Biodegradable Block Copolymer-Doxorubicin Conjugates via Different Linkages: Preparation, Characterization, and In Vitro Evaluation

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Doxorubicin (Dox) was conjugated onto a biodegradable block copolymer methoxy-poly(ethylene glycol)-block-poly(lactide-co-2,2-dihydroxymethylpropylene carbonate (mPEG-b-P(LA-co-DHP)) via a carbamate linkage and an acid-labile hydrazone linkage, respectively. Multifunctional mixed micelles consisting of Dox-containing copolymer mPEG-b-P(LA-co-DHP/Dox) and folic acid-containing copolymer mPEG-b-P(LA-co-DHP/FA) were successfully prepared by coassembling the two component copolymers. The mixed micelles had well-defined core shell structure and their diameters were in the range of 70–100 nm. Both Dox-conjugates (via carbamate or hydrazone linkage) showed pH-dependent release behavior, and the micelles with hydrazone linkage showed more pH-sensitivity compared to those with carbamate linkage. The in vitro cell uptake experiment by CLSM and flow cytometry showed preferential internalization of FA-containing micelles by human ovarian cancer cell line SKOV-3 than that without FA. Flow cytometric analysis was conducted to reveal the enhanced cell apoptosis caused by the FA-containing micelles. These results suggested that these micelles containing both chemotherapeutic and targeting ligand could be a promising nanocarrier for targeting the drugs to cancer cells and releasing the drug molecules inside the cancer cells.

Introduction

Doxorubicin (Dox) is a widely used anticancer drug in the treatment of many types of cancers. However, systemic administration of Dox itself elicits severe cardiac toxicity due to the lack of ability to target cancer cells, and also shows the multidrug resistance effect. To improve therapeutic efficacy of doxorubicin, various drug delivery systems, such as liposomes, polymeric nanoparticles, polymer conjugates, and dendrimer conjugates, have been reported. Recently, polymeric micelles of core–shell architecture based on amphiphilic AB diblock or ABA triblock copolymers have received much attention due to their unique structure and characteristics, such as improved solubility and bioavailability of hydrophobic drugs, low uptake by the reticuloendothelial system (RES), protecting the incorporated drug from fast degradation, blood clearance and elimination from the body, targeting to the tumor tissue in a passive manner via the “enhanced permeation and retention (EPR)” effect, and so on. Dox has been physically encapsulated into and/or chemically conjugated to polymers to prolong circulation time in the bloodstream as well as to increase the extent of extravascular accumulation in the tumor region. Physical encapsulation has certain advantages such as easy preparation and low cost, but also some disadvantages such as limited drug loading and difficulty in drug release rate control.

Conjugation of a drug with a polymer forms a so-called “polymeric prodrug”. The “prodrug” approach provides a powerful means of drug modification, for example, solubilization of hydrophobic drugs, elimination of initial burst release of drug micelles, and tuning of drug pharmacokinetics. Several prodrugs based on doxorubicin have been synthesized and evaluated. The primary amino and keto groups of Dox were used for covalent attachment of Dox onto block copolymer poly(ethylene oxide)-block-poly(aspartic acid) via amide bond and hydrazone bond or to terminal OH-activated poly(ethylene oxide)-block-poly(lactide). Ulbrich group has intensively investigated polymer-Dox conjugates using water-soluble macromolecular carriers poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) via amino or keto groups. Recent advances for these polymer—drug conjugates have been reviewed. According to the literature, the anticancer drug activity and toxicity of Dox is very sensitive to the modification sites and conjugation linkage. On the other hand, most of the investigated drug conjugates are based on nonbiodegradable polymers. Biodegradable polymers have special superiority in biomedical applications because they can be broken down into individual monomers, which are metabolized and removed from the body via normal metabolic pathways. In this paper, we chose amphiphilic block copolymer mPEG-b-P(LA-co-DHP) as the carrier. Both primary amino and keto groups of Dox were used as the conjugation sites. The carbamate linkage and hydrazone linkage thus formed between drug molecule and polymer were investigated.

Due to the development of biology, some specific interactions between antibody and antigen or between ligand and receptor have been found and used in drug delivery to realize site-specific and time-controlled delivery. For example, it is well-known that folic acid (FA) receptors are overexpressed in several human tumors, including ovarian and breast cancers, and rarely expressed in normal tissues; therefore, folic acid is covalently conjugated to anticancer drugs or polymer micelles to achieve selective targeting to tumors. We further prepared folic acid conjugated polymer mPEG-b-P(LA-co-DHP/FA) using the same carrier. Then multifunctional micelles containing both folic acid and Dox were prepared by coassembling the Dox-containing polymer and FA-containing
polymer. Distinct from other micellar systems, the present one exhibited the four-in-one features, that is, biodegradability, targeting, chemotherapy, and pH sensitivity were combined together in one micellar system. CLSM and flow cytometry were used to monitor the internalization of the micelle drug and free drug. MTT studies demonstrated the cytotoxic activity of the conjugate micelles against SKOV-3 and A549 cell lines.

**Experimental Section**

**Materials.** The pendant hydroxyl group carrying copolymer mPEG-b-(LA-co-DHP), that is, methoxy-poly(ethylene glycol)-b-poly(l-lactide-co-2,2-dihydroxymethyl-propylene carbonate), was prepared according to the literature. The polymerization degree of each segment was measured from the 1H NMR spectrum of copolymer. Distilled water to the organic phase, followed by dialyzing against water, was purified as polymeric micelles by adding doubly distilled before use. Folic acid was purchased from Beijing Aoboxing Pharmaceutical Co., Ltd. 4-Nitrophenyl chloroformate (NPC) was in the form of a hydrochloride salt was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. All other chemicals were of analytical or chromatographic grade.

**Synthesis of Doxorubicin Conjugated Block Copolymer mPEG-b-(LA-co-DHP/Dox).** Synthesis of NPC-Activated Copolymer. mPEG-b-(LA-co-DHP) copolymer was first activated with NPC. Briefly, to mPEG-b-(LA-co-DHP) block copolymer (0.8 g) dissolved in methylene chloride (20 mL), 4-nitrophenyl chloroformate (NPC; 0.195 g) and TEA (0.3 mL) were added dropwise at 0 °C (molar ratio of DHP/NPC/TEA is 1/1/2/4). The reaction mixture was stirred for 4 h at 0 °C, and finally, the NPC-activated mPEG-b-(LA-co-DHP) was obtained by precipitation in cold diethyl ether and dried in vacuo.

**Conjugation of Dox onto mPEG-b-(LA-co-DHP) Copolymer via Carbamate Linkage.** The NPC-activated mPEG-b-(LA-co-DHP) (0.2 g) dissolved in DMF (10 mL) was reacted with Dox (50 mg) in the presence of TEA (57 µL) for 48 h at room temperature under nitrogen. The product mPEG-b-(LA-co-DHP-cbm-Dox) (abbreviated as “cbm-Dox” hereafter) was purified by column chromatography using a Sephadex G-25 column, and the solution was dialyzed against DMSO with a cellulose membrane (cutoff: 3500) for 3 days. After dialysis, cbm-Dox was lyophilized and stored. The Dox content was determined to be 15% by measuring the UV absorbance of a DMSO solution of the conjugate at 480 nm. A calibration curve was constructed using a function of incubation time.

**Conjugation of Hydrazine on mPEG-b-(LA-co-DHP/NPC) Copolymer.** Hydrazine monohydrate (150 µL) was slowly dropped into NPC-activated mPEG-b-(LA-co-DHP) copolymer (0.89 g) dissolved in methylene chloride (10 mL), molar ratio of activated hydroxyl groups/hydrazine: 1/10. The reaction was carried out for 2 h at room temperature. By precipitation in diethyl ether, the resulting hydrazine conjugated mPEG-b-(LA-co-DHP) copolymer was obtained.

**Conjugation of Dox onto mPEG-b-(LA-co-DHP) Copolymer via Hydrazine Linkage.** The hydrazine conjugated mPEG-b-(LA-co-DHP) copolymer (0.68 g) dissolved in dimethylformamide (10 mL) was further reacted with doxorubicin (168 mg) in the presence of TEA (0.2 mL) for 48 h at room temperature under nitrogen. The Dox conjugate mPEG-b-(LA-co-DHP-hz-Dox) (abbreviated as hz-Dox hereafter) was purified by column chromatography using a Sephadex G-25 column. The doxorubicin content was determined to be 18%.

**Conjugation of Folic Acid onto mPEG-b-(LA-co-DHP) Copolymer.** mPEG-b-(LA-co-DHP) copolymer (1.0 g) was first dissolved in 10 mL of anhydrous DMSO; then folic acid (400 mg, 0.9 mmol), DCC (185.7 mg, 0.9 mmol), and DMAP (110 mg, 0.9 mmol) were added under stirring. The reaction mixture was stirred for 24 h at room temperature. Dicyclohexylurea (DCU) formed was filtered out and the solution was dialyzed against DMSO with a cellulose membrane (cutoff: Mw = 3500) to remove unreacted FA. Finally the product mPEG-b-(LA-co-DHP/FA) was obtained by precipitation in cold diethyl ether and dried in vacuo.

The FA content was determined to be 20% by measuring the UV absorbance of a DMSO solution of the conjugate at 288 nm. A calibration curve was constructed using different concentrations of FA in DMSO.

**Physicochemical Characterization of Dox-Conjugates.** 1H NMR spectra were measured in CDCI3 or DMSO-d6 at room temperature (20 ± 1 °C) by an AV-300 NMR spectrometer from Bruker. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC with CHCl3 as eluent (flow rate: 1 mL/min) at 35 °C. The molecular weights were calibrated against polystyrene (PS) standards.

**Measurement of the Micellar Size.** Size distribution of the micelles was determined by dynamic light scattering (DLS) with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology, U.S.A.). The scattering angle was fixed at 90° and the measurement was carried out at 25 °C.

**TEM Observation.** The morphology of the micelles was measured by transmission electron microscopy (TEM) performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV. To prepare specimens for TEM, a drop of micelle solution (1 mg/mL) was deposited onto a copper grid with a carbon coating. The specimens were air-dried, sputter-coated with a layer of gold, and measured at room temperature.

**In Vitro Doxorubicin Release Experiment.** Freeze-dried micelle samples (cbm-Dox and hz-Dox, 20 mg each) with either P(LA-co-DHP-cbm-Dox) cores or P(LA-co-DHP-hz-Dox) cores were suspended in acetate buffer (pH 5.0, pH 6.0) and phosphate buffered saline (PBS, pH 7.4) solutions (5 mL), respectively, sealed in a dialysis bag (MWCO: 3.5 kDa) and incubated in the release medium (25 mL) at 37 °C under oscillation at 90 r/min. At selected time intervals, buffer solution outside the dialysis bag was removed for UV–vis analysis and replaced with fresh buffer solution. The released amount of Dox was determined from the absorbance at 488 nm with the help of a calibration curve of Dox in the same buffer. Then the accumulative weight and relative percentage of the released Dox were calculated as a function of incubation time.

**Cell Lines.** Two cell lines, including SKOV-3 (human ovarian cancer cell line) and A549 (human lung adenocarcinoma cell line), were chosen for cell tests. SKOV-3 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies), 0.03% L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO2 at 37 °C. A549 cells were kindly supplied by the Medical Department of Jilin University in China and were first cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma), and the culture medium was replaced once every day.

**In Vitro Cytotoxicity.** The cytotoxicity test was used to evaluate in vitro antitumor activity of the Dox-conjugates. The folic acid receptor positive SKOV-3 cells [FR(+)] and folic acid receptor negative A549 cells [FR(−)] were used for experiment. The cytotoxicity of the Dox-conjugates on tumor cells was measured by MTT assay using Dox·HCl solution as a control. Briefly, SKOV-3 (or A549) cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 104 cells/well and incubated in RPMI 1640 (DMEM for A549) for 24 h. The medium was then replaced by the conjugate micelles or free Dox at various Dox concentrations from 0.01 to 10 µg/mL. At the designated time intervals (48 and 72 h), 20 µL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plate was incubated for another 4 h at 37 °C. After that, the medium containing MTT was
removed and 150 µL of DMSO was added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

**Cellular Uptake.** Cellular uptake by SKOV-3 human ovarian cancer cells was examined using flow cytometry and confocal laser scanning microscope (CLSM), respectively. For flow cytometry studies, approximately 10^6 SKOV-3 cells were seeded in 6-well culture plates and grown overnight. The cells were then treated with free Dox and Dox-conjugated micelles (equivalent Dox concentration: 10 µg/mL) at 37 °C for 0.5 and 2 h, respectively. After incubation, drug solutions were removed and the cells were washed three times with 0.1 M PBS buffer (pH 7.4). A total of 1 mL of 0.25% trypsin (GIBCO) solution was added thereafter, and the cells were detached from cell culture by incubation at 37 °C for 1.0 min. A single cell suspension was prepared by filtration through a 300 mesh filter. Finally, the cells suspended in 200 µL of PBS were subjected to flow cytometry analysis using a Becton Dickinson FACSCalibur cytometer equipped with an argon laser (488 nm) and emission filter for 570 nm (Cytomics FC 500, Beckmann-Coulter). Data were evaluated with the program WinMDI version.

For CLSM studies, SKOV-3 cells were seeded in 12-well culture plates (a clean coverslip was put in each well) at a density of 5 × 10^4 cells/well and allowed to adhere for 24 h. mPEG-b-(LA-co-DHP/Dox) micelles or free Dox (Dox concentration: 10 µg/mL) in culture media were added. After incubation 2 or 24 h at 37 °C, the supernatant was carefully removed and the cells were washed three times with ice-cold PBS. Subsequently, the cells were fixed with 4% formaldehyde in each well for 10 min at room temperature and washed twice with ice-cold PBS again. Samples were examined by CLSM using a Zeiss LSM 510 (Zurich, Switzerland) with a confocal plane of 300 nm. Dox was excited at 479 nm with emissions at 587 nm.

**Drug-Induced Apoptosis Assessed by Flow Cytometry.** SKOV-3 cells were seeded in sterile six-well plates at a density of 5 × 10^4 cells per well. Dox and micelles (equivalent Dox concentration: 10 µg/mL) were added into each well. After 24 h incubation, cells were collected, centrifuged at 1000 rpm for 5 min and washed consecutively with cell medium and PBS. Then cell pellets were resuspended in 1× Annexin V binding buffer and Annexin V-FITC, and incubated on ice for 15 min. After incubation, the cell suspension was transferred to a FACS tube containing propidium iodide and incubated on ice for 4 min. Cell samples were kept on ice until flow cytometry analysis. The first 10000 events were acquired by CXP analysis software V2.1.

**Results and Discussion**

**Construction and Physicochemical Properties of Dox-Conjugated Micelles.** The use of polymeric micelles for cancer treatment was first reported in the early 1980s by Ringsdorf and co-workers. In recent years, polymeric micelles have been extensively exploited to improve conventional cancer therapy. For targeting therapy, the coassembling method has been proven to be a good choice, for it is easier and more inexpensive than preparing the micelles from a block copolymer, which contains both drug molecules and targeting moieties. In this paper, folic acid (FA) was chosen as the targeting moiety. Pendant hydroxyl carrying copolymer mPEG-b-(LA-co-DHP) was chosen as the carrier (Scheme 1A). Figure 1A gave its 1H NMR spectra, and the polymerization degree of each segment was calculated using the known molecular weight of PEG to be PEG113-b-(LA12-co-DHP8). The anticancer drug doxorubicin was chemically conjugated to the pendant hydroxyl groups via two different covalent bonds, an acid-labile hydrazone linkage and a more stable carbamate linkage, by reacting with the amino and keto groups in Dox, respectively, as shown in Scheme 1B, C. The Dox content was determined to be 15% and 18% in the...
polymer conjugate with carbamate linkage and hydrazone linkage by measuring the UV absorbance of a DMSO solution of the conjugates at 488 nm. Folic acid conjugate mPEG-b-(LA-co-DHP/FA) was similar to Dox-conjugates in block backbones, amphiphilic nature, and comparable block lengths. The only difference was that FA and the carrier polymers were linked through an ester bond between the carboxyl group of FA and the hydroxyl group of the copolymer backbone using DCC and DMAP as the coupling agents (Scheme 1D). The obtained PEG-b-(LA-co-DHP/FA) was referred hereinafter as P-FA for short. The folic acid content was measured by 1H NMR as shown in Figure 1. 1H NMR supported that the conjugation of Dox and folic acid to PEG-(LA-co-DHP) was a little larger than that estimated from 1H NMR. This may be due to the existence of the more pendant hydroxyl groups in mPEG-b-(LA-co-DHP). The molecular size distribution of each conjugate was extremely narrow.

Because of the amphiphilic nature of the Dox-conjugates (hz-Dox and cbm-Dox), they can self-assemble into micelles using a solvent replacement method (Scheme 1E). The hydrophobic P(LA-co-DHP/Dox) segment constitutes the core of the micelles, and the hydrophilic PEG segment forms the “stealth” shell of the micelles, which improves the stability and circulation half-life of these drug-delivering micelles. Dynamic light scattering and transmission electron microscopy observation showed that the micelles had a spherical shape and had an average diameter less than 100 nm for hz-Dox and cbm-Dox (Figure 3).

A multifunctional micellar drug delivery system, which contains both drug and FA targeting moieties, was constructed by coassembling cbm-Dox and P-FA (or hz-Dox and P-FA), which carry the drug molecules and targeting moieties, respectively. The copolymer carriers, hz-Dox, cbm-Dox, and P-FA, had similar amphiphilic nature, identical PEG length, and comparable hydrophobic block length. Therefore, they were expected to coassemble into mixed micelles in aqueous media, not to form a mixture of micelles of individual copolymers. After assembling, Dox was wrapped in the core part of the micelles and got well protected because of its hydrophobic nature. The FA resided in between core and corona of the micelles to effectively play a role in targeting moieties, which has been proven by our previous study. The mass ratio of the two copolymers, hz-Dox and P-FA (or cbm-Dox and P-FA), was 90 to 10. This ratio was based on the following consideration: the micelles should contain as much as possible drug molecules and as little as possible targeting moieties if the targeting capability is high enough.

In Vitro Doxorubicin Release. The ideal drug delivery system for cancer targeting should hold the drug during circulation but release the drug exclusively in the target. The Dox release from the two types of conjugate micelles was studied in acetate buffer (pH 5.0, pH 6.0) and phosphate buffered saline (PBS, pH 7.4) solutions, respectively. As shown in Figure 4, there was no dramatic initial burst release for both cbm-Dox and hz-Dox conjugates. This was because the Dox molecules were chemically combined to the polymer chains and their release from the micelles due to physical diffusion was eliminated. However, these two drug conjugates were both pH-sensitive. The lower the pH value, the faster the drug was released. Take cbm-Dox as an example, its micelles showed sustained release of Dox at pH 7.4, only 23% was released in 20 days. In the same time period, cbm-Dox micelles released 47% of Dox at pH 6.0 and 90% at pH 5.0. hz-Dox showed even more significant pH sensitivity. Over 20 days, its micelles released only 11% of Dox at pH 7.4, but 63% at pH 6.0. At pH
5.0, 90% release of Dox took less than 10 days. These results were ascribed to the pH sensitivities of chain degradation of the carrier polymers and of breakage of the cbm- and hz-linkages. In the present study, the carrier polymers had the same structure, PEG-b-P(LA-co-DHP). As reported in our previous study,26 the PEG-b-P(LA-co-DHP) copolymer degraded more rapidly at lower pH value. Therefore, both cbm-Dox and hz-Dox showed pH sensitivity. It was reported that amide bond is more stable, while hydrazone is more acid sensitive in the case of PHPMA-Dox conjugates.20,25 In the present study, both carbamate and hydrazone linkages were employed. On the one hand, the hz-Dox was more basic than the carbamate bond, due to the hydrazone group and the NH2 group on the 3’ position of Dox molecule, leading to slower degradation of PEG-b-P(LA-co-DHP) backbone and slower release of Dox from hz-Dox micelles at pH 7.4 (Figure 4B vs A); on the other hand, hz-linkage was more acid sensitive than the cbm-linkage, and therefore, the hz-Dox micelles showed more significant pH sensitivity (Figure 4B vs A).

**Cellular Uptake.** To demonstrate uptake and internalization of the conjugate micelles, SKOV-3 human breast cancer cells were chosen as target cells. Autofluorescence of doxorubicin allows us to follow the internalization and intracellular distribution of the tested conjugates by confocal laser scanning microscopy (CLSM), as shown in Figure 5. The first five pictures in Figure 5 showed the CLSM image of SKOV-3 cells after 2 h incubation with (A) cbm-Dox, (B) (P-FA + cbm-Dox), (C) hz-Dox, (D) (P-FA + hz-Dox), and (E) the pristine Dox at the same 10 µg/mL equivalent Dox concentration. It can be seen that there are limited numbers of cbm-Dox micelles in the cells (Figure 5A). For (P-FA + cbm-Dox) micelles (Figure 5B), the fluorescent intensity improved obviously, indicating more micelles were internal-
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Figure 5. Confocal laser scanning microscopy of SKOV-3 cells (5 × 10⁴ cells/mL) after incubation with (A) cbm-Dox, (B) (P-FA + cbm-Dox), (C) hz-Dox, (D) (P-FA + hz-Dox), (E) free Dox for 2 h at 37 °C and with (F) cbm-Dox, (G) (P-FA + cbm-Dox), and (H) free Dox for 24 h at 37 °C, washed three times with PBS, fixed in 4% formaldehyde, and analyzed by confocal microscope (Dox-equivalent concentration 10 µg/mL for all formulations).

Figure 6. (A) Mean fluorescence intensity on SKOV-3 cells after treatment with Dox-conjugated micelles and free Dox at different incubation time measured by flow cytometry: (A) cbm-Dox, (B) (P-FA + cbm-Dox), (C) hz-Dox, (D) (P-FA + hz-Dox), (E) free Dox for 0.5 and 2 h.

After SKOV-3 cells were incubated with various conjugate micelles and free Dox, flow cytometry analysis was performed to determine the mean fluorescence intensity in cells. Figure 6 gives the flow cytometry results after treatment with cbm-Dox, (P-FA + cbm-Dox), hz-Dox, (P-FA + hz-Dox), and free Dox for 0.5 and 2 h, respectively. As shown in Figure 6, although identical Dox concentration (10 µg/mL) was used for these formulations, the SKOV-3 cells incubated with FA-containing micelles displayed higher Dox uptake compared to their corresponding Dox conjugate micelles without P-FA. This result was in agreement with those of CLSM observations. It implies that FA is responsible for the enhanced uptake of the micelles.

Meanwhile, hz-Dox and its FA-containing micelles displayed higher Dox uptake compared to corresponding cbm-Dox micelles.

In Vitro Antitumor Activity. The A549 and SKOV-3 cell lines were employed to investigate the cytotoxicity of cbm-Dox, (P-FA + cbm-Dox), hz-Dox, and (P-FA + hz-Dox) micelles, with free Dox as a positive control and blank culture medium without drugs as a negative control. Figure 7 shows the cell viability after (A,C) 48 h and (B,D) 72 h culture with various conjugated micelles and free Dox at various Dox concentrations, respectively. It can be seen that cytotoxicities of the tested formulations were all concentration dependent, and the plot of cell viability versus logarithm of Dox concentration is nearly linear. For the four different drug formulations [cbm-Dox, (P-FA + cbm-Dox), hz-Dox, (P-FA + hz-Dox)], as shown in Figure 7A,B, the A549 cell viability level did not show an obvious difference over the drug concentration range of 0.01 to 10 µg/mL and free Dox showed the best cytotoxicity after incubation for 48 and 72 h. No preferred internalization for FA containing micelles was observed because A549 cells were folate receptor negative. No appreciable difference was observed between cbm-Dox and hz-Dox. Both of them displayed less cytotoxicity than free Dox. This was because free Dox molecules were internalized and transported to the nucleus more quickly than the conjugate micelles.

To demonstrate the contribution of FA-targeting to the cytotoxicity, folate receptor positive SKOV-3 cells were used for the MTT assay. As shown in Figure 7C,D, the plot of cell viability versus logarithm of Dox concentration is linear except the data point of free Dox at 1 µg/mL in Figure 7D. Different from the A549 cell, the SKOV-3 cell viability level exhibited difference between the test groups over the Dox concentration range of 0.01 to 10 µg/mL. The order was (P-FA + hz-Dox) < pristine Dox < hz-Dox < (P-FA + cbm-Dox) < cbm-Dox. Based on the linear relationship, the IC₅₀ of each drug sample can be calculated and the corresponding IC₅₀ was 0.27, 0.49, 1.95, 7.38, and 25.3 (estimated) µg/mL. In Figure 7D, this order changed to (P-FA + hz-Dox) < (P-FA + cbm-Dox) < hz-Dox < cbm-Dox ≈ pristine Dox. The
Figure 7. Cell viabilities of A549 cells (A,B) and SKOV-3 cells (C,D) after 48 (A,C) and 72 h (B,D) incubation with various drug conjugate micelles and free Dox at various equivalent Dox concentrations (mean ± SD and n = 4).

Figure 8. Apoptotic cell populations determined by flow cytometric analysis with Annexin V-FITC and propidium iodide (PI) staining after incubating SKOV-3 cells in RPMI1640 media, with (A) media alone; (B) cbm-Dox; (C) (P-FA + cbm-Dox); (D) hz-Dox; (E) (P-FA + hz-Dox); and (F) free Dox. The lower-left and upper-left quadrants in each panel indicate the populations of normal cells and necrotic cells, respectively. While the lower-right and upper-right quadrants in each panel indicate the populations of early and late apoptotic cells, respectively.
corresponding IC50 was 0.31, 0.48, 0.64, 1.26, and 2.33 µg/mL. Considering the fact that PEG-b-(LA-co-DHP) itself does not show appreciable cytotoxicity (data are not shown), the following conclusions can be drawn: (1) cytotoxicity of the drug-containing micelles is dependent on the linkage between carrier polymer and drug molecule and on the FA-moieties in the micelles; (2) hz-linkage is more efficient than cbm-linkage in causing cancer cell cytotoxicity; (3) P-FA + hz-Dox) micelles exhibited the highest cytotoxicity against folic acid receptor overexpressed cell line because both hz-linkage and FA-targeting were combined together in them. These results were in agreement with the above drug release and cell uptake data. The cytotoxicity of the Dox–containing micelles was the final consequence of the following steps: (1) internalization of the micelles, (2) Dox release from the micelles, (3) Dox escape from the endosome, and (4) Dox diffusion into the nucleus. The FA-moieties enhanced the targeting and endocytosis of the micelles. In the acidic environment of endosome, the hz-linkage was easy to break down and, thus, Dox was released from hz-Dox micelles and escaped from the endosome more efficiently. Because free Dox can diffuse into the nucleus quickly, the last two steps were not the determining steps. Therefore, the above dependences of cell cytotoxicity on linkage bonds and FA moieties were observed. In the above experiment, performance of pristine Dox was special. On the one hand, because it was internalized very effectively by cancer cells and it diffused into the nucleus quickly, as indicated in the cell uptake experiment, it exhibited quite good cell cytotoxicity in a shorter time scale, as shown in Figure 7C; on the other hand, because free Dox was consumed relatively rapidly, its cytotoxicity decreased with time and finally became less than the Dox-containing micelles (Figure 7D).

Drug-Induced Apoptosis. To investigate how the polymer conjugated-Dox impacts its ability to induce apoptosis in vitro, flow cytometry was conducted to evaluate the extent to which samples of drug conjugated micelles or free drug induce apoptosis in SKOV-3 cells. Cells were double stained for viability (negative for propidium iodide (PI)) and apoptosis (positive for Annexin V-FITC). Incubated with SKOV-3 cells at a concentration of 10 µg/mL Dox-equivalent for 24 h, the four micelles (cbm-Dox, (P-FA + cbm-Dox), hz-Dox, (P-FA + hz-Dox)) and free Dox resulted in 31.6, 58.6, 59.8, 64.3, and 45.9% early apoptotic cells (LR, Annexin+/PI-) and 65.2, 35.5, 37.4, 31.2, and 51.6% normal cells (LL, Annexin-/PI-) respectively (Figure 8). These results indicate that the biological function of Dox was retained following conjugation to the polymer. However, cbm-Dox micelles induce lower apoptosis than free Dox. Taking into account the CLSM and in vitro drug release result, this can be ascribed to the comparably slower internalization and slower drug liberation from the carrier. As compared to free Dox, (P-FA + cbm-Dox), hz-Dox, and (P-FA + hz-Dox) micelles showed enhanced apoptosis, which is likely due to the enhanced intracellular uptake associated with FA-receptor-mediated endocytosis and the accelerated release of the drug molecules from micelles with acid-labile hydrazone linkage by sensing the acidic environment of the endosomal compartments.

Conclusions

Two Dox-conjugates (i.e., hz-Dox and cbm-Dox) were synthesized from biodegradable block copolymer mPEG-b-P(LA-co-DHP). A higher loading of doxorubicin could be achieved by using pendant hydroxyl-carrying copolymer instead of traditional terminal functional copolymer. Multifunctional micelles consisting of Dox-containing copolymer and folic acid-containing copolymer P-FA were successfully prepared by coassembling the two component copolymers. The drug molecule was wrapped in the core part of the micelles and the hydrophilic PEG segments constituted the corona of the micelles, leading to effective solubilization of the Dox, while FA resided in between core and corona of the micelles to effectively play a role of targeting moieties. In vitro drug release studies showed that both Dox-conjugates exhibited pH-dependent release behavior, and the micelles with hydrazone linkage showed more pH-sensitivity compared to that with carbamate linkage. The in vitro cell uptake experiment by CLSM and flow cytometry showed preferential internalization of FA-containing micelles over that without FA, in agreement with higher cytotoxicity against SKOV-3 cells tested by MTT. Flow cytometric analysis was conducted to reveal the enhanced cell apoptosis caused by the FA-containing micelles. These results suggested that these micelles could be a promising drug delivery system.

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References and Notes


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