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Bile acid-based drug delivery systems for enhanced doxorubicin encapsulation: Comparing hydrophobic and ionic interactions in drug loading and release

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ABSTRACT

Doxorubicin (Dox) is a drug of choice in the design of drug delivery systems directed towards breast cancers, but is often limited by loading and control over its release from polymer micelles. Bile acid-based block copolymers present certain advantages over traditional polymer-based systems for drug delivery purposes, since they can enable a higher drug loading via the formation of a reservoir through their aggregation process. In this study, hydrophobic and electrostatic interactions are compared for their influence on Dox loading inside cholic acid-based block copolymers. Poly(allyl glycidyl ether) (PAGE) and poly(ethylene glycol) (PEG) were grafted from the cholic acid (CA) core yielding a star-shaped block copolymer with 4 arms (CA-(PAGE-*b*-PEG)₄) and then loaded with Dox via a nanoprecipitation technique. A high Dox loading of 14 wt% was achieved via electrostatic as opposed to hydrophobic interactions with or without oleic acid as a cosurfactant. The electrostatic interactions confer a pH responsiveness to the system. 50% of the loaded Dox was released at pH 5 in comparison to 12% at pH 7.4. The nanoparticles with Dox loaded via hydrophobic interactions, did not show such a pH responsiveness. The systems with Dox loaded via electrostatic interactions showed the lowest IC₅₀ and highest cellular internalization indicating the pre-eminence of this interaction in Dox loading. The blank formulations are biocompatible and did not show cytotoxicity up to 0.17 mg/mL. The new functionalized star block copolymers based on cholic acid show great potential as drug delivery carriers.

Keywords: Bile acids, doxorubicin, pH-responsive, drug delivery systems

INTRODUCTION

Breast cancer is the second most prevalent and deadly form of cancer in women. It is currently treated with a combination of surgery and chemotherapy.¹ Over the course of chemotherapy, not only will the patient suffer greatly from adverse effects, but the cancer often develops resistance

mechanisms limiting the therapeutic outcome of the treatment.² A common strategy to diminish the side effects inherent to chemotherapy is the encapsulation of the cytotoxic molecules into a drug delivery system. Doxorubicin (Dox) is currently used for breast cancer treatment, but is characterized with severe toxicity due to its off-target effects. Over the past decades, a great deal of research effort has been directed at encapsulating chemotherapeutics in drug delivery systems to increase their safety and transport efficiency to the cancer tissues. Toward this end, polymer-based micelles have attracted much attention with several formulations reaching clinical trials.^{3, 4}

Polymer-based micelles of a core-shell structure are typically aggregates of polymer chains with a hydrophobic core and hydrophilic corona. They are often the preferred platform for antitumor drug formulations due to their unique core-shell structure, tunable size and shape, ease of synthesis and freedom in design and structure.^{5, 6} Much research work has focused on the loading of therapeutically relevant molecules with hydrophobic character inside the core of these micelles.⁷⁻¹⁰ For these systems, the drug loading level is greatly influenced by the hydrophobic interaction of the core-forming block and the drug.¹¹ Unfortunately, these formulations are often plagued with a low drug loading and instability in vivo.¹²⁻¹⁴ Strategies to circumvent this limitation include adjustment of the hydrophobic-hydrophilic balance,¹⁵⁻¹⁷ improvement of the polymer-drug interactions,¹⁸⁻²¹ cross-linking,²²⁻²⁵ or conjugation of the drug to the polymer via covalent bonding.^{15, 26-29} However, these strategies are often met with challenges, particularly slow, insufficient release. For example, release of a drug conjugated to the polymer necessitates the cleavage of a covalent bond, which may slow down the release. Non-covalent interactions such as ionic interactions and hydrogen bonding may also favour drug loading.³⁰⁻³⁸ Dox bears a positive charge at physiological pH and is composed of aromatic rings favoring non-covalent interactions via aromatic stacking. Many reports demonstrate the superiority of ionic interactions offering higher drug loading and better stability, and stimuli-sensitive release in response to the environmental pH.^{30, 32-34} Despite recent advancements in polymer-based systems, achieving high drug loading and improving tissue biodistribution remain limiting factors.^{6, 15, 39} In an effort to address the first issue, we intend to study the interaction and forces that govern Dox loading in the context of bile acid-based polymer micelles.

We have previously shown the advantage of using bile acids in the design of drug delivery systems.⁴⁰⁻⁴² Micelles formed by bile acid-based polymers are less densely packed than linear

polymers of similar structure,⁴³ creating an internal reservoir that may hold a larger amount of hydrophobic therapeutic molecules. We have studied the loading of hydrophobic itraconazole in bile acid-based nanoparticles.⁴¹ These formulations reached high loading contents (35 wt%) and high encapsulation efficiencies (>89 %) as well as improved bioavailability and pharmacokinetics of the drug. Dox presents a different challenge with different interaction forces. Therefore, we modified the design of the copolymer based on cholic acid. Hydrophobic poly(allyl glycidyl ether) (PAGE) and hydrophilic poly(ethylene glycol) (PEG) blocks were attached sequentially via anionic polymerization onto a cholic acid (CA) core to yield a star-shaped copolymer of 4 arms, CA-(PAGE-*b*-PEG)₄ (Scheme 1). The star-shaped polymers based on bile acids have demonstrated their advantages in biocompatibility and drug loading.⁴⁴ The allyl groups of PAGE can be functionalized post-polymerization. Incorporating COOH moieties in the polymer backbone can facilitate Dox loading via electrostatic interactions and improve the stability of the complex. The different interactions between Dox and the polymer aggregates (hydrophobic, intrinsic electrostatic, and extrinsic electrostatic) are studied to compare their impact on drug loading, stability, drug release, and in vitro cytotoxicity of the new formulations.

MATERIALS AND METHOD

Materials

All chemical reagents were purchased from Sigma-Aldrich and used without further purification unless stated otherwise. For the anionic polymerization, dimethyl sulfoxide (DMSO) and allyl glycidyl ether (AGE) was dried overnight with calcium hydride and distilled immediately prior to use. Ethylene oxide gas was passed through a column of calcium hydride and condensed with dry ice and acetone for quantification before adding to the reaction vessel. Tetrahydrofuran (THF) was dried using sodium under reflux, and methanol was dried using magnesium sulphate. Doxorubicin hydrochloride was purchased from Tecoland.

Synthesis of CA-(PAGE-*b*-PEG)₄ via anionic polymerization

The synthesis and functionalization of the polymers are shown in Scheme 1. For the anionic polymerizations, all glassware was flame-dried under vacuum and purged three times with argon before use. The ethanolamine derivative of cholic acid with four hydroxyl groups was

synthesized according to a previous method.^{45, 46} For anionic polymerization, 5 β -cholanoamide (1.66 mmol, 0.75 g) was dissolved in 40 mL of dry DMSO. A potassium naphthalenide solution in THF was added (0.44 mol/L, 1.66 mmol, 1 eq., 3.74 mL) dropwise using a canula under high pressure. AGE was distilled and added (7.6 g, 66.4 mmol, 40 eq.) dropwise using a canula under high pressure. The anionic polymerization was initiated by immersing in an oil bath at 40 °C and allowed to proceed for 24 h to allow the consumption of all the AGE monomers. Then, dry ethylene oxide (17 mL, 332.2 mmol, 200 eq.) chilled in dry ice/acetone was introduced into the flask and polymerized for another 24 h. The reaction was stopped by quenching the reaction with concentrated hydrochloric acid. The DMSO solution was extracted with hexane (3 x 10 mL) to remove the naphthalenide. Distilled water was added to the DMSO solution and the mixture was dialyzed against distilled water (48 h) through a membrane with 3,500 Da molecular weight cut-off (MWCO) to remove all unreacted monomers.

Scheme 1. Synthesis of cholic acid-based star polymers via anionic polymerization and the functionalization of the PAGE block through thiol-ene reactions.

Functionalization of CA-(PAGE-*b*-PEG)₄ to CA-(PAGE-COOH-*b*-PEG)₄

The purified and dried CA-(PAGE-*b*-PEG)₄ polymers (2 g, 0.14 mmol) were dissolved in dry methanol (20 mL). Azobisisobutyronitrile (0.5 g, 3.2 mmol), and 3-mercaptopropionic acid (9.0 g, 84.4 mmol) were dissolved in the solution which was refluxed at 70 °C overnight. The reaction was stopped by cooling and methanol was removed. Finally, distilled water was added to dissolve product and remaining reactants and the solution was dialyzed against distilled water for 48 h and dried to yield CA-(PAGE-COOH-*b*-PEG)₄.

Characterization methods

The molar mass of the polymers was determined by size exclusion chromatography (SEC) on a Breeze system from Waters equipped with a 717 plus autosampler, a 1525 Binary HPLC pump, and a 2410 refractive index detector and three consecutive styragel columns with a flow rate of 1.0 mL/min. Polystyrene standards were used for calibration. All samples were filtered on a PTFE 0.2 µm filters prior to injection. ¹H-NMR spectra were recorded on a Bruker AV400 spectrometer operating at 400 MHz. Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer NanoZS instrument equipped with a He-Ne laser with a wavelength of 633 nm, and at a scattering angle of 173.5°. Intensity-averaged hydrodynamic diameters of the dispersions were obtained using the non-negative least-squares algorithm (NNLS). Disposable cuvettes were used, and the samples were filtered using 0.45 µm Nylon filters prior to measurements. Sample concentration used was 1 mg/mL for both blank and loaded micelles. Dox concentration was determined by recording the fluorescence spectra on a Cary eclipse fluorescence spectrophotometer with excitation and emission wavelengths of 485 and 590 nm, respectively, and a bandwidth of 5 nm. The transmission electron microscope (TEM) images were obtained on a FEI Tecnai 12 at 80kV and equipped with an AMT XR80C CCD camera system. TEM samples were prepared by drop-casting blank micelles on a carbon-coated copper grid (300 mesh, Carbon Type-B, Ted Pella Inc.).

Doxorubicin loading and release

Dox was encapsulated into the micelles by a nanoprecipitation method. Briefly, for the CA-(PAGE-COOH-*b*-PEG)₄ sample, the copolymers (10 mg) were dissolved in methanol (1 mL)

along with Dox (2 mg) with gentle stirring. The solution was added dropwise into 10 mL PBS buffer (pH 7.4, 10 mM) over a period of 10 min using a syringe pump. The resulting PBS solution was stirred gently for 10 min before purification via dialysis (MWCO 6,000-8,000 Da) against 1 L PBS (pH 7.4, 10 mM) for 24 h, changing the outer media twice. Samples of the outer media were taken before replenishment to quantify the amount of Dox released. The same procedure was repeated for the CA-(PAGE-*b*-PEG)₄ + OA sample, with a slight modification by using 10 mg of copolymer and 0.126 mg of OA. For the CA-(PAGE-*b*-PEG)₄ system, the same procedure was followed by using 10 mg of CA-(PAGE-*b*-PEG)₄ alone and Dox which was reacted overnight with 1.5 eq TEA prior to the nanoprecipitation. Dox loading was measured by quantifying the amount of Dox released during the purification and subtracting this value from the amount of Dox in the feed. The quantification was achieved by taking 1 mL of the outer water, mixing with 1 mL DMSO, measuring the fluorescence intensity on a Cary fluorescence spectrometer (excitation 485 nm, emission 590 nm), and comparing with a calibration curve for Dox in DMSO/PBS (1:1 Vol.).

$$DLC \text{ (wt\%)} = \frac{\text{weight of loaded drug}}{\text{weight of copolymer}} \times 100\%$$

$$EE \text{ (\%)} = \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \times 100\%$$

The Dox release from the different formulations was determined with a dialysis method at different pH. Briefly, 2 mL of the drug-loaded formulations were placed in a dialysis bag (MWCO 6,000-8,000 Da) and dialyzed against 225 mL of the appropriate buffer at 37 °C; PBS pH 7.4 or acetate buffer pH 5 10 mM for the experiments conducted at pH 7.4 and 5, respectively. 1 mL of the outer media was taken at regular intervals and replenished with fresh buffer. The amount of Dox released was quantified by fluorescence spectroscopy with the appropriate calibration curve. All experiments were conducted in triplicates and the results are presented as average values.

In vitro cytotoxicity assay

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS). MTT assay was used to determine the

cytotoxicity of the blank formulations and the IC_{50} for free Dox and all three Dox-loaded formulations. MTT assay was conducted according to manufacturer's protocol. HeLa cells were plated on a 96-well plate at a seeding density of 5,000 cells per well in 200 μ L DMEM and allowed to adhere overnight (37°C, 5% CO_2 , 12 h). 40 μ L of the formulation to be tested was added to each well. The plates were incubated for 48 h (37°C, 5% CO_2). Medium was removed and replaced with 100 μ L of fresh medium. 10 μ L of MTT solution (5 mg/mL) was added to each well and incubated (37°C, 5% CO_2) for 4 h. DMEM medium was carefully removed from each well and replaced with DMSO. MTT crystals were dissolved and the absorbance was read at 590 nm using a plate reader. Cytotoxicity is reported as compared to absorbance measured for control (untreated cells). To calculate the IC_{50} , a linear regression was intrapolated by drawing a straight line through the inflection in the viability curve. The IC_{50} value was obtained by setting $y = 50\%$ from the linear equation obtained and solving for x .

Fluorescence activated cell sorting (FACS) assay

HeLa cells were grown in DMEM medium supplemented with 10% FBS. Cells were seeded in a 24-well plate at a seeding density of 5×10^4 cells/well and allowed to adhere overnight (37°C, 5% CO_2). The cells were washed with PBS and replenished with fresh medium. Free Dox and Dox-loaded formulations were added to the cells to yield a final concentration of 1 and 10 μ M Dox in 550 μ L DMEM. After 1 h incubation, the cells were washed with PBS, trypsinized (100 μ L of 0.25 % Trypsin/EDTA), and suspended in FACS buffer (400 μ L, 95% PBS, 5% FBS, 1 mM EDTA). The cells were observed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Dox concentration internalized in the cells was calculated with respect to control samples (untreated cells). The experiment was done in triplicates.

RESULTS AND DISCUSSION

Drug formulation

To study the interactions driving the Dox loading, three formulations were prepared (Figure 1). The first is composed of CA-(PAGE-*b*-PEG)₄ alone, in which only hydrophobic interactions are present to ensure Dox loading inside the core of the micelles. The hydrophobicity is conferred from the hydrophobic face of the cholic acid and the hydrophobic PAGE block, while the

hydrophilic PEG block promotes micelle formation and stability in the aqueous environment. The second formulation is composed of functionalized PAGE blocks bearing carboxylic acid groups (CA-(PAGE-COOH-*b*-PEG)₄). Here, electrostatic interactions between the NH₂ groups of Dox and the COOH groups attached on PAGE drives the loading of Dox into the middle block in addition to the hydrophobic core. In this situation, the electrostatic interactions may be tuned by varying the PAGE block length. The third formulation is composed of CA-(PAGE-*b*-PEG)₄ supplemented with oleic acid (OA). OA is used to mimic the carboxylic acid content on the CA-(PAGE-COOH-*b*-PEG)₄ formulation. The carboxylic acid groups of OA can complex electrostatically to Dox and the aliphatic chain of OA imparts an enhanced hydrophobicity thereby increasing Dox loading while reducing its premature release at physiological pH.⁴⁷ Here the electrostatic interactions are extrinsic to the system; the COOH group of OA and the increased hydrophobicity imparted by OA drive the loading of Dox inside the nanoparticles.

Figure 1. Formulations based on different drug-polymer interactions. For CA-(PAGE-*b*-PEG)₄, Dox is loaded via hydrophobic interactions between the drug and the core of the micelle

composed of cholic acid and hydrophobic PAGE. For CA-(PAGE-COOH-*b*-PEG)₄, Dox is loaded via intrinsic electrostatic interactions between the drug and the pendant COOH groups. For CA-(PAGE-*b*-PEG)₄ + OA, the electrostatic interactions are perceived as extrinsic because they appear on OA only. The hydrophobicity of the OA aliphatic chains may drive the loading of Dox more toward the hydrophobic core.

Polymer synthesis and characterization

The star-shaped block copolymers were synthesized as shown in Scheme 1. An ethanolamine derivative of CA with four hydroxyl groups was used as initiator for the anionic polymerization of allyl glycidyl ether (AGE), followed by the addition of ethylene glycol (EG) to afford a star-shaped CA-(PAGE-*b*-PEG)₄ with 4-arms composed of hydrophobic PAGE and hydrophilic PEG blocks. The copolymer obtained was analyzed by SEC and ¹H-NMR (Figures S1 and S2). The SEC traces show monomodal, narrow distributions and an increase in *M_n* when going from CA-(PAGE)₄ to CA-(PAGE-*b*-PEG)₄. The NMR signals at 0.58, 0.81, and 0.92 ppm were assigned to the 18-CH₃, 19-CH₃, and 21-CH₃ groups, respectively, on the cholic acid backbone. These peaks were used as reference for the analysis of the peaks at 5.79-5.92 and 3.35-3.60 ppm, which correspond respectively to the CH₂ protons on the PAGE block and on both PAGE and PEG. The block copolymer was determined to be CA-(AGE₁₉-*b*-EG₃₀)₄ with 19 monomer units of AGE and 30 monomer units of EG (Table 1). The presence of four arms was confirmed by reacting the polymers with trifluoroacetic anhydride and by the appearance of the methylene protons at 4.5 ppm, corresponding to protons of the PEG terminal CH₂ unit coupled with the trifluoroacetic group. Then, the allyl side chains from the PAGE block were functionalized with mercaptopropionic acid with close to 100% functionalization as confirmed by ¹H-NMR (Figure S2).

Table 1. SEC and ¹H-NMR results for the anionic polymerization of CA with AGE and EG monomers.

Polymer	Molecular Weight (<i>M_n</i> , g/mol)		Đ
	¹ H-NMR	SEC	
CA-(PAGE) ₄	9,100	3,900	1.10
CA-(PAGE ₁₉ - <i>b</i> -PEG ₃₀) ₄	14,400	8,400	1.13

The critical micellar concentration (CMC) of the bile acid derivative in water was measured with fluorescence spectroscopy using pyrene as a probe.⁴⁸ Pyrene fluorescence is measured with increasing concentration of the block copolymers and the CMC is calculated from a plot of the excitation intensity ratio I_{383}/I_{373} as a function of polymer concentration. The CMC for CA-(PAGE-*b*-PEG)₄ is determined to be 20 µg/mL (Figure S3), comparable to typical polymer-based micellar systems found in the literature.^{43, 49-51}

Particle size and distribution were analyzed with dynamic light scattering (DLS) and transmission electron microscopy (TEM), before and after Dox loading. The DLS results show that CA-(PAGE-*b*-PEG)₄ forms larger aggregates ($d = 62 \pm 18$ nm) than (CA-(PAGE-*b*-PEG)₄ with OA, and CA-(PAGE-COOH-*b*-PEG)₄) (Figure 2). CA-(PAGE-*b*-PEG)₄ mixed with OA, a surfactant, forms mixed micelles smaller in diameter (27 ± 10 nm). The negatively charged COOH moieties may induce an electrostatic repulsion between individual polymer chains leading to smaller micelles. These results are corroborated with the TEM images showing spherical aggregates with a dense core (Figure 3).

Figure 2. The volume-size distribution of (A) CA-(PAGE-*b*-PEG)₄ with and without Dox, (B) CA-(PAGE-*b*-PEG)₄ + OA with and without Dox, and (C) CA-(PAGE-COOH-*b*-PEG)₄ with and without Dox as determined by DLS. Blank micelles and formulations are obtained at 1 mg/mL and filtered with 0.45 μ m PES filters prior to DLS measurements.

Table 2 shows the zeta potentials measured for the nanoparticles. The CA-(PAGE-*b*-PEG)₄ micelles have a zeta potential close to neutral with a value of -3.9 ± 0.4 mV, whereas the presence of the negatively charged COOH moieties lead to larger negative values, -9.2 ± 0.7 mV for CA-(PAGE-*b*-PEG)₄ with OA and -17.2 ± 0.1 mV for the CA-(PAGE-COOH-*b*-PEG)₄ micelles where the COOH groups in the middle block are more exposed to the surface.

Table 2. Aggregate size and Dox-loading of the formulations based on star-shaped block copolymers.

Drug formulation	Diameter blank micelle and Dox-loaded micelle (nm)		Micelle dispersity from DLS	Zeta potential (mV)	DLC (wt%) [‡]	EE (%) [†]
	DLS	TEM				
CA-(PAGE- <i>b</i> -PEG) ₄	62 ± 18	57 ± 29	0.2	-3.9 ± 0.4	10	50
CA-(PAGE- <i>b</i> -PEG) ₄ + Dox	54 ± 27	29 ± 7	0.2	-0.29 ± 0.06		
CA-(PAGE- <i>b</i> -PEG) ₄ + OA	27 ± 14	26 ± 7	0.1	-9.2 ± 0.7	11.6	58
CA-(PAGE- <i>b</i> -PEG) ₄ + OA + Dox	31 ± 10	34 ± 14	0.3	-5.4 ± 0.2		
CA-(PAGE-COOH- <i>b</i> -PEG) ₄	27 ± 10	28 ± 11	0.2	-17.2 ± 0.09	14	72
CA-(PAGE-COOH- <i>b</i> -PEG) ₄ + Dox	59 ± 32	40 ± 11	0.1	-17.4 ± 0.9		

* All samples were examined at a 1 mg/mL concentration in 10 mM PBS at 7.4 pH with 154 mM NaCl.

[‡] Drug loading content.

[†] Encapsulation efficiency.

Doxorubicin loading and release

We hypothesize that electrostatic interactions enhance the loading of Dox in comparison to Dox via hydrophobic interaction. To study these interaction forces, the three different formulations were loaded with equal amount of Dox using the same loading procedure. The encapsulation efficiencies and drug loading contents are listed in Table 1. The CA-(PAGE-*b*-PEG)₄ aggregates showed drug loading content of 10 wt% and an encapsulation efficiency of 50%. Adding OA to the formulation enabled a higher drug loading and encapsulation efficiency, 11.6 wt% and 58%, respectively, most likely due to an increased hydrophobicity of the Dox complex.⁴⁷ Functionalizing the bile acid-based polymers with pendant COOH moieties resulted in an improvement of the drug loading to 14 wt% and loading efficiency to 72%.

In the case of CA-(PAGE-*b*-PEG)₄ and CA-(PAGE-*b*-PEG)₄ with OA, there is a small size increase upon Dox loading (Figure 2). TEM images in Figure 3 show that upon Dox loading, both CA-(PAGE-*b*-PEG)₄ and CA-(PAGE-*b*-PEG)₄ with OA retain their spherical shape. In contrast, the Dox-loaded CA-(PAGE-COOH-*b*-PEG)₄ micelles showed the formation of aggregates larger in size (Figure 2) and the spherical micelles changed to vesicular-shaped polymersomes (Figure 3). Zeta potential measurements showed a slight increase in the surface charge of the micelles upon Dox loading when compared to the blank micelles (Table 1) due to the presence of positively charged Dox loaded in the reservoir of the polymersome and adsorbed on the surface of the micelles.

Figure 3. Transmission electron micrograph images of the micelles formed: (top) blank formulations and (bottom) Dox-loaded formulations (1 mg/mL samples).

In vitro Dox release profiles at 37 °C were obtained at two different pH values, mimicking the endosomal pH at 5 encountered by the nanoparticles upon cellular entry and the physiological pH at 7.4 sensed by the nanoparticles during transport. The objective of this experiment is to compare the release kinetics of the different formulations to assess the impact of the interaction forces on the release profile. Moreover, the presence of the COOH moieties in the CA-(PAGE-COOH-*b*-PEG)₄ formulation bestows pH-responsiveness to the system, an advantage compared to hydrophobic entrapment alone. The results shown in Figure 4 compare the Dox release

profiles at both pH values for all three formulations. As a control, free Dox was loaded in the dialysis bag and its release was monitored over time. The declivity observed for free Dox at pH 7.4 can be explained by the possible degradation of the drug as reported previously.^{52, 53} At pH 5 the free Dox is more stable.⁵⁴ The degradation of Dox was also observed for the CA-(PAGE-*b*-PEG)₄ with OA formulation at pH 7.4, but not for the other two formulations for which the quantity of Dox released was small so that the degradation was not measured at an appreciable amount.

All formulations show a biphasic release with an initial burst release within the first 8 h, followed by a slower, continuous release. The CA-(PAGE-*b*-PEG)₄ formulation should be pH-irresponsive but shows an increase in the release from 7.2 to 23% when the pH changes from 7.4 to 5. The higher solubility of Dox at pH 5 facilitates its release.⁵⁵ For the CA-(PAGE-*b*-PEG)₄ with OA formulation, the release increased from 24 to 35% when pH varies from 7.4 to 5 (Table 3). In this case, Dox interacts electrostatically with the negative COOH moieties on the OA and the long aliphatic chain of OA provides a higher hydrophobicity for Dox to remain in the core.⁴⁷ At pH 5, the COOH groups are partially protonated weakening their electrostatic interaction with Dox, while the core remains hydrophobic and keeps the drug inside. Therefore, lowering the pH does not significantly impact the release of Dox from the core of the micelle.⁵⁶ Finally, the CA-(PAGE-COOH-*b*-PEG)₄ formulation shows the highest difference between the two pHs, with a change in release from 16 to 51% when the pH changed from 7.4 to 5. This demonstrates the pH responsiveness of the formulation. The COOH groups on the polymer protonate at pH 5, thereby disrupting the electrostatic interaction between the polymer and the drug helping with its release. Moreover, this formulation shows a more stable drug loading at physiological pH than the other two formulations.

Table 3. The maximum amount of Dox released in response to pH change.

Drug formulation	Dox release	
	pH 7.4 ¹	pH 5 ²
CA-(PAGE- <i>b</i> -PEG) ₄	7.2	23.0
CA-(PAGE- <i>b</i> -PEG) ₄ + OA	24.2	35.3
CA-(PAGE-COOH- <i>b</i> -PEG) ₄	16.3	51.5

¹Maximum Dox released at pH 7.4 and ²maximum Dox released at pH 5, the values at 48 h were used. This value gives a measure of the pH responsiveness of the system.

Figure 4. Cumulative release of Doxorubicin from formulations at 37 °C (A) in PBS buffer at pH 7.4 and (B) in acetate buffer at pH 5. Results show pH-responsiveness for the CA-(PAGE-COOH-*b*-PEG)₄ formulation but not for the other two formulations.

In vitro cytotoxicity

The toxicity of the blank formulations was tested in HeLa cells and the results are presented in Figure S6. All the formulations are non-toxic up to 0.17 mg/mL with over 80% cell viability; consistent with the previous reports on bile acid micelles.⁴⁴ To determine the efficiency of the new bile acid-based drug delivery system, the IC_{50} of the different formulations were obtained on HeLa cells and compared with that of free Dox. The IC_{50} of free Dox is 2.2 μ M, in agreement with values reported in the literature.^{22, 57} The CA-(PAGE-*b*-PEG)₄ formulation has an IC_{50} of 13 μ M which is ca. 6 times higher than that of free Dox. For the CA-(PAGE-*b*-PEG)₄ with OA formulation, no IC_{50} was observed at the concentrations tested. Finally, for the CA-(PAGE-COOH-*b*-PEG)₄ formulation, the IC_{50} obtained is 11.4 μ M, comparable to that of the CA-(PAGE-*b*-PEG)₄ formulation.

Figure 5. In vitro cytotoxicity obtained on HeLa cells after 48 h incubation of (A) free Dox compared with the three blank formulations: (B) CA-(PAGE-*b*-PEG)₄ with OA, (C) CA-(PAGE-COOH-*b*-PEG)₄, and (D) CA-(PAGE-*b*-PEG)₄. The results are presented as mean $\pm$ S.D.

To clarify these results, cellular entry for the formulation was studied and compared with that of free Dox using flow cytometry (Figure 6). Free Dox is readily internalized inside the cell, but Dox in aggregates of both CA-(PAGE-*b*-PEG)₄ and CA-(PAGE-COOH-*b*-PEG)₄ is less readily internalized. Although previous reports indicate the advantage of spherical over vesicular-shaped nanoparticles for cellular entry, the negative charge of the nanoparticles had a greater impact.^{58, 59} Presumably, the negative charge of CA-(PAGE-COOH-*b*-PEG)₄ may interact with extracellular proteins to facilitate membrane interaction and internalization, and consequently slightly lower the IC₅₀.⁵⁹ The higher IC₅₀ observed may be a result of cellular entry. Therefore, the pH responsiveness of the CA-(PAGE-COOH-*b*-PEG)₄ formulation does not bring an advantage over the neutral, non-responsive system. The pH-responsiveness was unfavorable due to kinetics of Dox release; Dox release was not sufficiently rapid to promote endosomal escape and cellular uptake was the determining factor for lowering of the IC₅₀. Surprisingly, the formulation with OA showed even less cellular entry than the other two formulations. The presence of OA affected the cellular entry hence increasing further the IC₅₀, probably due to its impact on the structure of the micelles or the surface properties. The TEM images shows that the micelles prepared from OA are much smaller than those of the other two formulations, but are also dense and irregular in shape. Reports demonstrated the advantage of smaller nanoparticles for cellular entry, but also the effect of particle shape on cellular entry.^{58, 59} It is believed that the irregular shape of the Dox-loaded nanoparticles caused by the presence of the OA reduced the cellular internalization process observed via FACS. The proposed drug delivery systems achieve a higher stability avoiding unspecific uptake of Dox, but further studies are necessary to improve the specific nanoparticle internalization in cancer cells and optimize the pH-responsiveness to enable adequate endosomal escape.

Figure 6. Flow cytometry uptake profiles for formulations of free Dox and with CA-(PAGE-*b*-PEG)₄, CA-(PAGE-*b*-PEG)₄ with OA, and CA-(PAGE-COOH-*b*-PEG)₄. Experiment was performed in triplicates with HeLa cells with 2 h incubation; non-treated HeLa cells were used as control.

CONCLUSION

Cholic acid-based micelles formed stable micellar aggregates with high Dox loading. Electrostatic interactions in the case of the CA-(PAGE-COOH-*b*-PEG)₄ formulation led to higher loading than the CA-(PAGE-*b*-PEG)₄ formulation based on hydrophobic interaction. The loading of Dox via electrostatic interactions confers pH-responsiveness to the drug delivery

system, lowering the pH from 7.4 to 5 led to an increase in Dox release from 12 to 50%. Moreover, the new nanoparticles are non-toxic with over 80% cell viability at concentrations up to 0.17 mg/mL. IC₅₀ values for both CA-(PAGE-*b*-PEG)₄ and CA-(PAGE-COOH-*b*-PEG)₄ formulations are higher than that for free Dox and this has been linked to lower cellular internalization of the Dox-loaded micellar aggregates. The CA-(PAGE-*b*-PEG)₄ formulation with OA did not show an IC₅₀ at the measured concentration range and had lower cellular internalization than the other two formulations. Cholic acid-based drug delivery systems present advantages of higher Dox loading and pH-responsiveness in release. Challenges related to cellular entry need to be further addressed to achieve an overall successful drug delivery platform. To achieve higher cellular internalization and increased efficiency, the addition of targeting ligand on the surface of the nanoparticle is currently being tested. Further studies are underway to determine the efficiency of the new bile acid-based drug delivery system on drug resistant cancer cell lines and cytotoxic drugs with poor cellular entry.

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ASSOCIATED CONTENT

Supporting information including the following figures is available. SEC traces and ¹H-NMR spectra of block copolymers, critical micellar concentration, Dox calibration curves and in vitro cytotoxicity of formulations.

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SUPPORTING INFORMATION

Bile acid-based drug delivery systems for enhanced doxorubicin encapsulation: Comparing hydrophobic and ionic interactions in drug loading and release

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Critical micellization concentration (CMC) determination

The CMC was determined according to a previously published protocol.⁴⁸ Briefly, pyrene was dissolved in methanol to give a final concentration of 10^{-6} M in 5 mL PBS, followed by the evaporation of methanol. A stock solution at 8 mg/mL of copolymer micelles was prepared via a nanoprecipitation method. This stock solution was diluted with buffer to obtain a series of

solutions with copolymer concentrations ranging from 10^{-4} to 8 mg/mL and added to the vials containing pyrene. The solutions were stirred for 48 h to allow the pyrene to equilibrate. Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrometer (Agilent). The emission wavelength was set to 335 nm with a slit width of 2.5 for both excitation and emission. The I_3/I_1 ratio was obtained from the ratio of fluorescence intensity at 383 nm (I_3) and 373 nm (I_1).

Figure S1. SEC traces of homopolymer CA-(PAGE)₄ and block copolymer CA-(PAGE-*b*-PEG)₄. THF was used as eluent with a flow rate of 1 mL/min at 35°C and analyzed with a calibration curve based on PS standards. Figure shows increase in molecular weight following polymerization of PEG and narrow dispersity.

Figure S2. ^1H NMR spectra of homopolymer $\text{CA}-(\text{PAGE})_4$, and block copolymers $\text{CA}-(\text{PAGE}-b\text{-PEG})_4$ and $\text{CA}-(\text{PAGE}-\text{COOH}-b\text{-PEG})_4$ in $\text{d}_6\text{-DMSO}$. The disappearance of the methine and methylene peaks at 5.8 – 6.0 ppm and 5.1 – 5.3 ppm of pendant allyl group on PAGE backbone testifies to the successful functionalization.

Figure S3. Critical micellar concentration measured by fluorescence spectroscopy using pyrene as a probe. The ratio I_3/I_1 (obtained from fluorescence spectra) is plotted as a function of CA-(PAGE-*b*-PEG)₄ concentration. CMC is obtained by the intercept of the two linear regressions. In this case, CMC = 20 $\mu\text{g/mL}$.

Figure S4. Dox calibration curves for release experiment. Dox was quantified in outer water for the release experiment by taking 1 mL of outer water, measuring fluorescence intensity, and quantifying the corresponding Dox amount using this calibration curve. Calibration curve was obtained by measuring fluorescence intensity of Dox in (a) 1/1 DMSO/PBS (pH 7.4) solution and (b) 1/1 DMSO/Acetate (pH5) solution.

Figure S6. In vitro cytotoxicity of blank formulations was tested in HeLa cells using the MTT assay with a 48 h incubation. The results are expressed as mean $\pm$ S.D. Results show the formulations are non-toxic up to 0.17 mg/mL.