Published in final edited form as:

Colloids Surf B Biointerfaces. 2014 April 1; 116: 128–137. doi:10.1016/j.colsurfb.2013.12.041.

Effects of Cholesterol Incorporation on the Physicochemical, Colloidal, and Biological Characteristics of pH-sensitive AB₂ Miktoarm Polymer-Based Polymersomes

Haiqing Yin^{a,1}, Han Chang Kang^{b,1}, Kang Moo Huh^c, and You Han Bae^{a,d,*}

^aDepartment of Pharmaceutics and Pharmaceutical Chemistry, The University of Utah, 30 S 2000 E, Room 2972, Salt Lake City, Utah 84112, USA

^bDepartment 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

^cDepartment of Polymer Science and Engineering, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon 305-764, Republic of Korea

^dUtah-Inha Drug Delivery Systems (DDS) and Advanced Therapeutics Research Center, 7-50 Songdo-dong, Yeonsu-gu, Incheon, 406-840, Republic of Korea

Abstract

In our previous study, a histidine-based AB₂ 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 μ L/mg for 0 wt% *vs.* 1.42 μ L/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

© 2013 Elsevier B.V. All rights reserved.

*Correspondence to: Professor You Han Bae, Department of Pharmaceutics and Pharmaceutical Chemistry, The University of Utah, 30 S 2000 E, Room 2972, Salt Lake City, Utah 84112, USA, Tel: +1-801-585-1518, Fax: +1-801-585-3614. you.bae@utah.edu.

¹HY and HCK contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

stability, lower drug loss during circulation, acidic pH-induced drug release, and endosomal disruption.

Keywords

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

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 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 [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 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 has 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- $N_{(\text{im})}$ -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 AB₂ 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 AB₂ 3-miktoarm polymer, mPEG-*b*-(polyHis)₂ (Fig. 1). The synthesis of mPEG-*b*-(polyHis)₂ was confirmed by ¹H-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 μ L), 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 hr, and the formed polymersomes (CL-free polymersome) were recovered at 24 hr. 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_{0wt%}-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 μ m 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 k and η are the Boltzmann constant and the viscosity of water respectively at T. The polydispersity index (PDI) of the sample is calculated from the relative variance of the intensity-weighted distribution at 90° by the integrated software.

The SLS mode was measured using angles from 30° to 120°. The partial Zimm equation ($1/I_{ex}(q) = P(1+R_g^2 q^2/3)$) related $I_{ex}(q)$ (the excess intensity of scattered light) and R_g ,

where q and P is the magnitude of the scattering wave vector and an experimental parameter, respectively. R_g is determined from the slope and intercept of a plot of $1/I_{\text{ex}}(q)$ vs. q^2 .

The structural morphology of the polymersomes was observed by transmission electron microscopy (TEM, a Philips TECNAI T12 electron microscope). To prepare a sample without staining, one drop of the aqueous polymersome solution was deposited onto a Formvar-carbon-coated copper grid.

2.4. Polymersome encapsulation and in vitro release of the hydrophilic dye 5(6)-carboxyfluorescein (CF)

To prepare CF-loaded polymersomes, 0.5 mL of polymer solution (4 mg/mL in DMSO) and 2 mL of borate buffer solution (10 mM, pH 9.0) containing 150 mM CF were prepared separately, and then the two solutions were mixed by vigorous stirring for 2 hr to obtain a homogenous solution. The solution was dialyzed in a dialysis bag (MWCO 15 kDa) against 1 L of borate buffer (10 mM, pH 9.0) containing 140 mM NaCl for 4 hr. Using a gel filtration technique with a Sephadex G-25 column (1.5 cm×40 cm), CF-containing polymersomes (*i.e.*, CF-loaded CL-free polymersome and CF-loaded CL-Polymersome) and free CF were separated using borate buffer as the eluent at a flow rate of 0.5 mL/min.

As previously reported [5], the purified CF-containing polymersomes (50 μ L) were mixed with an equivalent volume of 2 wt% Triton-X 100. The mixed solution was further diluted by adding borate buffer (pH 9.0, 5 mL) and was sonicated for 10 min to disrupt the polymersome for complete release of the encapsulated CF. The resulting fluorescence intensity was monitored at $\lambda_{\text{ex}}/\lambda_{\text{em}}=490\text{ nm}/520\text{ nm}$ using a SPECTRAMax M2 spectrofluorometer. The encapsulated amount of CF in the polymersome was calculated from a linear regression equation relating the fluorescence intensity (I) to the CF concentration (C , ng/mL) using a series of CF standard solutions (5–200 ng/mL) in borate buffer (pH 9.0): $C = 0.0265 \times I - 0.70$ (correlation coefficient $R^2=0.999$). The encapsulated volume is calculated with the following equation: $1000 \times (m_E V) / (m_F m_P)$, where m_E and m_F are the mass (mg) of the encapsulated and feed CF, respectively; m_P is the mass (mg) of the polymer used; and V is the volume (mL) of the CF-loaded polymersome solution.

When a high concentration of CF was trapped in a vesicle, the CF self-quenched. However, upon release of the CF from the vesicle, the CF fluorescence was readily detected.[23] Immediately after gel filtration of the CF-loaded polymersomes, the purified polymersome (0.2 mL) was mixed with 1.8 mL of PBS pre-adjusted to different pH values and incubated at 37°C with shaking (100 rpm). At predetermined intervals, 0.1 mL of the mixture was withdrawn, and the fluorescence intensity (I_t) was measured. The percentage of CF released from the polymersome was calculated using the following equation: $(I_t - I_0) / (I_{\text{tot}} - I_0) \times 100$, where I_0 is the fluorescence intensity immediately after gel filtration and I_{tot} is the fluorescence intensity after disrupting the vesicle and complete release of the dye upon addition of Triton X-100.

2.5. Biological characterization of polymersomes

A2780 cells (human ovarian carcinoma), A2780/ADR cells (a DOX-induced multidrug resistant subline of human ovarian carcinoma A2780 cells), and MCF7/ADR-RES cells (a DOX-induced multidrug resistant subline of human breast adenocarcinoma MCF7 cells) were used to evaluate cellular uptake and anti-tumor effects of the polymersomes and to record confocal images. The cells were cultured in RPMI-1640 medium supplemented with glutamine (2 mM), glucose (1 g/L), penicillin (100 U/mL), streptomycin (100 µg/mL) and FBS (10 %) at 37°C under humidified air containing 5% CO₂. For MCF7/ADR-RES cells, insulin (4 mg/L) was also added to the culture medium. To maintain the MDR characteristics, the two MDR cell lines (*i.e.*, MCF7/ADR-RES cells and A2780/ADR cells) were treated with DOX (400 ng/mL) weekly.

For measuring cellular uptake and recording confocal images of polymersomes, A2780 cells were plated at a density of 5×10^4 cells/well in a 6-well plate. After 24 hr, the culture medium was replaced with 160 µL of fresh culture medium and 40 µL of free CF or CF-encapsulated polymersomes in borate buffer solution (pH 7.4) with the same CF concentration (10 µg/mL), while non-dye-treated cells were used as the control. After 4 hr of incubation, the cells were washed with DPBS. For flow cytometry analysis, the cells were detached with a trypsin-EDTA treatment and then fixed with 4% PFA solution. CF-containing cells were monitored by a Becton-Dickinson FACScan analyzer equipped with an Ar laser (488 nm) and a fluorescence detector (530 ± 15 nm), and at least 5,000 cells were counted. For confocal laser scanning microscopy (CLSM), the cells were seeded on coverslips and mounted on a glass microscope slide with anti-fading agent after the cells were fixed using 4% PFA solution. The sample was observed using an Olympus FV1000-XY Confocal Microscope with a step size of 0.5 µm.

To evaluate the drug efficacy of the drug-encapsulated polymersomes, DOX was loaded into the polymersomes. After purifying the polymersomes to remove unloaded free DOX, DOX-loaded polymersomes were used to measure anti-tumor effects. Two types of MDR cells were seeded at a density of 5×10^3 cells/well in 96-well plates. The seeded cells were cultured for 24 hr prior to adding various concentrations of DOX and DOX-loaded polymersomes. After a 3 d incubation period, the culture medium was replaced with fresh medium. MTT solution (10 µL; 5 mg/mL) was added to the cells (0.1 mL of culture medium) on day 5 of the incubation. Following additional 4 hr incubation, the MTT-containing medium was removed. The formazan crystals produced by living cells were dissolved in DMSO, and their absorbance was measured at 570 nm using a microplate reader.

3. Results and Discussion

To study the effects of CL incorporation on pH-sensitive polymersomes, CL-incorporating polymersomes (*i.e.*, CL-Polymersome) were constructed by the addition of CL during vesicle preparation, as shown in Fig. 1. A CL-Polymersome having x wt% CL, which indicates the weight percentage of CL compared to the entire polymersome weight, was denoted as CL _{x wt%}-Polymersome. In this study, the CL content in CL-incorporating polymersomes was either 1 wt% (*i.e.*, 17 mol%) or 5 wt% (*i.e.*, 52 mol%) because typical

amounts of CL introduced into conventional liposomes range from about 22 mol% for CL-poor liposomes to about 50 mol% for CL-rich liposomes [24]. Thus, the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were compared with the CL-free polymersome (*i.e.*, CL_{0wt%}-Polymersome).

3.1. Effects of CL incorporation on physicochemical characteristics of polymersomes

After preparing the CL-Polymersome, the effects of CL incorporation on the hydrodynamic radius (R_h), the radius of gyration (R_g), and their ratio (R_g/R_h) were evaluated. CL-incorporating polymersomes were narrowly distributed spherical vesicles, as measured by TEM (Fig. 1), and this result is similar to the CL_{0wt%}-Polymersome, as demonstrated in our previous report [5]. From the intercept (D_0) of the D_{app} -axis of the plot of D_{app} vs. q^2 (Fig. 2), the calculated hydrodynamic radii of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were 91 nm and 134 nm, respectively, and both were larger than the CL_{0wt%}-Polymersome (70 nm) [5] (Table 1). Thus, the R_h of the polymersomes increased as the CL wt% in the polymersomes increased. However, their size distributions were narrow as confirmed by DLS, and their PDI values were 0.03 and 0.09 for the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome, respectively, similar to that of the CL_{0wt%}-Polymersome (0.05) [5] (Table 1). The results indicate that when polymersomes incorporate CL, their size increases without a significant change in their PDI.

In addition, using SLS, the R_g values of the polymersomes were calculated from the slope and $1/I_{ex}$ -intercept of the plot of $1/I_{ex}$ vs. q^2 (Fig. 3). The radii of gyration of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were 92 nm and 129 nm, respectively, and were larger than the CL_{0wt%}-Polymersome (72 nm) [5] (Table 1). Similar to the hydrodynamic radius of the polymersomes, the R_g also increased as the weight content of incorporated CL increased.

The ratio R_g/R_h of a particular structure is related to its shape, known as a shape factor, and is dependent on its inner density profile [25, 26]. When the density distribution in a spherical particle is shifted to the surface of the particle, its shape factor increases [27]. In theory, the shape factor is 0.775 for monodisperse solid spheres and ~1.5 for a Gaussian random coil [25, 26]. The R_g/R_h ratio of an elliptical structure is strongly affected by its axial ratio, and for a specific cylindrical particle having length L and thickness d , the ratio of $R_g/R_h = 0.57735\{\ln(L/d)+0.38\}$ [25]. Typical values of the shape factor are close to 0.77 for spherical micelles [27], 0.9–1.1 for spherical vesicles (*i.e.*, liposomes and polymersomes) [27–29], and >1.2 for cylindrical micelles [25, 26, 30]. In our studies, the shape factors of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were 1.01 and 0.96, respectively, and were similar to the CL_{0wt%}-Polymersome (1.03) [5]. The values indicate that the formed CL_{1wt%}-Polymersome and CL_{5wt%}-Polymersome are thin-walled hollow spheres (*i.e.*, polymeric vesicles or polymersomes) because the R_g/R_h value is close to the unity. Based on the confirmed vesicular nanostructures of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome, their size increase could be explained as follows: the hydrophobic CL molecules might be largely buried in the hydrophobic polyHis segment, which can lead to a decrease in the preferred curvature of the bilayer [31].

3.2. Effects of CL incorporation on the colloidal stability of polymersomes

Binding of plasma protein is thought to be one primary mechanism for the instability of self-assembled nanostructures in blood [32]. The kinetic stability of polymersomes in the presence of BSA, a model serum protein, was studied using DLS at 37°C, pH 7.4, and physiological osmolarity (300 mOsm/L) because scattered light intensity is roughly proportional to the mass and the concentration of particles in the solution [21]. In addition, the relative change in the scattered light intensity reflects the change in the colloidal stability of the polymersomes.

In our previous study, the CL_{0wt%}-Polymersome had a monomodal $R_{h,app}$ distribution with an average 70 nm [5]. Upon adding CL_{0wt%}-Polymersomes (0.1 mg/mL) into a BSA (1 mg/mL)-containing aqueous solution, the DLS histogram showed a bimodal size distribution due to the newly formed size distribution (approximately 4 nm in $R_{h,app}$) of BSA and the existing size distribution of the CL_{0wt%}-Polymersome (Fig. 4(a)). The same bimodal distribution was maintained until 7 hr of exposure of the CL_{0wt%}-Polymersome to BSA, but a remarkable decrease in the relative scattered light intensity of the peak for the CL_{0wt%}-Polymersome was observed. Thus, the CL_{0wt%}-Polymersome became destabilized by BSA with time, and its contribution to the intensity of the existing $R_{h,app}$ distribution (with average 70 nm) decreased. This decrease subsequently increased the intensity of the $R_{h,app}$ distribution (average of 4 nm) because a partial disruption of the vesicular structure caused the molecular mPEG-*b*-(polyHis)₂ to form a unimer (Fig. 4(a)). After 24 hr of exposure, a trimodal $R_{h,app}$ size distribution was detected by DLS with a newly formed additional peak appearing from 10 to 30 nm. The CL_{0wt%}-Polymersome might be mostly disrupted, and the newly appearing particles may be the fragments of the disrupted CL_{0wt%}-Polymersome rearranged into either small vesicles or micelles. These results suggest that the CL_{0wt%}-Polymersome is gradually destabilized and disrupted in the presence of BSA.

In addition, the half-life ($t_{1/2}$) of the polymersome was estimated to be the time period at which the scattered light intensity of the polymersome decreased to the half of its original value following incubation with BSA. To estimate the $t_{1/2}$ of the polymersome, the scattered light intensity of the CL_{0wt%}-Polymersome at 0 hr of exposure to BSA was set to 100 as an arbitrary unit (a.u.) of the normalized intensity. The change over time in the scattered light intensity of the CL_{0wt%}-Polymersome was normalized, and the percentage of the CL_{0wt%}-Polymersome that remained (e.g., 66% and 35% at 1 hr and 24 hr exposure, respectively) is shown in Fig. 4(b). The estimated $t_{1/2}$ of the CL_{0wt%}-Polymersome was approximately 5 hr in the presence of BSA (1 mg/mL) (Table 1).

The CL effects on the colloidal stability of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome in the presence of BSA were evaluated. Similar to the CL_{0wt%}-Polymersome, the CL_{1wt%}-Polymersome showed a bimodal size distribution caused by the scattered light intensities of the CL_{1wt%}-Polymersome (average of 90 nm) and BSA (average of 4 nm) at 0 hr of exposure to BSA (1 mg/mL) (Fig. 4(a)). However, the scattered light intensity of the CL_{1wt%}-Polymersome showed a much slower decrease than the CL_{0wt%}-Polymersome because the scattered light intensity of the CL_{1wt%}-Polymersome did not change significantly until after 72 hr of exposure (Fig. 4(a)). The estimated $t_{1/2}$ of the CL_{1wt%}-

Polymersome was already beyond the studied time scale (72 hr), indicating a significant enhancement of polymersome stability upon the addition of CL. The study using the CL_{5wt%}-Polymersome was excluded because the vesicles were even more stable than the CL_{1wt%}-Polymersome.

The colloidal stability of the polymersomes was further tested in the presence of BSA (40 mg/mL) because the serum albumin concentration normally ranges from 34~54 mg/mL in human blood. As summarized in Table 1, the estimated $t_{1/2}$ of the CL_{0wt%}-Polymersome decreased at 1 hr and was much shorter than in the presence of BSA (1 mg/mL). The $t_{1/2}$ values of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were 12 hr and 15 hr, respectively. This could indicate that the difference between the colloidal stability of CL_{1wt%}-Polymersome and the colloidal stability of CL_{5wt%}-Polymersome may be negligible in blood.

Studying the colloidal stability of the polymersomes showed that mPEG-*b*-(polyHis)₂ polymersomes remained stable for several hours in the presence of a low concentration of BSA (1 mg/mL), but the polymersomes were quickly disrupted when the BSA concentration was high (40 mg/mL). The results indicate that the effect of BSA is dependent on the BSA/mPEG-*b*-(polyHis)₂ polymer ratio. In general, serum proteins can insert into liposome bilayers via electrostatic and hydrophobic interactions to disrupt the membrane [11, 12]. For mPEG-*b*-(polyHis)₂ polymersomes, the presence of the PEG corona could largely suppress the interactions with BSA via steric hindrance [32] at a lower BSA/polymer ratio. However, such steric protection of the PEG was overwhelmed when the BSA/polymer ratio became sufficiently high, leading to the short half-life of the polymersome. Cholesterol (CL) was chosen as the second constituent of the polymersome system to increase the bilayer stability because the inclusion of CL in many liposome systems has been demonstrated to improve the *in vitro/vivo* half-lives by preventing serum protein absorption [33, 34]. The incorporation of a small amount of CL did significantly prolong the half-life of the polymersome, and the $t_{1/2}$ was still as high as 12 hr even in the presence of 40 mg/mL BSA. The presence of CL in the vesicle bilayer might prohibit the insertion of BSA by decreasing the permeability and increasing the rigidity of the membrane, similar to its effect on liposomes [7, 8]. Thus, CL incorporation may be used as another facile approach, in addition to surface PEGylation, for fabricating long-circulating polymersome carriers.

3.3. Effects of CL incorporation on the drug loading/releasing characteristics of polymersomes

To estimate the encapsulation capability of the mPEG-*b*-(polyHis)₂ polymersome, the self-quenching fluorescent probe 5(6)-carboxyfluorescein (CF), was used as a hydrophilic model dye, which physically accumulated into the aqueous lumen of the polymersomes [5]. In our previous study [5], the encapsulation efficiency of the CL_{0wt%}-Polymersome, which is defined as the encapsulated volume per milligram of polymer used [35], was 0.92 ± 0.10 $\mu\text{L}/\text{mg}$. The value was comparable to the encapsulation efficiency of liposomes with similar sizes (1–3 $\mu\text{L}/\text{mg}$) [36]. When CL was incorporated into the polymersomes, the encapsulation volumes of the CL_{1wt%}-Polymersome and the CL_{5wt%}-Polymersome were 1.24 ± 0.16 $\mu\text{L}/\text{mg}$ and 1.42 ± 0.09 $\mu\text{L}/\text{mg}$, respectively (Table 1). The encapsulation volumes

of the CL-incorporating polymersomes were also higher than for CL-free polymersomes because the former vesicles were larger than the latter, although their bilayer thicknesses may be similar.

Subsequently, the CL effects on drug release from the polymersomes were evaluated using the *in vitro* release of the encapsulated dye at different pH values at 37°C and 300 mOsm/L. As previously mentioned, the CL_{1wt%}-Polymersome showed sufficient colloidal stability in the presence of BSA (40 mg/mL) and its colloidal stability was not significantly different from CL_{5wt%}-Polymersome. As a model system of CL-incorporating polymersomes, the CL_{1wt%}-Polymersome was tested and compared to the CL_{0wt%}-Polymersome. In our previous study, the CL_{0wt%}-Polymersome quickly released 70~90% of the encapsulated CF at pH 7.2~7.4 over the 72 hr incubation time [5]. However, the CL_{1wt%}-Polymersome showed much slower release profiles of the encapsulated CF at pH values of 9.0, 7.4 and 7.2 during the studied time scale (72 hr) (Fig. 5(a)). At pH 7.4 and pH 7.2, the CL_{1wt%}-Polymersome released approximately 30% and 60% of the encapsulated CF, respectively, over 72 hr of incubation. Although the pH-sensitivity of CL_{1wt%}-Polymersome could have decreased because of the increase in CL content and decrease in the weight content of the pH-sensitive components in the polymersome compared to the CL_{0wt%}-Polymersome, interestingly a significantly accelerated release was observed at pH 6.8 and pH 6.0, and the encapsulated dye was almost completely released within 24 hr (Fig. 5(a) and (b)). The acidic pH-induced accelerated release of the encapsulated CF in the CL_{1wt%}-Polymersome is caused by a pH-sensitive nanostructure transition from polymersomes to cylindrical and then spherical micelles as the pH decreases from 6.8 to 5.0 (data not shown), similar to the results from our previous TEM studies [5].

In general, the incorporation of CL into liposome bilayers has been found to decrease the permeability and increase the rigidity of membranes [7, 8]. A similar effect may occur for the polymersome system, where the release pattern was more retarded. Thus, the release pattern of the CL_{1wt%}-Polymersome also indicates that the polymersome may have a desirable pH-responsive property for intracellular delivery: the system is able to contain the majority of its payload during circulation, whereas the acidic environment of endosomes (pH 5.5–6.5) in the cell can trigger the rapid release of the drug encapsulated in the polymersomes [37, 38]. However, although the exact prediction of CL_{5wt%}-Polymersome could be difficult because pH-dependent release profiles of CL_{5wt%}-Polymersome were not investigated, pH-sensitive drug release from CL_{5wt%}-Polymersomes could be somewhat retarded or partially lost due to the increased CL content and decreased pH-sensitive components' content in the polymersome.

3.4. Effects of CL incorporation on the biological characteristics of polymersomes

The *in vitro* biological characteristics of polymersomes were evaluated for cellular uptake/internalization and anti-tumor effects using two different fluorescent molecules: 5(6)-carboxyfluorescein (CF) dye and doxorubicin (DOX) drug. CF was only used for cellular uptake/internalization because CF is not an anti-tumor drug. Although DOX can be used for cellular uptake/internalization because it has fluorescence, DOX was only used for anti-tumor effects because fluorescence intensity of DOX was dependent on its subcellular

localization and its degradation products [39]. DOX can readily intercalate with DNA in the nucleus so the fluorescence intensity of DOX-intercalated DNA was much higher than that of free DNA in the cytosol [40]. Also, some DOX degraded derivatives had higher fluorescence than non-degraded DOX [39]. Thus, in this study, we determined that DOX was not a good model drug for cellular internalization. Instead of DOX, we used CF for cellular internalization.

First, using A2780 cells, the cellular uptake of the CF-loaded CL_{1wt%}-Polymersome was monitored using flow cytometry and compared to the uptake of the CF-loaded CL_{0wt%}-Polymersome and free CF. In general it is difficult for a hydrophilic drug to be internalized into cells without the expression of specific receptors/transporters. Thus, after 4 hr of incubation, a low fluorescence intensity caused by the free CF was observed in the A2780 cells, and the value was slightly higher than in the untreated A2780 cells (control cells) (Fig. 6(a)). For the CF-loaded polymersomes (*i.e.*, the CF-loaded CL_{0wt%}-Polymersome and the CF-loaded CL_{1wt%}-Polymersome), it was expected that their cellular internalization would not be much higher than free CF because the polymersomes had a hydrophilic PEG shell. However, although the CF-loaded CL_{1wt%}-Polymersome caused slightly more cellular internalization than the CF-loaded CL_{0wt%}-Polymersome, the A2780 cells took up much more CF-loaded polymersomes than free CF (Fig. 6(a)). In addition, the flow cytometry results were further confirmed by CLSM evaluation. Fig. 6(b) indicates that stronger and more diffusive fluorescence inside the cell was observed for the CF-loaded CL_{0wt%}-Polymersome and the CF-loaded CL_{1wt%}-Polymersome than for the free CF.

To evaluate the therapeutic efficacy of the polymersomes, DOX was selected as a hydrophilic anticancer drug because its loading content in polymersomes can be easily estimated from its fluorescence intensity. However, unlike free CF, free DOX could show strong cellular internalization because DOX (weakly basic) possesses is relatively hydrophobic at pH 7.4. Thus, this study was performed using drug-resistant cell lines, such as MCF7/ADR-RES and A2780/ADR cells, to possibly reduce any favorable cellular internalization effects of DOX. In addition, the anti-tumor effect of DOX-loaded CL_{0wt%}-Polymersome was excluded in this study. To study the anti-tumor effects, the medium used contained 10% serum which contains about 4 mg/mL of serum albumin. The serum condition could destabilize DOX-loaded CL_{0wt%}-Polymersome and cause DOX release into the culture medium prior to cellular internalization of DOX-loaded CL_{0wt%}-Polymersome because the half-life of CL_{0wt%}-Polymersome was disrupted following incubation with 1 mg/mL of BSA within 5 hr. These facts indicate that both DOX-loaded CL_{0wt%}-Polymersome and its released DOX can co-exist. Therefore any anti-tumor results from the DOX-loaded CL_{0wt%}-Polymersome would not be accurate and thus the anti-tumor study using DOX-loaded CL_{0wt%}-Polymersome was excluded.

As shown in Fig. 7(a) and 7(b), the DOX-loaded CL_{1wt%}-Polymersome showed IC₅₀ values of approximately 45 μ M and 30 μ M for MCF7/ADR-RES and A2780/ADR cells respectively, which contrasted with the IC₅₀ of free DOX (45 μ M and 5 μ M for MCF7/ADR-RES and A2780/ADR cells, respectively). Although the DOX-loaded CL_{1wt%}-Polymersome had a 6-fold lower IC₅₀ than free DOX in A2780/ADR cells, the polymersome demonstrated a killing power that was almost equivalent to free DOX in

MCF7/ADR-RES cells. This result might be linked with the slower cellular uptake of the DOX-loaded CL_{1wt%}-Polymersome compared to the relatively hydrophobic DOX.

Poor cellular uptake of the hydrophilic CF dye in A2780 cells is mainly because the lipid membrane of the cell is largely impermeable to the hydrophilic dye, whereas the weakly basic DOX drug could be internalized to a larger extent than a hydrophilic drug because DOX can undergo cellular uptake via adsorptive endocytosis. This discrepancy might be dependent on the hydrophilicity and hydrophobicity of the drug, which cause different interactions with the cellular membrane. The cellular uptake rates of polymersomes made from the mPEG-*b*-(PolyHis)₂ polymer can be faster or slower than that of the free drug, depending on whether the drug is hydrophilic or hydrophobic because PEGylated polymersomes generally circulate in the blood for a long time and their weak electrostatic interactions with the plasma membrane cause slow cellular internalization.

However, it is possible for cellular internalization of the DOX-loaded CL_{1wt%}-Polymersome to be slower than for free DOX. The DOX-loaded CL_{1wt%}-Polymersome showed equivalent or slightly weaker killing power against drug-resistant cancer cells compared to free DOX. The potential anti-tumor effects of DOX-loaded CL_{1wt%}-Polymersome may be linked to its pH-sensitive nanostructure transition as well as to its proton buffering capacity for endosome breakage [5]. That is, once inside the relatively acidic endosomal compartments, rapid release of the payload from the polymersomes can be triggered as a result of the vesicle to micelle transition. Moreover, histidine-based polymers were found to cause endosomal membrane disruption via the so-called “proton-sponge” effect due to their pH buffering capability [5, 41–43]. Therefore, the synergy between the endosomal pH-triggered release and the endosomolytic property of the polymersomes may have enabled efficient trafficking of the encapsulated drug into the cytosolic compartment. As a result, the CL_{1wt%}-Polymersome may have contributed to the slower release of the encapsulated cargo into the culture medium at pH 7.4 as well as improved colloidal stability in the serum-containing medium relative to the corresponding properties for the CL_{0wt%}-Polymersome. In particular, if the CL_{1wt%}-Polymersome was decorated with a targeting moiety for a specific cell type, the polymersome could be quickly internalized via receptor-mediated endocytosis.

Unlike most classical polymersomes made of diblock copolymers, this study employed Y-shaped block copolymers having one hydrophilic block and two hydrophobic blocks because this polymer architecture mimics the structure of phospholipids, a vesicle forming component found in nature. We proved that the miktoarm architecture has a significantly superior capability of forming polymersomes compared to the linear block copolymer counterparts[4]. However, the miktoarm architecture may influence some properties of the polymersome membrane such as thickness, packing density, and permeability. For example, if the hydrophobic blocks in 3-miktoarm copolymers and diblock copolymers have a similar molecular weight with identical chemical structure, the length of one hydrophobic block in the 3-miktoarm block copolymer is half the length of the hydrophobic block in the diblock copolymer. This may lead to a thinner membrane in 3-miktoarm polymersomes compared to diblock copolymer-based polymersomes. The 3-miktoarm architecture would result in a less dense membrane compared to diblock copolymers due to the angle between two

hydrophobic blocks, yielding more free volume which then weakens the mechanical property and causes higher permeability.

Filling the free volume by inserting cholesterol will result in improved mechanical strength and lower permeability as observed in liposomes. In liposomes, cholesterol insertion generally causes increased bending modulus and decreased membrane permeability. This observation supports our results of increased colloidal stability in the presence of serum protein and the decreased release rate of payload at neutral-to-basic pHs, respectively. Thus, the similarity between the CL incorporation results for 3-miktoarm polymer-based polymersomes and liposomes may open some possibility that the lessons from liposome-based research can be used to estimate various characteristics of newly developed polymersomes when introducing similar functions, such as cholesterol incorporation, molecular insertion, and lyophilization ability into polymersomes.

4. Conclusion

Compared to CL-free polymersomes, mPEG-*b*-(PolyHis)₂-based polymersomes in which CL has been incorporated formed larger particles and had enhanced colloidal stability in the presence of serum protein, which were further manifested as a higher encapsulation volume and slower payload release, respectively. The CL_{1wt%}-Polymersome showed better cellular internalization than the free hydrophilic dye (e.g., CF) and demonstrated similar or somewhat lower anti-tumor effects compared to the well-known relatively hydrophobic drug DOX. In particular, CL incorporation may improve intracellular drug delivery because of the resulting improved colloidal stability, lowered drug loss at pH 7.4 or during blood circulation, and improved endosomal pH-induced drug release and endosomal disruption. Thus, mPEG-*b*-(PolyHis)₂-based polymersomes that incorporate CL could have potential for intracellular drug delivery of chemical drugs (especially hydrophilic drugs).

Acknowledgments

This work was supported by NIH grant CA101850. HCK and KMH also appreciate a financial support by Basic Science Research Program through National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, & Future Planning (NRF-2013R1A2A2A04015914.). The authors are grateful to Kwonhyeok Yoon (Chungnam National University) and Hana Cho (The Catholic University of Korea) for technical assistance. This work was supported

References

1. Christian DA, Cai S, Bowen DM, Kim Y, Pajerowski JD, Discher DE. Polymersome carriers: From self-assembly to siRNA and protein therapeutics. *Eur J Pharm Biopharm.* 2009; 71:463–474. [PubMed: 18977437]
2. Levine DH, Ghoroghchian PP, Freudenberg J, Zhang G, Therien MJ, Greene MI, Hammer DA, Murali R. Polymersomes: a new multi-functional tool for cancer diagnosis and therapy. *Methods.* 2008; 46:25–32. [PubMed: 18572025]
3. Meng F, Zhong Z, Feijen J. Stimuli-responsive polymersomes for programmed drug delivery. *Biomacromolecules.* 2009; 10:197–209. [PubMed: 19123775]
4. Yin H, Kang SW, Bae YH. Polymersome formation from AB(2) type 3-miktoarm star copolymers. *Macromolecules.* 2009; 42:7456–7464.

5. Yin H, Kang HC, Huh KM, Bae YH. Biocompatible, pH-sensitive AB₂ miktoarm polymer-based polymersomes: preparation, characterization, and acidic pH-activated nanostructural transformation. *J Mater Chem*. 2012; 22:19168–19178.
6. Rao J, Zhang Y, Zhang J, Liu S. Facile preparation of well-defined AB₂ Y-shaped miktoarm star polypeptide copolymer via the combination of ring-opening polymerization and click chemistry. *Biomacromolecules*. 2008; 9:2586–2593. [PubMed: 18611048]
7. Papahadjopoulos D, Cowden M, Kimelberg H. Role of cholesterol in membranes. Effects on phospholipid-protein interactions, membrane permeability and enzymatic activity. *Biochim. Biophys. Acta*. 1973; 330:8–26. [PubMed: 4128465]
8. Liu D, Huang L. Role of cholesterol in the stability of pH-sensitive, large unilamellar liposomes prepared by the detergent-dialysis method. *Biochim. Biophys. Acta*. 1989; 981:254–260. [PubMed: 2730903]
9. Hunt CA. Liposomes disposition in vivo. V. Liposome stability in plasma and implications for drug carrier function. *Biochim Biophys Acta*. 1982; 719:450–463. [PubMed: 7150653]
10. Gabizon A, Chemla M, Tzemach D, Horowitz AT, Goren D. Liposome longevity and stability in circulation: effects on the in vivo delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. *J Drug Target*. 1996; 3:391–398. [PubMed: 8866658]
11. Liu D, Huang L, Moore MA, Anantharamaiah GM, Segrest JP. Interactions of serum proteins with small unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid: high-density lipoprotein, apolipoprotein A1, and amphipathic peptides stabilize liposomes. *Biochemistry*. 1990; 29:3637–3643. [PubMed: 2111162]
12. Sabin J, Prieto G, Ruso JM, Messina PV, Salgado FJ, Nogueira M, Costas M, Sarmiento F. Interactions between DMPC liposomes and the serum blood proteins HSA and IgG. *J. Phys. Chem B*. 2009; 113:1655–1661. [PubMed: 19159271]
13. Chen C, Han D, Cai C, Tang X. An overview of liposome lyophilization and its future potential. *J Control Release*. 142:299–311. [PubMed: 19874861]
14. Wang D, Kong L, Wang J, He X, Li X, Xiao Y. Polymyxin E sulfate-loaded liposome for intravenous use: preparation, lyophilization, and toxicity assessment in vivo. *PDA J Pharm Sci Technol*. 2009; 63:159–167. [PubMed: 19634354]
15. Yadava P, Gibbs M, Castro C, Hughes JA. Effect of lyophilization and freeze-thawing on the stability of siRNA-liposome complexes. *AAPS PharmSciTech*. 2008; 9:335–341. [PubMed: 18431646]
16. Lim SK, de Hoog H-P, Parikh AN, Nallani M, Liedberg B. Hybrid, nanoscale phospholipid/block copolymer vesicles. *Polymers*. 2013; 5:1102–1114.
17. Chemin M, Brun P-M, Lecommandoux S, Sandre O, Le Meins J-F. Hybrid polymer/lipid vesicles: fine control of the lipid and polymer distribution in the binary membrane. *Soft Matter*. 2012; 8:2867–2874.
18. Nam J, Vanderlick TK, Beales PA. Formation and dissolution of phospholipid domains with varying textures in hybrid lipo-polymersomes. *Soft Matter*. 2012; 8:7982–7988.
19. Winzen S, Bernhardt M, Schaeffel D, Koch A, Kappl M, Koynov K, Landfester K, Kroeger A. Submicron hybrid vesicles consisting of polymer-lipid and polymer-cholesterol blends. *Soft Matter*. 2013; 9:5833–5890.
20. Olubummo A, Schulz M, Lechner BD, Scholtysek P, Bacia K, Blume A, Kressler J, Binder WH. Controlling the localization of polymer-functionalized nanoparticles in mixed lipid/polymer membranes. *ACS Nano*. 6:8713–8727. [PubMed: 22950802]
21. Chu, B. *Laser light scattering*. 2 ed.. New York: Academic Press Inc.; 1991.
22. Provencher SW. A Fourier method for the analysis of exponential decay curves. *Biophys J*. 1976; 16:27–41. [PubMed: 1244888]
23. Lelkes, PI. *Liposome Technology*. Gregoriadis, G., editor. Boca Raton: CRC Press; 1984. p. 225–256.
24. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochem J*. 1980; 186:591–598. [PubMed: 7378067]
25. Massey J, Power KN, Manners I, Winnik MA. Self-assembly of a novel organometallic-inorganic block copolymer in solution and the solid state: nonintrusive observation of novel wormlike

- poly(ferrocenyldimethylsilane)-b-poly(dimethylsiloxane) micelles. *J Am Chem Soc.* 1998; 120:9533–9540.
26. Gohy JF, Lohmeijer BG, Alexeev A, Wang XS, Manners I, Winnik MA. U.S. Schubert, Cylindrical micelles from the aqueous self-assembly of an amphiphilic poly(ethylene oxide)-b-poly(ferrocenylsilane) (PEO-b-PFS) block copolymer with a metallo-supramolecular linker at the block junction. *Chem Eur J.* 2004; 10:4315–4323. [PubMed: 15352114]
 27. Liu T, Diemann E, Li H, Dress AW, Muller A. Self-assembly in aqueous solution of wheel-shaped Mo₁₅₄ oxide clusters into vesicles. *Nature.* 2003; 426:59–62. [PubMed: 14603315]
 28. Barz M, Tarantola M, Fischer K, Schmidt M, Luxenhofer R, Janshoff A, Theato P, Zentel R. From defined reactive diblock copolymers to functional HEMA-based self-assembled nanoaggregates. *Biomacromolecules.* 2008; 9:3114–3118. [PubMed: 18855476]
 29. Melzak KA, Bender F, Tsortos A, Gizeli E. Probing mechanical properties of liposomes using acoustic sensors. *Langmuir.* 2008; 24:9172–9180. [PubMed: 18642856]
 30. Cohen DE, Thurston GM, Chamberlin RA, Benedek GB, Carey MC. Laser light scattering evidence for a common wormlike growth structure of mixed micelles in bile salt- and straight-chain detergent-phosphatidylcholine aqueous systems: relevance to the micellar structure of bile. *Biochemistry.* 1998; 37:14798–14814. [PubMed: 9778354]
 31. Antonietti M, Forster S. Vesicles and liposomes: A self-assembly principle beyond lipids. *Adv Mater.* 2003; 15:1323–1333.
 32. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* 2001; 53:283–318. [PubMed: 11356986]
 33. Senior J, Gregoriadis G. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. *Life Sci.* 1982; 30:2123–2136. [PubMed: 7109841]
 34. Semple SC, Chonn A, Cullis PR. Influence of cholesterol on the association of plasma proteins with liposomes. *Biochemistry.* 1996; 35:2521–2525. [PubMed: 8611555]
 35. Perkins WR, Minchey SR, Ahl PL, Janoff AS. The determination of liposome captured volume. *Chem Phys Lipids.* 1993; 64:197–217. [PubMed: 8242834]
 36. Zhang XM, Patel AB, de Graaf RA, Behar KL. Determination of liposomal encapsulation efficiency using proton NMR spectroscopy. *Chem Phys Lipids.* 2004; 127:113–120. [PubMed: 14706745]
 37. Grabe M, Oster G. Regulation of organelle acidity. *J Gen Physiol.* 2001; 117:329–344. [PubMed: 11279253]
 38. Watson P, Jones AT, Stephens DJ. Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells. *Adv Drug Deliv Rev.* 2005; 57:43–61. [PubMed: 15518920]
 39. Hovorka O, Subr V, Vetvicka D, Kovar L, Strohalm J, Strohalm M, Benda A, Hof M, Ulbrich K, Rihova B. Spectral analysis of doxorubicin accumulation and the indirect quantification of its DNA intercalation. *Eur J Pharm Biopharm.* 76:514–524. [PubMed: 20638475]
 40. Bagalkot V, Farokhzad OC, Langer R, Jon S. An aptamer-doxorubicin physical conjugate as a novel targeted drug-delivery platform. *Angew Chem Int Ed Engl.* 2006; 45:8149–8152. [PubMed: 17099918]
 41. Lodge TP, Rasdal A, Li Z, Hillmyer MA. Simultaneous, segregated storage of two agents in a multicompartment micelle. *J Am Chem Soc.* 2005; 127:17608–17609. [PubMed: 16351082]
 42. Li Z, Kesselman E, Talmon Y, Hillmyer MA, Lodge TP. Multicompartment micelles from ABC miktoarm stars in water. *Science.* 2004; 306:98–101. [PubMed: 15459387]
 43. Kim D, Lee ES, Park K, Kwon IC, Bae YH. Doxorubicin loaded pH-sensitive micelle: antitumoral efficacy against ovarian A2780/DOXR tumor. *Pharm. Res.* 2008; 25:2074–2082. [PubMed: 18449626]

Highlights

Cholesterol (CL) was incorporated into pH-sensitive polymersomes.

The CL incorporation enhanced colloidal stability of the polymersomes.

The CL incorporation did not damage on pH sensitivity of the polymersomes.

CL-incorporating polymersomes played a role as an intracellular drug carrier.

Fig. 1.

Schematic illustration of a unilamellar vesicle (CL-Polymersome) formed by the AB₂ 3-miktoarm star polymer (one mPEG block (2 kDa) and two polyHis (2.9 kDa) blocks) and cholesterol (CL) and its TEM image. The degree of polymerization (DP) for ethylene glycol (k) and histidine (l) in each arm and the weight fraction (f) of PEG are indicated.

Fig. 2.

Angle-dependent relaxation times of CL-Polymersome solutions (0.3 mg/mL, pH 9.0) measured by DLS. D_0 is the intercept of the regression line (dash line) against the D_{app} -axis, and R_h is calculated based on D_0 . The CL_{0wt%}-Polymersome is the CL-free polymersome, and its plot used as a control was taken from Yin *et al.*, J. Mater. Chem. 22 (2012) 19168–19178.

Fig. 3.

Partial Zimm plots of CL-Polymersome solutions (0.3 mg/mL, pH 9.0) measured by SLS. R_g is calculated from the slope and $1/I_{ex}$ -intercept of the regression line (dash line). The $CL_{0wt\%}$ -Polymersome is the CL-free polymersome, and its plot, used as a control was taken from Yin *et al.*, J. Mater. Chem. 22 (2012) 19168–19178.

(a)

(b)

Fig. 4.

(a) Time-dependent $R_{h,app}$ distribution and (b) time-dependent colloidal stability of the CL_{0wt%}-Polymersome and the CL_{1wt%}-Polymersome in the presence of BSA (1 mg/mL). The colloidal stability of CL-Polymersome (0.1 mg/mL) was determined by subtracting the portion of BSA from the total scattered light intensity of the mixtures (CL-Polymersome and BSA) at 90°.

(a)

(b)

Fig. 5.

In vitro pH-dependent 5(6)-carboxyfluorescein (CF) release profiles of the CF-loaded CL_{1wt%}-Polymersome: (a) 72 hr release profile and (b) the comparison of CF release between the CF-loaded CL_{1wt%}-Polymersome and the CF-loaded CL_{0wt%}-Polymersome at pH 6.8 and pH 6.0. The CF release data of the CF-loaded CL_{0wt%}-Polymersome, used as the control, was taken from Yin *et al.*, J. Mater. Chem. 22 (2012) 19168–19178. (Mean ± standard deviation; n=3)

(a)

(b)

Fig. 6.

Cellular uptake of CF-loaded CL-Polymersome in A2780 cells cultured at pH 7.4: (a) FACS histograms and (b) confocal microscopy images. The data were obtained at 4 hr post-incubation.

(a)

(b)

Fig. 7.

In vitro anti-tumor effect of the DOX-loaded CL-Polymersome in (a) MCF7/ADR-RES and (b) A2780/ADR cells. Cells were incubated for 5 days following treatment with the DOX-loaded CL-Polymersome or DOX. (Mean $\pm$ standard error; n=6)

Table 1Physicochemical parameters of the mPEG-*b*-(polyHis)₂ polymersomes with or without cholesterol (CL)

	mPEG- <i>b</i> -(polyHis) ₂ polymersome (CL _{0wt%} -Polymersome)	mPEG- <i>b</i> -(polyHis) ₂ /Cholesterol _{1wt%} polymersome (CL _{1wt%} -Polymersome)	mPEG- <i>b</i> -(polyHis) ₂ /Cholesterol _{5wt%} polymersome (CL _{5wt%} -Polymersome)
$D_0 [a]$	3.40×10^{-8} cm ² /sec $[e]$	2.64×10^{-8} cm ² /sec	1.79×10^{-8} cm ² /sec
Polydispersity index (PDI) $[a]$	0.05 $[e]$	0.03	0.09
R_h (nm) $[a]$	70 nm $[e]$	91 nm	134 nm
R_g (nm) $[b]$	72 nm $[e]$	92 nm	129 nm
R_g/R_h	1.03 $[e]$	1.01	0.96
Half-life ($t_{1/2}$) in BSA (1 mg/mL) $[c]$	5 hr	> 72 hr	N.T.
Half-life ($t_{1/2}$) in BSA (40 mg/mL) $[c]$	1 hr	12 hr	15 hr
Encapsulated volume (μL/mg) $[d]$	0.92±0.10 μL/mg $[e]$	1.24±0.16 μL/mg	1.42±0.09 μL/mg

 $[a]$ determined by DLS; $[b]$ determined by SLS; $[c]$ polymer solution (0.1 mg/mL) under a condition of pH 7.4, 37°C, and 300 mOsm/L; $[d]$ determined from encapsulation study of 5(6)-carboxyfluorescein (CF); $[e]$ cited from Yin et al., J. Mater. Chem. 22 (2012) 19168–19178. N.T. means “not tested”. All the parameters were given as the average value from at least three measurements.