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Hydrogen bonding-enhanced micelle assemblies for drug delivery

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ABSTRACT

Ring-opening polymerization (ROP) of functionalized cyclic carbonates derived from 2,2-bis(methylol) propionic acid (bis-MPA) allows for incorporation of H-bonding urea-functional groups into block copolymers with a potential application of supramolecular drug-delivery systems. The strong H-bonding functionalities of poly(ethylene glycol)-*block*-poly(ethyl-*random*-urea carbonate) (PEG-P(E_{1-x}-U_x)C) block copolymers not only lowered critical micelles concentration (cmc) of the block copolymer (to 1/4×) in aqueous environment compared to conventional PEG-poly(trimethylene carbonate) (PEG-PTMC) block copolymer without the non-covalent stabilization, but also improved kinetic stability of micelles and Dox-loaded micelles in the presence of a destabilizing agent. It was observed that the incorporation of anticancer drug doxorubicin affected the micellization process of block copolymers in water and caused a sudden increase in sizes of drug-loaded micelles above 200 nm. This phenomenon that can be a significant drawback in drug delivery applications was considerably mitigated in urea-bearing block copolymer/Dox micelles with simultaneously accompanying a significant improvement in drug loading. *In vitro* drug release profile showed that the increase in urea content led to a slight decrease in Dox release rate. Block copolymer did not have any significant cytotoxicity against HEK293 and HepG2 cells up to 400 mg/L. Importantly, Dox-loaded micelles exerted cytotoxic effect against HepG2 cells.

1. Introduction

Research at the interface of polymer chemistry and biomedical science opens a new era of the nanosized polymer-based pharmaceuticals [1–3]. The majority of clinically used drugs are low-molecular-weight compounds that exhibit a short half-life in the blood stream and a high overall clearance rate. Thus, a number of drug-delivery systems have been proposed to overcome these transport problems including limited solubility, stability, and toxicity of drugs or potent drug candidates. Polymer therapeutics includes polymeric drugs (sequestrant), polymer–drug conjugates, polymer–protein conjugates, polymer–DNA complexes (polyplexes), and polymeric micelles to which drugs are covalently bound or physically incorporated [4–9]. Conventionally, nanoscale therapeutics are derived from polymer–drug (or polymer–protein) conjugates, in which a drug is covalently linked to polymers through a cleavable linker such as a lysosome-dependent Gly-Phe-Leu-Gly tetrapeptide and pH sensitive *cis*-aconityl, hydrazone or acetal linkages [4–9]. On the other hand, supramolecular drug-delivery systems based on block copolymer micelles or dendritic polymer nanocarriers also show great promise for tumor targeting and drug delivery. Both covalent and non-covalent systems can utilize the enhanced permeability and retention (EPR) effect in disorganized and leaky angiogenic tumor vasculature for targeting [10–12].

Self-assembled block copolymer micelles are typically several tens of nanometers in diameter with a relatively narrow size distribution and have long been explored because they are expected to be a simple, economic and versatile approach to nanosized drug carriers [10–12]. The major obstacles for supramolecular drug-delivery systems based on non-covalent entrapment of drugs into core-shell architectures are the lack of kinetic stability of polymer micelles that are susceptible to infinite dilution arising from their administration and poor drug loading capacity. Critical micelle concentration (CMC) of polymers is an important parameter to anticipate *in vivo* kinetic stability of the micelles. It was reported that 74% of micelles made from poly(ethylene glycol)-*b*-poly-caprolactone (PCL) with CMC of 38 mg/L was still found in mouse blood after 24 h of circulation as compared to 33% of micelles formed from Pluronic P85 (poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide)) with CMC of 300 mg/L [13,14].

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Efforts to bolster the weak intermolecular interactions that effect micelle formation and stability include selective crosslinking of either the interior (core) or exterior (corona), crosslinking throughout the micelle, or stabilizing non-covalent interactions [15,16]. Despite the improved stability of the chemical crosslinking, this approach may not be optimal in the encapsulation of a guest molecule or biodegradability. The precisely-tunable structure of block copolymers combined with new synthetic methodologies can allow the use of non-covalent interactions in polymeric assemblies [17–19]. The role of non-covalent interactions is particularly pronounced as a collective driving force to the formation of stable aggregates [20–23]. For example, Weaver et al. explored complexation of oppositely charged poly(ethylene glycol) (PEG) block copolymers with cationic quaternized amino methacrylates or anionic styrene sulfonates for preparation of ionically shell crosslinked micelles [20]. Borsali et al. reported that specific acid–base interaction between hydrophobic drug molecules (R_1-COOH) and polymer segments (NH_2-R_2) improved the drug loading capacity of block copolymer micelles [21]. Leroux et al. showed that stereocomplex block copolymer micelles formed from mixture of poly (ethylene glycol)-poly(lactide) have the enhanced micelle stability with a potential for the delivery of drugs [22]. Motivated by these reports, we and others have been interested in a simple yet versatile synthetic methodology that employs reversible non-covalent interactions for mediating the formation of macromolecular assemblies that encapsulate, transport, and release therapeutic agents [24–28]. Ureas are known to associate *via* bifurcated hydrogen bonds [29]. In addition, ureas are also known to bind carboxylate derivatives that are present in many anticancer drugs such as doxorubicin and paclitaxel, and their isosteres (such as sulfonates, phosphonates, and phosphates) [30,31]. In this study, we report the first urea-functional block copolymers with various urea contents and molecular weights, and investigate the effect of incorporating hydrogen-bonding urea groups into the hydrophobic block of polymers on kinetic stability of their self-assembled micelles, and the ability of the micelles to encapsulate doxorubiucin (Dox, a model anticancer drug) for potential drug delivery applications. Drug release profiles from Dox-loaded micelles made from block polymers with various urea contents are studied under a simulated physiological condition (PBS: phosphate-buffered saline, pH 7.4, 37 °C). Cytotoxicity of polymer micelles and Dox-loaded micelles are also investigated against a model cancer cell line, i.e. HepG2 liver carcinoma cell line.

2. Materials and methods

2.1. Materials

Cyclic carbonate monomers of MTC-OBn [32], MTC-Cl [32], and MTC-Et [25] and thiourea catalyst [33] were described elsewhere. Monomethylether-PEG (Fluka) and trimethylene carbonate (Bohringer-Ingelheim) were azeotropically distilled and recrystallized from toluene prior of use. Sparteine was distilled from calcium hydride prior of use. Benzoic acid, ethanolamine, and phenylisothiocyanate were used as received. Dry THF and CH_2Cl_2 were obtained by using a solvents drying system from Innovative.

HepG2 liver carcinoma and HEK293 human embryonic kidney cell lines were purchased from ATCC and cultured according to ATCC's recommendation. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma.

2.2. General characterization

^{1}H NMR spectra were obtained on a Bruker Avance 400 instrument at 400 MHz. Gel permeation chromatography (GPC) was performed in THF using a Waters chromatograph equipped with four 5 μm Waters columns (300 mm × 7.7 mm) connected in series with increasing pore size (10, 100, 1000, 10^5, 10^6 Å), a Waters 410 differential refractometer and a 996 photodiode array detector, and calibrated with polystyrene standards (750–2 × 10^6 g mol^{-1}).

2.3. Synthesis of phenylureaethyl cyclic carbonate (MTC-Urea)

Urea functional cyclic carbonate (MTC-Urea) was synthesized by tagging phenylureaethanol with cyclic carbonate. In order to prepare phenylureaethanol, ethanolamine (2.96 g, 48.5 mmol, 1 eq) was dissolved in dry THF (30 mL) and cooled to 0 °C using an ice bath. Phenylisocyanate (5.19 g, 4.74 mL, 43.6 mmol, 0.9 eq) in dry THF (30 mL) was added dropwise during a period of 30 min. The resulting solution was allowed to warm to ambient temperature and stirred for an additional 16 h. Then, THF was evaporated. The crude product was recrystallized from ethyl acetate and then stirred rigorously for an additional 4 h. The solids thus formed were removed by filtration, washed with further ethyl acetate and dried until a constant weight was reached, yield 7.0g (~86%). ^{1}H NMR (DMSO-d_6): δ 8.59 (s, 1H, NH), 7.39 (d, 2H, ArH), 7.21 (t, 2H, ArH), 6.95 (t, 1H, ArH), 6.10 (t, 1H, NH), 4.78 (t, 1H, OH), 3.43 (q, 2H, CH_2), 3.17 (q, 2H, CH_2).

For the coupling of phenyureaethanol to cyclic carbonate, MTC-OH (3.04 g, 19 mmol) was initially converted to MTC-Cl using standard procedures with oxalyl chloride [32]. The formed intermediate was dissolved in dry methylene chloride (50 mL) and charged in an addition funnel. In a dry 500 mL round bottom flask equipped with a stir bar was charged phenylureaethanol (4.10 g, 22.8 mmol), pyridine (1.81 g, 1.85 mL, 22.8 mmol) and dry methylene chloride (150 mL) and cooled to 0 °C using an ice bath. The MTC-Cl solution was added dropwise during a period of 30 min and stirred for 30 min. Then, the ice bath was removed, and the solution was allowed to gently heat to ambient temperature and left under stirring for an additional 16 h. The crude product was purified by column chromatography. Ethyl acetate/hexane (1/1) was initially used as eluent before gently increasing the polarity and finishing with ethyl acetate. The product fractions were collected, and the solvent removed through rotational evaporation. The isolated product was dried under vacuum until a constant weight was reached yielding 6.0 g (~80%) of an off-white/slight yellow oil which crystallized upon standing. ^{1}H NMR ($CDCl_3$) δ: 7.39 (d, 2H, ArH), 7.25 (m, 2H, ArH), 7.02 (t, 1H, ArH), 4.68 (d, 2H, CH_2), 4.30 (t, 2H, CH_2), 4.20 (d, 2H, CH_2), 3.55 (t, 2H, CH_2), 1.30 (s, 3H, CH_3). HR-MS-ESI: m/z calculated for $C_{15}H_{18}N_2O_6$ + Na 345.31 found 345.10.

2.4. Synthesis of poly(ethylene glycol)-block-polycarbonate block copolymers

Block copolymers used in this study were prepared from ring-opening polymerization of different cyclic carbonate monomers by using monomethylether-PEG (PEG-OH, M_n 5000 g/mol) as a macroinitiator. The ring-opening polymerization was performed in a glove box using thiourea and tertiary amine catalysts designed for bifunctional activation of both monomer and initiator [33]. The compositions of block copolymers were controlled by changing feed ratios of monomers. In the synthesis of PEG-*b*-poly(ethyl-*random*-urea carbonate) such as PEG-P($E_{0.8}$-$U_{0.2}$)C (5k–5k) as a typical example, PEG-OH (0.5g, 0.1 mmol), MTC-Et (0.35g, 1.86 mmol), and MTC-Urea (0.15g, 0.47 mmol) were dissolved in dry methylene chloride (~1 g) and kept under stirring in a glove box. Thiourea catalyst (37 mg, 0.1 mmol) and sparteine co-catalyst (24 mg, 0.1 mmol) were added and kept under stirring for 1 day. At the end of reaction determined from ^{1}H NMR, benzoic acid (15 mg, 0.12 mmol) was added to quench the catalyst, and the crude polymer precipitated in cold diethyl ether (500 mL). The white solids were collected and dried under vacuum until a constant weight was reached. Yield 0.75 g (75%). ^{1}H NMR ($CDCl_3$) δ: 7.38 (br, 2H, polyMTC(urea)–ArH), 7.22 (br, 2H, polyMTC(urea)–ArH), 6.95 (br, 1H, polyMTC(urea)–ArH), 4.30 (br, 4H, polyMTC; 2H, polyMTC(urea)–$COOCH_2$), 4.10 (br, 2H, polyMTC(ethyl)–CH_2), 3.68 (s, 4H, PEG), 3.38 (s, 3H, PEG-α-end), 1.38 (br, 3H, polyMTC–CH_3; 3H, polyMTC(ethyl)–CH_3). GPC (THF, PS standard): PDI = 1.11.

PEG-*b*-Poly(trimethylene carbonate) (PEG-PTMC) block copolymers were prepared from ROP of commercial trimethylene carbonate according to a similar procedure to the PEG-P(E-U)C block copolymer and used as a non-functionalized PEG block copolymer.

PEG-*b*-Poly(ethyl-*random*-benzyl carbonate) (PEG-P(E-B)C) block copolymers were prepared from ROP of MTC-Et and MTC-OBn according to a similar procedure to the PEG-P(E-U)C block copolymer and regarded as a comparison with a strong hydrphobic interaction. A solution of MTC-OBn (0.075 g, 0.3 mmol) in CH_2Cl_2 (1 mL) was mixed with the solution of MTC-Et (0.1128 g, 0.6 mmol) and thiourea (0.06 mmol) in CH_2Cl_2 (1 mL), then the mixture was transferred to the solution of PEG-OH (0.3 g, 22 mg, 0.06 mmol) and DBU (9.2 mg, 0.06 mmol) in CH_2Cl_2 (1 mL) under stirring. After reacting for 3 h, benzoic acid (5–10 mg) was added to quench the polymerization. The reaction mixture was then precipitated into diethyl ether (40 mL) and the precipitate was centrifuged and dried *in vacuo*. Finally, the crude product was purified by column chromatography on a Sephadex LH-20 column with THF as eluent, to give PEG-P(E-B)C as colorless viscous liquid. Yield 0.35 g (83%). ^{1}H NMR ($CDCl_3$) δ: 7.33 (m, 5H, polyMTC–ArH), 5.14 (s, 2H, polyMTC–CH_2), 4.27 (br, 6H, polyMTC–CH_2O and polyMTC–OCH_2), 3.65 (m, 4H, PEG), 3.38 (s, 3H, PEG-α-end), 1.22 (s, 6H, polyMTC–CH_3 and polyMTC(Ethyl)–CH_2CH_3). GPC (THF, PS standard): PDI = 1.09.

2.5. Fluorescence measurement

Critical micelle concentration (CMC) of the polymer in de-ionized (DI) water was determined using pyrene as a probe on a LS 50B luminescence spectrometer