Colloids and Surfaces B: Biointerfaces 116 (2014) 128–137

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Effects of cholesterol incorporation on the physicochemical, colloidal, and biological characteristics of pH-sensitive AB_2 miktoarm polymer-based polymersomes

Haiqing Yin[a,1], Han Chang Kang[b,1], Kang Moo Huh[c], You Han Bae[a,d,*]

[a] Department of Pharmaceutics and Pharmaceutical Chemistry, The University of Utah, 30 S 2000 E, Room 2972, Salt Lake City, UT 84112, USA
[b] Department of Pharmacy and Integrated Research Institute of Pharmaceutical Sciences, College of Pharmacy, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon-si, Gyeonggi-do 420-743, Republic of Korea
[c] Department of Polymer Science and Engineering, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon 305-764, Republic of Korea
[d] Utah-Inha Drug Delivery Systems (DDS) and Advanced Therapeutics Research Center, 7-50 Songdo-dong, Yeonsu-gu, Incheon 406-840, Republic of Korea

ARTICLE INFO

Article history:
Received 23 April 2013
Received in revised form
24 November 2013
Accepted 20 December 2013
Available online 30 December 2013

Keywords:
Cholesterol
Colloidal stability
Miktoarm polymer
pH-sensitive
Poly(histidine)
Polymersome

ABSTRACT

In our previous study, a histidine-based AB_2 miktoarm polymer, methoxy poly(ethylene glycol)-*b*-poly(L-histidine)$_2$ (mPEG-*b*-(PolyHis)$_2$), was designed to construct pH-sensitive polymersomes that transform in acidic pH; the polymer self-assembles into a structure that mimics phospholipids. In this study, the polymersomes further imitated liposomes due to the incorporation of cholesterol (CL). The hydrodynamic radii of the polymersomes increased with increasing CL wt% (*e.g.*, 70 nm for 0 wt% *vs.* 91 nm for 1 wt%), resulting in an increased capacity for encapsulating hydrophilic drugs (*e.g.*, 0.92 μL/mg for 0 wt% *vs.* 1.42 μL/mg for 1 wt%). The CL incorporation enhanced the colloidal stability of the polymersomes in the presence of serum protein and retarded their payload release. However, CL-incorporating polymersomes still demonstrated accelerated release of a hydrophilic dye (*e.g.*, 5(6)-carboxyfluorescein (CF)) below pH 6.8 without losing their desirable pH sensitivity. CF-loaded CL-incorporating polymersomes showed better cellular internalization than the hydrophilic CF, whereas doxorubicin (DOX)-loaded CL-incorporating polymersomes presented similar or somewhat lower anti-tumor effects than free hydrophobic DOX. The findings suggest that CL-incorporating mPEG-*b*-(PolyHis)$_2$-based polymersomes may have potential for intracellular drug delivery of chemical drugs due to their improved colloidal stability, lower drug loss during circulation, acidic pH-induced drug release, and endosomal disruption.

1. Introduction

Liposomes, which are spherical vesicles formed from natural and synthetic lipids, have been a dominant class of drug carriers. Several formulations have been approved for clinical usage, and many candidates are in clinical trials. However, many significant problems must still be solved, including the lack of a controlled release mechanism, colloidal stability, and pharmacokinetic shortcomings, which mainly originate from their low molecular weight. These problems with conventional liposomes have triggered rapidly growing interest in polymer-based vesicles (*i.e.*, polymersomes) as drug delivery carriers [1,2]. Polymersomes demonstrate some advantages over liposomes in colloidal stability, controllable molecular weights, and easy modification [1,2]. Therefore, various efforts have been made to develop clinically efficacious polymersomes to mimic natural structures, introduce stimulus responsiveness, endow biocompatibility, and generate other desirable characteristics [3].

Like polymer micelles, most polymersomes are constructed by the self-assembly of amphiphilic block copolymers [3]. Polymersomes have promising properties and structures that are useful for drug carriers, but the polymersome structure can only be constructed within a narrow range of the hydrophilic block fraction of a linear block copolymer structure, which significantly limits its carrier development. Because of the favorable geometry and polymeric nature of polymersomes that are based on Y-shaped amphiphilic miktoarm polymers with a phospholipid-mimic structure, these polymers were proposed as an alternative to linear block copolymers as a material for forming polymersomes [4–6]. Y-shaped miktoarm polymers exhibited a greater ability to form vesicles than typical amphiphilic diblock copolymer structures

* Corresponding author at: Department of Pharmaceutics and Pharmaceutical Chemistry, The University of Utah, 30 S 2000 E, Room 2972, Salt Lake City, UT 84112, USA. Tel.: +1 801 585 1518; fax: +1 801 585 3614.
E-mail address: you.bae@utah.edu (Y.H. Bae).
[1] HY and HCK contributed equally to this work.

0927-7765/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.colsurfb.2013.12.041

[4]. In addition, pH-sensitive and biocompatible polymersomes were constructed using methoxy poly(ethylene glycol)-*b*-poly(L-histidine)$_2$ (mPEG-*b*-(PolyHis)$_2$) miktoarm polymers, and these polymersomes showed negligible cytotoxicity [5]. In particular, mPEG-*b*-(PolyHis)$_2$-based polymersomes showed pH-triggered morphological nanostructure transitions in the following sequence as the pH decreased: polymersomes (pH $\geq$ 7.4), cylindrical micelles, spherical micelles, and finally unimers. These transitions suggested the suitability of these polymersomes as an intracellular delivery vehicle for therapeutics [5].

A series of extensive studies on the physicochemical modification and characteristics of liposomes, such as the colloidal stability [7–10], insertion of biologically active molecules [11,12], and lyophilization ability [13–15], have been performed to overcome the inherent shortcomings of liposomes. As a result, abundant information has been produced on liposomal formulations which have influenced the development of polymersomes with various distinctive carrier properties, such as tunable membrane properties, versatility in chemical composition, biodegradability, and high colloidal stability [3]. Significant interest in hybrid vesicles composed of block polymers and lipid/phospholipid/cholesterol has arisen because hybrid systems can combine advantageous features from both polymersomes and liposomes [16–20]. In polymer/lipid hybrid vesicles, lipids form their own domains (lipid-rich domains) which are "raft-like" domains on the polymer membrane that allow incorporation of membrane proteins into vesicles [17]. When cholesterol was inserted into polymer vesicles, it caused reduced membrane thickness and increased bending stiffness due to its higher packing density compared to pure polymersomes [19]. The study of hybrid vesicles has focused on physical, biophysical or physicochemical properties related to lipid-rich domains, and these studies have been performed with polymersomes formed from linear block copolymers. Hybrid vesicles would have potential in drug delivery, diagnostics, and biochemical reactions and assist in understanding the function of cellular membranes [16–20].

Although the physicochemical modification and the characteristics of polymersomes, especially Y-shaped polymer-based stimuli-responsive polymersomes have been of great interest as biofunctional nanocarriers for modulated drug release, their systemic study has been not performed because this formulation has only recently been developed. Thus, as a starting point, this study is to report the effects of cholesterol (CL) incorporation into polymersomes because CL, as an essential structural component of mammalian cell membranes, influences various characteristics (*e.g.*, size, encapsulation volume, and colloidal stability) of liposomes. The effects of cholesterol incorporation on various physicochemical, colloidal, and biological characteristics of polymersomes were investigated.

2. Materials and methods

2.1. Materials

N_α-Boc-$N_{(im)}$-2,4-dinitrophenyl-L-histidine (Boc-His(DNP)-OH) isopropanol solvate was purchased from BACHEM Co. (Torrance, CA). Thionyl chloride, propargylamine, methyl acrylate, ethylenediamine, methoxy poly(ethylene glycol) (mPEG-OH, M_n 2 kDa), *p*-toluenesulfonyl chloride, ethylenediaminetetraacetic acid (EDTA) tetrasodium salt hydrate, copper (II) sulfate pentahydrate, sodium L-ascorbate, 2-mercaptoethanol, pyrene, RPMI1640 medium, 4-(2-hydroxy-ethyl)-1-piperazine (HEPES), L-glutamine, D-glucose, acetone, dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol, 1,4-dioxane, diethyl ether, phosphate buffered saline (PBS), 5(6)-carboxyfluorescein (CF), cholesterol (CL), paraformaldehyde (PFA), sodium tetraborate, boric acid, and Triton X-100 were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, and trypsin-EDTA solution were purchased from Invitrogen Co. (Carlsbad, CA).

2.2. Preparation of the AB_2 3-miktoarm polymer and its polymersomes

As previously reported [5], the hydroxyl group of mPEG was modified into azide-functionalized mPEG (A block), and then the A block was linked with the alkyne group of polyamidoamine (PAMAM) type trifunctional dendron (D1) with an alkyne group and two primary amine groups by click chemistry, resulting in the synthesis of mPEG-D1. The remaining primary amines of mPEG-D1 initiated ring-opening polymerization (ROP) of α-amino acid *N*-carboxyanhydrides (NCA) (B block), finally resulting in the synthesis of the AB_2 3-miktoarm polymer, mPEG-*b*-(polyHis)$_2$ (Fig. 1). The synthesis of mPEG-*b*-(polyHis)$_2$ was confirmed by ^{1}H-NMR spectroscopy and FT-IR spectroscopy, and the molecular weights of the A block (mPEG) and two B blocks (polyHis) were 2 kDa (degree of polymerization (DP) of ethylene oxide: 45) and 5.8 kDa (DP of L-histidine in each polyHis chain: average 21), respectively, as previously reported [5].

To prepare mPEG-*b*-(polyHis)$_2$ polymersomes as previously reported [5], mPEG-*b*-(polyHis)$_2$ polymer (5 mg) dissolved in DMSO (10 mL) was neutralized by adding 5 M NaOH (5 μL), and the polymer solution was vigorously stirred overnight. Then, the polymer solution was dialyzed against 10 mM borate buffer (pH 9.0) with 140 mM NaCl using dialysis membrane with a molecular weight cut-off (MWCO) of 2 kDa. The buffer phase was replaced with fresh solution at 2, 4, and 12 h, and the formed polymersomes (CL-free polymersome) were recovered at 24 h. For CL-incorporating polymersomes (*i.e.*, CL-polymersome), CL was dissolved with the polymer in DMSO, and then prepared following the same method used for CL-free polymersomes.

2.3. Physicochemical characterization of polymersomes

To measure the radius of gyration (R_g), the hydrodynamic radius (R_h), colloidal stability, and other properties of the CL-free polymersome (*i.e.*, $CL_{0\,wt\%}$-polymersome) and the CL-polymersome, as previously reported [5], dynamic light scattering (DLS) and static light scattering (SLS) experiments were performed using a Brookhaven Instruments Corp. system consisting of a BI-200SM goniometer and a BI-9000AT autocorrelator at a temperature (T) of 25 °C, if not otherwise specified. Prior to the light scattering (LS) measurement, samples were filtered with a 0.80 μm disposable membrane filter to remove any dust.

Using DLS mode, the normalized first-order autocorrelation function $g_1(t)$ was determined from the measured second order autocorrelation function $G_2(t)$ [21] and, with further analysis using the constrained regularized CONTIN algorithm, was used to estimate the normalized distribution of the relaxation time $G(t_R)$ [22]. In the case of simple diffusion, the angular dependence of the relaxation time (t_R) is defined by $D_{app} = 1/(t_R q^2)$, where D_{app} and q are the apparent translational diffusion coefficient and the magnitude of the scattering wave vector, respectively. The magnitude ($q = 4\pi n \sin(\theta/2)$) is calculated using n (the refractive index of the solvent), λ (the wavelength of the incident beam *in vacuo* (632.8 nm)), and θ (the scattering angle). When $qR_g \sim 1$ for a dilute solution, the angular dependence of D_{app} can be expressed as $D_{app} = D_0(1 + CR_g^2 q^2)$ using the intrinsic translational diffusion coefficient (D_0), R_g, and a characteristic parameter of the molecular architecture (C). From a plot of D_{app} *vs.* q^2, D_0 is obtained by extrapolation to determine the intercept at the zero angle. Based on the Stokes–Einstein equation ($D = kT/6\pi\eta R_h$), R_h is defined, where