tissues in a passive targeting manner through an EPR effect [25,26]. However, the pH at both primary and metastasised tumours (pH 6.5–6.8) is slightly lower than the pH of normal tissue, and intracellular components such as the endosomes (pH 6.0–6.5) or lysosomes (pH 5.0–5.5) are more acidic [27,28]. Therefore, the poly(ethylene glycol) (PEG) shells of PEG-BC@PBLG micelles would be detached in tumour tissues. The exposed boronic acid segment would then realise receptor-mediated endocytosis. Furthermore, the micelles would liberate the loaded drug and achieve improved cancer therapy (Scheme 1). The structure of PEG-BC-PBLG copolymer was characterised by ^{1}H NMR and gel permeation chromatography (GPC). The physicochemical properties of PEG-BC@PBLG micelles, such as the particle size, critical micelle concentration (CMC) and drug accumulative release, were studied. Subsequently, cellular uptake and cell toxicity were evaluated and compared with that of PEG@PBLG micelles that do not contain boronate ester bonds. The *in vivo* anti-tumour activity of PEG-BC@PBLG and Dox-loaded micelles in tumour-bearing nude mice were later evaluated in detail.

2. Experimentals

2.1. Materials

N, *N*-diisopropylethylamine (DIPEA, 99%), 1-hydroxybenzotriazole (HOBT, 99%), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 99%) and 3-aminobenzeneboronic acid (98%) were purchased from Sigma–Aldrich. Methoxypolyethylene glycol amine (CH_3O-PEG-NH_2, Mn = 2000, Fluka) was dried by azeotropic distillation from toluene. 3, 4-Dihydroxyphenylacetic acid (DA, 99%), fluorescein isothiocyanate (FITC, 97%) and 2, 4-dihydroxybenzoic acid (98%) were obtained from Aladdin. 5-Benzyl L-glutamate *N*-carboxyanhydride (BLG–NCA, 98%) was obtained from Alfa Aesar. Dialysis bags and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT, 98%) were purchased from Vita Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water was prepared using a Milli-Q system (Millipore, USA). Toluene and tetrahydrofuran (THF) were dried by refluxing over sodium wire and distilled before use. Triethylamine (TEA), dichloromethane (DCM), and dimethyl sulfoxide (DMSO) were dried by refluxing over CaH_2 and distilled prior to use. Doxorubicin hydrochloride (>99%, Energy Chemical) and other reagents were used as received.

2.2. Cell line and culture

Human hepatoma cell line HepG2 and human hepatocyte cell line HL-7702 was supplied by the Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. HepG2 cells were cultured in minimum essential medium (MEM), and HL-7702 cells were cultured in RPMI 1640 (Gibco BRL, Paris, France), both of which were supplemented with 10% foetal bovine serum (FBS, HyClone, Logan, UT), streptomycin at 100 μg/mL, and penicillin at 100 U/mL. All cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The confluent cells were dissociated using a pre-warmed trypsin-EDTA solution at 37 °C.

2.3. Synthesis of PEG-BC-PBLG

DA was conjugated to the polymer mPEG-NH_2 by an amidation reaction [29]. Briefly, mPEG-NH_2 (1.000 g, 0.500 mmol) was first dissolved in 30 mL of anhydrous DCM; then, EDC·HCl (0.154 g, 0.800 mmol), HOBT (0.108 g, 0.800 mmol), DIPEA (0.260 mL, 1.500 mmol), and DA (0.126 g, 0.750 mmol) were added and protected by nitrogen. After stirring for 12 h at 25 °C, the mixture was purified by column chromatography (dichloromethane:methanol = 30:1). The organic solvent was removed by low-pressure evaporation. The product (PEG-DA) was then precipitated into cold ethyl ether, isolated by filtration, and dried under high vacuum (yield: 78%).

3-Aminobenzeneboronic acid (0.137 g, 1.000 mmol) and PEG-DA (0.430 g, 0.200 mmol) were dissolved in 100 mL of toluene and placed in a reactor fitted with a Dean-Stark apparatus for water removal [30–33]. The mixture was stirred and refluxed at 120 °C for 8 h, and the reaction was then terminated by cooling to room temperature. The crude product was dissolved in anhydrous THF and dialysed for 48 h (molecular weight cut-off size: 1000). The resulting polymer was precipitated in cold ethyl ether, isolated by filtration, and dried under vacuum; this product was named PEG-BC (yield: 80%).

PEG-BC-PBLG was synthesised by ring-opening polymerisation [34]. BLG-NCA (0.740 g, 2.800 mmol) and PEG-BC (0.897 g, 0.400 mmol) was dissolved in 30 mL of anhydrous THF. The reaction mixture was stirred for 24 h at 25 °C and protected by N_2. The crude product was dissolved in anhydrous THF and dialysed for 48 h (molecular weight cut-off size: 3500). The resulting polymer was isolated by precipitation in cold ethyl ether, filtered, washed three times, and then dried under vacuum (yield: 95%). PEG-PBLG was synthesised by mPEG-NH_2 and BLG-NCA in the same manner as described above.

2.4. Synthesis of PEG-BC-PBLG-FITC

FITC (0.089 g, 0.230 mmol) and PEG-BC-PBLG (0.500 g, 0.150 mmol) were dissolved in10 mL of anhydrous THF and stirred for 12 h at 25 °C [35]. The crude product was then evaporated and added to cold ethyl ether, washed several times to remove excess FITC, and dried under vacuum (yield: 91%). PEG-PBLG-FITC was synthesised in the same way.

2.5. Characterization of PEG-BC-PBLG diblock copolymer

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Bruker Avarice TM 500 NMR spectrometer. The molecular weights of samples were measured using an Agilent 1200 gel permeation chromatography (GPC) system (Agilent Technologies Inc., Shanghai Branch). An Agilent 1200 refractive index detector and aqueous SEC start-up kit were used. Chromatography columns (PL aquagel-OH MIXED columns; Polymer Laboratories Ltd., Amherst, MA, USA) were calibrated using a poly (ethylene glycol) kit. The col-