Colloids and Surfaces B: Biointerfaces 126 (2015) 178–187

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Reductively-sheddable cationic nanocarriers for dual chemotherapy and gene therapy with enhanced release

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ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form
29 November 2014
Accepted 5 December 2014
Available online 18 December 2014

Keywords:
Block copolymer
Co-delivery
Drug delivery
Oligonucleotide delivery
Reduction-responsive degradation
Enhanced release

ABSTRACT

The development of a versatile strategy to synthesize cationic nanocarriers capable of co-delivery and enhanced release of drugs and oligonucleotides is promising for synergic dual chemotherapy and gene therapy. Herein, we report a novel cationic amphiphilic diblock copolymer having a single reduction-responsive disulfide linkage at a junction between a FDA-approved polylactide (PLA) block and a cationic methacrylate block (C-ssABP). The amphiphilic design of the C-ssABP enables the formation of cationic micellar aggregates possessing hydrophobic PLA cores, encapsulating anticancer drugs; cationic coronas, ensuring complementary complexation with negatively-charged oligonucleotides through electrostatic interactions; and disulfides at interfaces, leading to enhanced release of both encapsulated drugs and complexed oligonucleotides. The reduction-responsive intracellular trafficking results from flow cytometry, confocal laser scanning microscopy, and cell viability, as well as *in vitro* gene transfection assay suggest that C-ssABP offers versatility as an effective nanocarrier platform for dual chemotherapy and gene therapy.

1. Introduction

Amphiphilic block copolymer (ABP)-based self-assembled micelles have been extensively explored as effective candidates of polymer-based nanocarriers in pharmaceutical science [1–5]. Well-designed ABP-based micelles consist of hydrophobic cores enabling encapsulation of hydrophobic therapeutics to deliver targeted sites. Polylactide (PLA) and its copolymers have been considered as effective building blocks in constructing hydrophobic cores. This is due to their unique features being biocompatible, FDA-approved for clinical use, and biodegradable by enzymatic reaction or hydrolysis under physiological conditions [6–8]. To promote their applicability toward biomedical applications, an introduction of dynamic covalent linkages, particularly disulfide linkages, into PLA-based ABPs and their self-assembled structures has been proposed [9–12]. The reductive cleavage of the disulfide linkages caused the disintegration of PLA-based nanocarriers, exhibiting the enhanced release of encapsulated anticancer drugs. Furthermore, hydrophobic PLA cores are engineered with hydrophilic surfaces to minimize opsonization, leading to prolonged circulation in the blood [13,14]. Typical hydrophilic polymers that have been used include poly(ethylene glycol) (PEG) [15–19] and polymethacrylates [20–24]. Besides these neutral sheaths, ionic shells can also be appealing because therapeutic biomolecules having relatively high molecular weight such as nucleic acids, proteins, and polysaccharides are ionic compounds (either cationic or anionic) [25–27]. In particular, nanocarriers with positive charges (cationic nanocarriers) facilitate the delivery of anionic nucleic acids (DNA, RNA, and chemically modified oligonucleotides) though electrostatic interactions [28,29].

Gene therapy including gene silencing mostly utilizes nonviral vectors based on positively-charged cationic polymers that grant nucleic acids with protection against enzymatic degradation. They form polyplexes with negatively-charged phosphate groups of nucleic acids through ionic complexation. After internalization inside targeted cells, the nucleic acids can escape from endosomes and transfer to the nuclei; such endosomal escape that leads to effective gene transfection is facilitated by proton-sponge or pH buffering effect inside endosomes (called endosomal escape) [30–32]. Of several cationic polymers that have been extensively explored as gene carriers, poly(N,N-dimethylaminoethyl methacrylate) (PDMA) has shown its relatively lower cytotoxicity driven by tertiary amine [33–36]. The quaternized PDMA (cPDMA) can imbibe nucleic acids with protection against enzymatic degradation as they form polyplexes through ionic complexation with negatively charged phosphate groups of nucleic acids. Further,

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http://dx.doi.org/10.1016/j.colsurfb.2014.12.010
0927-7765/

the PDMA block is easily incorporated into the block copolymers through various controlled radical polymerization methods as well as converted to the corresponding quaternized cationic block. In order to enhance nucleic acid transfection for the success of nucleic acid-based therapies, disulfide reduction chemistry has been explored. Two typical approaches based on the location of disulfide linkages in cationic (co)polymers include reduction-responsive main-chain degradation and PEG deshedding. For the reduction-responsive main-chain degradation, disulfides are formulated in polycation backbones. This approach enhances endosomal escape inside cells while minimizing cytotoxicity as well as increasing transfection efficiency [37–40]. For the reduction-responsive PEG, disulfides are positioned at block junctions between polycation backbones and PEG blocks. This approach is desired to circumvent what is known as PEG dilemma, thus enhancing both circulation time in the blood and transfection efficiency [41–43].

Herein, we report on novel PLA-based cationic micelles self-assembled from cationic PLA-ss-cPDMA ABP (C-ssABP) for development of reduction-responsive co-delivery and enhanced intracellular release of encapsulated drugs in hydrophobic PLA cores and oligonucleotides in cationic coronas (Scheme 1). The cationic ABP was synthesized by a combination of ring-opening polymerization (ROP), atom transfer radical polymerization (ATRP), and post-functionalization through quaternization. The single disulfide linkages are positioned at block junctions in C-ssABP; thus self-assembled micelles had the disulfides located at interfaces between PLA cores/drugs and cPDMA coronas/DNA. Different from the conventional methods previously mentioned to circumvent the PEG dilemma, here the reductive cleavage of the interfacial disulfide linkages resulted in shedding cPDMA coronas, causing the destabilization of the integrity of micellar aggregates, thus leading to both enhanced release of drugs and prompted endosomal escape of oligonucleotides. Intracellular release of encapsulated doxorubicin (DOX) and DNA transfection were confirmed by *in vitro* results from flow cytometry, confocal laser scanning microscopy, and cell viability assay as well as gene transfection assay.

2. Materials and methods

2.1. Instrumentation and analyses

^{1}H NMR spectra were recorded using a 500 MHz Varian spectrometer. The $CDCl_3$ singlet at 7.26 ppm, DMSO-d_6 multiplet at 2.5 ppm, and DMF-d_7 singlet at 8.03 ppm were selected as the reference standards. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). An Agilent GPC was equipped with a 1260 Infinity Isocratic Pump and a RI detector. Two Agilent PLgel mixed-C and mixed-D columns were used with DMF containing 0.1 mol% LiBr at 50 °C at a flow rate of 1.0 mL/min. Linear poly(methyl methacrylate) standards from Fluka were used for calibration. Aliquots of polymer samples were dissolved in DMF/LiBr. The clear solutions were filtered using a 0.25 μm PTFE filter to remove any solvent-insoluble species. A drop of anisole was added as a flow rate marker. Monomer conversion was determined using ^{1}H NMR.

2.2. Dynamic light scattering (DLS)

The size of micelles in hydrodynamic diameter by volume was measured by dynamic light scattering (DLS) at a fixed scattering angle of 175° at 25 °C with a Malvern Instruments Nano S ZEN1600 equipped with a 633 nm He–Ne gas laser. UV–vis spectra were recorded on an Agilent Cary 60 UV–vis spectrometer using a 1 cm wide quartz cuvette.

2.3. Transmission electron microscope (TEM) images

TEM images were taken using a Philips Tecnai 12 TEM, operated at 120 kV and equipped with a thermionic LaB6 filament. An AMT V601 DVC camera with point to point resolution and line resolution of 0.34 nm and 0.20 nm respectively was used to capture images at 2048 × 2048 pixels. To prepare specimens, the micellar dispersions were dropped onto copper TEM grids (400 mesh, carbon coated), blotted and then allowed to air dry at room temperature.

2.4. Materials

3,6-Dimethyl-1,4-dioxane-2,5-dione (DL-lactide, LA), tin(II) 2-ethylhexanoate ($Sn(EH)_2$, 95%), copper(I) bromide (CuBr, >99.99%), *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA, >98%), iodomethane (MeI), L-glutathione reduced (GSH), and doxorubicin hydrochloride (DOX, $-NH_3^+Cl^-$ salt form, >98%) from Aldrich, DL-dithiothreitol (DTT, 99%) from Acros Organics, and 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites and protected 2′-deoxyribonucleoside-CPG from Glen Research (Sterling, Virginia) were purchased and used as received. N,N-dimethylaminoethyl methacrylate (DMA, >98%) purchased from Aldrich was purified by passing it through a column filled with basic alumina to remove the inhibitors. 2-Hydroxyethyl-2′-(bromoisobutyryl)ethyl disulfide (HO-ss-Br) was synthesized according to our previous publication [44].

2.5. Synthesis of ssDNA

ssDNA were assembled using an Applied Biosystems Model 3400 synthesizer on a 1 μmol scale employing standard β-cyanoethylphosphoramidite cycles supplied by the manufacturer. The oligomer-derivatized CPG beads were transferred from the reaction column to screw cap microfuge tubes fitted with teflon lined caps and the oligomer released from the support and protecting groups removed by treatment with a mixture of concentrated ammonium hydroxide/EtOH (0.3/0.1, v/v) for 4 h at 55 °C. The crude oligonucleotide were transferred and concentrated in a speed-vac concentrator followed by purification from pre-terminated products by strong anion exchange HPLC using a Dionex DNAPAC PA-100 column (0.4 cm × 25 cm) purchased from Dionex Corp (Sunnyvale, CA) with a linear gradient of 0–50% buffer B over 30 min (buffer A: 100 mM Tris HCl, pH 7.5, 10% acetonitrile and buffer B: 100 mM Tris HCl, pH 7.5, 10% acetonitrile, 1 M NaCl) at 40 °C. The column was monitored at 260 nm for analytical runs or 280 nm for preparative runs. The purified oligomer was desalted using C-18 SEP PAK cartridges (Waters Inc.) and quantified using a Varian CARY Model 3E spectrophotometer.

2.6. Synthesis of PLA-ss-Br using ROP

HO-ss-Br (300.3 mg, 0.99 mmol), LA (10.0 g, 69.4 mmol), $Sn(EH)_2$ (20.1 mg, 0.05 mmol), and toluene (6 mL) were added to a 25 mL Schlenk flask. The resulting mixture was deoxygenated four times by freeze–pump–thaw cycles. The reaction flask was filled with nitrogen, thawed, and then immersed in an oil bath preheated to 120 °C to start the polymerization. After 2.5 h, the polymerization was stopped by cooling down to room temperature. The resulting PLA homopolymers were precipitated from MeOH and dried in a vacuum oven at room temperature for >12 h.

2.7. Synthesis of PLA-ss-PDMA (ssABP) using ATRP

The purified, dried PLA-ss-Br (1.0 g, 87 μmol), DMA (0.68 g, 4.34 mmol), PMDETA (9.05 μL, 0.04 mmol), and THF (1.07 mL) were