Self-Assembled Micelles Prepared From Amphiphilic Dextran-Polylactide Diblock Copolymers for Antitumor Drug Release

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ABSTRACT

Fully biobased diblock copolymers were synthesized by reducing amination from dextran (DEX) and amino terminated polylactide (PLA). The resulted copolymers were characterized by using nuclear magnetic resonance, Fourier-transform infrared spectroscopy, gel permeation chromatography, and Kaiser test. Self-assembled copolymer micelles were characterized by transmission electron microscopy and dynamic light scattering. The micelles are spherical in shape, and the particle size increases with increase of both DEX and PLA block length. The critical micellar concentration (CMC) of copolymers was determined by fluorescence spectrometry. Data showed that the CMC decreases with increase of PLA block length. Drug loading and drug release properties of DEX-PLA micelles were evaluated using curcumin as model drug. An increase in drug loading content is obtained with increase of the PLA block length. In vitro drug release from DEX-PLA micelles was performed at 37°C in phosphate buffered saline. Biphasic release was observed with an initial burst followed by slower release. The drug release rate from DEX-PLA micelles was mainly related to copolymer composition, and reached 84.17% at 120 h for the copolymer with the highest hydrophilic/hydrophobic ratio (DEX_{10K}-PLA_{20}). DEX-PLA micelles present good cytocompatibility as evidenced by MTT assay, and drug loaded micelles exhibit significant cytotoxicity to HeLa cells. The half maximum inhibitory concentration (IC_{50}) of drug loaded DEX_{10K}-PLA_{20} micelles was 9.12 μg/mL, which was lower than that of free curcumin (11.9 μg/mL). Therefore, self-assembled micelles prepared from fully biobased DEX-PLA copolymers could be a promising nanocarrier for hydrophobic antitumor drugs.

Keywords : Dextran; polylactide; self-assemby; amphiphilic block copolymer; micelle; drug release

1. Introduction

Amphiphilic block copolymers can spontaneously self-assemble in water to form nano-size micelles composed of a hydrophilic shell and a lipophilic core.[1] The hydrophilic segments form the outer layer of micelles, which allows to maintain the structural stability, and prolong the blood circulation time.[2, 3] On the other hand, the hydrophobic segments form a solid core of micelles which serves as reservoir for drug loading through physical or electrostatic interactions.[4,5] In the past decades, polymeric micelles have attracted more and more attention as nanocarrier in the field of controlled drug release due to their good stability, high drug loading, improved bioavailability of drugs, and reduced side effects.[6-10]

Poly(lactic acid) or plylactide (PLA) is a biobased polymer with outstanding biodegradability and biocompatibility. PLA has been widely investigated for applications in food packaging, mulch film in agriculture, medical implants, drug carrier, etc.[11-15] When PLA is used as a drug carrier, drug release is generally controlled by drug diffusion and polymer degradation. PLA is also used as a hydrophobic block to build amphiphilic block copolymers with many hydrophilic polymers such as poly(ethylene glycol) (PEG),[16-18] poly(2-ethyl-2-oxazoline) (PEtOx),[19] poly(N-isopropylacrylamide) (PNIPAAm),[20] etc. The resulting copolymers, and in particular PLA-PEG, present great interest as drug carrier in the form of self-assembled micelles. Nevertheless, the widespread use of PEG in drug delivery systems is limited by its immunogenicity, particularly the induction of anti-PEG antibodies which accelerate the clearance of PEGylated systems.[21]

Polysaccharides such as cellulose, chitosan, alginate and dextran, are biopolymers derived from renewable resources, including plants, seaweeds, microorganisms and crustacean wastes. They are largely used as biomaterials due to their excellent biocompatibility, non-toxicity; hydrophilicity and biodegradability.[22-24]

Polysaccharides are also used as a hydrophilic block to build amphiphilic block copolymers with hydrophobic polymers. Recently, Lu et al. synthesized a series of amphiphilic block copolymers by reductive amination between the hemiacetal reducing end of hydroxypropyl methyl cellulose (HPMC) and the amine terminal group of Jeffamine,[25] amino terminated PLA,[26] and amino terminated poly(ε-caprolactone) (PCL).[27] The resulted copolymers are able to self-assemble into spherical micelles in aqueous medium, showing great potential as nanocarrier of hydrophobic drugs.

Dextran is a bacterial polysaccharide composed of a main chain of α-1,6-linked glucose and α-1,3 branches with a large number of hydroxyl groups. Dextran has been used to conceive drug carriers in the form of conjugates, nanoparticles, and hydrogels.[28] Both PLA and DEX are biobased materials with good biocompatibility. Amphiphilic block polymers have been synthesized from PLA and DEX for the preparation of drug release systems. Verma et al. synthesized DEX-PLA block copolymer in three steps: (1) reductive amination between dextran and N-Boc-ethylenediamine, (2) removal of the Boc group, and (3) conjugation of amino terminated dextran and carboxy-terminated PLA in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS).[29] On the other hand, in the work of Wang et al.,[30] DEX was end modified by reaction with ethylenediamine, and PLA was modified with acryloyl chloride. Finally, DEX-PLA amphiphilic diblock copolymer was synthesized by coupling of amino-terminated dextran and acryloyl-terminated PLLA via Michael addition reaction. In both cases, excess DEX was used, and the reaction process was complex and tedious.

Curcumin is a natural polyphenol derived from curcuma longa. It exhibits a wide spectrum of pharmacological activities including anti-oxidant, anti-inflammatory, anti-microbial, and anti-amyloid properties. Importantly, curcumin presents an effective therapeutic effect on cancers such as breast cancer, lung cancer and blood tumors due to its diverse range of molecular targets.[31,32] Nevertheless, the therapeutic efficacy of curcumin is limited by its extremely low water solubility, rapid metabolism, and low bioavailability. Nanotechnology based drug delivery systems have been developed to enhance the therapeutic efficacy of curcumin, including nanoparticles, liposomes and

phospholipids. [33]

In this work, DEX-PLA copolymers with different DEX and PLA chain lengths were synthesized by reductive amination between the amino group of NH_2-PLA and the hemiacetal end group of DEX according to the synthesis method reported by Lu et al.[26] The size and morphology, zeta potential and critical micelle concentration of self-assembled micelles were determined. The drug loading and release capacity of the micelles was studied using curcumin as a drug model. Finally the cytocompatibility of blank micelles and the antitumor activity of drug loaded micelles were studied to evaluate their potential as drug carrier in cancer treatment.

2. Materials and methods

2.1. Materials

Sodium triacetoxyborohydride ($NaBH(OAc)_3$), DEX with molecular weight of 5000 and 10000, trifluoroacetic acid (TFA), EDC, NHS and curcumin were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). L-lactide and DL-lactide were purchased from Daigang Biotechnology Co., Ltd. (Shandong, China) and purified by recrystallization in ethyl acetate. Tert-butyl-N-(3-hydroxypropyl) carbamate (3-(Boc-amino)-1-propanol) was obtained from Sahn Chemical Technology Co. Ltd. (Shanghai, China). Tin (II) 2-ethyl hexanoate ($Sn(Oct)_2$) purchased from Alfa Aesar Co., Ltd. (Shanghai, China). Toluene and dichloromethane were obtained from Sigma-Aldrich, dried over by calcium chloride ($CaCl_2$) and distilled prior to use. Mouse fibroblasts L-929 cells and HeLa cells were provided by Qilu Stem Cell Engineering Co., Ltd. (Shandong, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and other reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All other reagents were of analytic grade from Sigma-Aldrich and used without further purification.

2.2. Synthesis of amino-terminated PLA

Amino-terminated PLA was synthesized by ring-opening polymerization of lactide using 3-(Boc-amino)-1-propanol as initiator and $Sn(Oct)_2$ as catalyst, followed

by removal of Boc protective group under the action of TFA.[26] Typically, L-lactide (2.592 g, 18 mmol), D, L-lactide (0.288 g, 2 mmol), 3-(Boc-amino)-1-propanol (0.35 g, 2 mmol), and $Sn(Oct)_2$ (0.0932 g, 0.23 mmol) were added to a three-mouth flask. 20 mL anhydrous toluene was added under N_2 atmosphere, and the solution was degassed through five freeze-pump-thawing cycles. Then, the reaction proceeded at 80 °C for 24 h. BOC-NH-PLA was then obtained by precipitation with ethanol, followed by centrifugation and vacuum drying.

In a flask under N_2 purge, 1.0 g BOC-NH-PLA was dissolved in 5 mL freshly distilled dichloromethane. 5 mL anhydrous TFA was then added, and the reaction proceeded for 1 h at room temperature. The crude product was obtained after removal of all the liquid. It was then dissolved in dichloromethane, successively washed with 5% $NaHCO_3$ aqueous solution and distilled water, dried with $MgSO_4$. Finally the solution was filtrated, precipitated in cold ethanol, and vacuum dried up to constant weight to yield NH_2-PLA.

2.3. Synthesis of DEX-PLA block copolymer

Six DEX-PLA block copolymers were synthesized by reduction amination from dextran with molar mass of 5000 and 10000 and PLA with degree of polymerization (DP) of 20, 30 and 40, respectively.[26, 34] Typically, under N_2 atmosphere, DEX_{10k} (0.5 g, 0.05 mmol), NH_2-PLA (0.3 g, 0.05 mmol) and $NaBH(OAc)_3$ (0.042 g, 0.2 mmol) were dissolved in 20 mL anhydrous DMSO. The reaction proceeded at 35 ℃ under N_2 atmosphere for 72 h. The solution was then dialyzed against distilled water for 72 h, and freeze-dried to obtain the final product.

2.4. Characterization

A Bruker AVANCE 500 spectrometer with an operating frequency of 500 MHz was used for nuclear magnetic resonance (NMR) analysis with tetramethylsilane as internal reference and $CDCl_3$, DMSO or D_2O as solvent.

Gel permeation chromatography (GPC) was performed on a Shimazu instrument equipped with a Waters 410 refractive index detector. Polystyrene standards were used for calibration, and tetrahydrofuran (THF) as the mobile phase. Samples at a

concentration of 5 mg/mL were filtered through a PTFE filter of 0.22 μm before analysis at a flow rate of 1.0 mL/min.

Fourier transform infrared spectroscopy (FT-IR) was realized on TENSOR 27 FT-IR spectrometer (Bruker Optics, Germany). Samples were prepared using potassium bromide (KBr) pellet method. 32 scans were made for each measurement in the wavenumber range of 400 - 4000 cm^{-1}.

Kaiser test was performed to detect the presence of primary amine from color changes,[35-37] using three color development agents, i.e. ethanol solution containing 6% ninhydrin (reagent A), ethanol solution containing 80% phenol (reagent B) and pyridine solution containing 2% 0.001M KCN (reagent C). A small amount of polymer was added into a test tube, followed by addition of 2-3 drops of the above reagents A, B and C. The mixture was heated at 110 ℃ for 5 min. The color change of the solution in the test tube was then observed. The experiment was repeated three times.

The CMC of copolymers was determined by fluorescence spectroscopy using pyrene as fluorescent probe.[38,39] A series of copolymer solutions were prepared with concentrations varying from 1.0×10^{-3} to 5.0 mg/mL, whereas the concentration of pyrene was fixed at 2.0×10^{-6} M. The excitation spectra of the above solutions were recorded on Perkin Elmer LS55 fluorescence spectrometer at an excitation wavenumber of 390 nm. The CMC value was estimated from the intersection of regression lines of intensity ratio at 339.2 and 336 nm *versus* the logarithm of concentration plots.

The hydrodynamic size and zeta potential of copolymer micelles were determined by using Nano-ZS90 nanosizer (Malvern, Worcestershire, UK). Dynamic light scattering (DLS) was performed at 25 °C at a scattering angle of 90°. Copolymer solutions at 1.0 mg/mL were filtered through a 0.45 μm cellulose acetate membrane before measurements.

The morphology of micelles was observed by TEM on a JEM-1200EX microscope with an acceleration voltage of 80 kV. A copper mesh was immersed in 1.0 mg/mL micelle solution for 1 min and then removed. A small volume of 2.0% phosphotungstic acid solution was dropped at the copper mesh for staining, and air dried at room temperature prior to measurement.

2.5. In vitro drug release

Drug-loaded micelles were prepared in two steps. 20 mg copolymer was first dissolved in 20 mL distilled water to yield blank micelles. 2 mg curcumin dissolved in 50 μL acetone was then added to the micellar solution under vigorous stirring. Drug-loaded micelles were obtained after evaporation of the solvent overnight. Unloaded drug was eliminated by filtration using 0.8 μm cellulose acetate filter. Finally the solution was lyophilized and stored at 4 °C.

Lyophilized micelles were dispersed in pH 7.4 phosphate buffered saline (PBS) to prepare a micellar solution at 2.0 mg/mL, and 3 mL micellar solution was placed in a dialysis bag (molecular weight cut-off 3500). The dialysis bag was placed in 30 mL PBS containing 0.5% Tween 80. Drug release studies were performed under constant shaking at 37 ℃, and the release medium was changed regularly. 35 μL solution was taken at predetermined time points, and replaced with fresh PBS of the same volume.

The drug loading concentration was determined by HPLC. The mobile phase was a mixture of acetonitrile and water 50/50 (v/v). The column temperature was 30 ℃, and the detection wavelength was 425 nm. 10 μL of samples were injected for each analysis at a flow rate of 1.0 mg/min. A calibration curve was established from a series of curcumin solutions at concentrations from 0.001 to 0.08 mg/mL. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated from the following formulas :

$$\text{DLC}\ (\%) = \frac{\text{weight of loaded drug}}{\text{weight of polymeric micelles}} \times 100 \quad (1)$$

$$\text{DLE}\ (\%) = \frac{\text{weight of loaded drug}}{\text{therotical drug load}} \times 100 \quad (2)$$

2.6. Assay of cytotoxicity of DEX-PLA micelles

Mouse fibroblasts (L-929) were co-cultured with micellar samples to evaluate the cytotoxicity in vitro. After sterilization by exposure to 60 W UV light for 24 h, micellar samples were dispersed in DMEM containing 15% FBS, and then diluted to obtain micellar solutions with final concentrations of 5, 2, 1, 0.5, 0.25 mg/mL, respectively. L929 cells were suspended in DMEM containing 10% FBS, 100 U/mL penicillin and

100 μg/mL streptomycin at a concentration of 1×10^4 cells/mL. 100 μL cell suspension was added in each well of a 96-well plate. After that, the medium was incubated for 24 h in an environment of 5% CO_2 and 95 % humidity to allow cell adhesion. 100 μL micellar solution was then added to replace the original medium. 100 μL phenol solution at 0.5 mg/mL was used as positive control, and 100 μL fresh medium was used as negative control. After 24, 48 and 72 h, 20 μL MTT solution was added, and cell culture proceeded for 6 h. Finally, all the medium was removed, followed by addition of 150 μL DMSO. The absorbance at 490 nm was measured after gently shaking the solution for 10 min. Cell survival rate or relative activity was calculated as follows:

$$\text{Relative activity (\%)} = \frac{\text{OD}_{\text{text sample}}}{\text{OD}_{\text{negative sample}}} \times 100 \quad (3)$$

2.7. In vitro antitumor activity

HeLa cells were used to evaluate the antitumor activity of free curcumin and curcumin-loaded micelles by MTT assay. Blank micelles and drug-loaded micelles were dispersed in the medium. Free curcumin dissolved in DMSO was diluted to desired concentrations using the medium. The curcumin concentrations of free drugs and drug loaded micelles ranged from 0.25 to 25 μg/mL. Blank cells served as a negative control. MTT assay was performed using the same protocol described above. The anticancer activity of drug-loaded micelles was evaluated by cell inhibition rate after 48 h. Cell inhibition rate was calculated as follows:

$$\text{Cell inhibition rate (\%)} = \frac{\text{OD}_{\text{negative sample}} - \text{OD}_{\text{text sample}}}{\text{OD}_{\text{negative sample}}} \times 100 \quad (4)$$

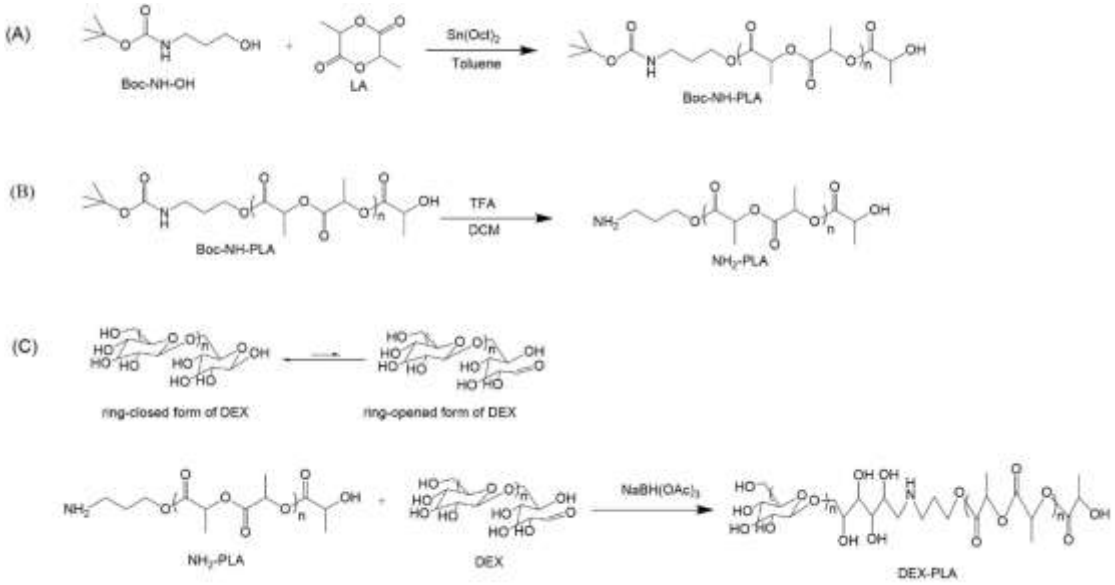
2.8. Statistical analysis

Statistical analysis was carried out by using the sample t-test for in vitro drug release, cytotoxicity of DEX-PLA micelles, and in vitro antitumor activity experiments. All the experiments were performed in triplicates. The data were shown as mean ± standard deviation. Values with $p > 0.05$ were considered not significant, $^*p < 0.05$ statistically significant, $^{**}p < 0.01$ very significant.

3. Results and discussion

3.1. Synthesis of amphiphilic block copolymers

The synthesis of DEX-PLA copolymers was realized in 3 steps according to the method reported by Lu et al.,[26] as illustrated in **Scheme 1**. At first, 3-(Boc-amino)-1-propanol was used to initiate the ROP of lactides, yielding Boc-NH-PLA (Scheme 1A). A mixture of L- and DL-lactides with L/DL ratio of 90/10 was used to ensure the solubility of the resulting PLA in DMSO as PLLA with degree of polymerization (DP) above 20 is hardly soluble in DMSO. Then, the protective Boc group at the chain end was removed by treatment using TFA to obtain amino terminated PLA (Scheme 1B).



Scheme 1. Synthesis route of DEX-PLA block copolymers by reductive amination in three steps: (A) ring opening polymerization; (B) deprotection to remove the protective Boc group; (C) reductive amination.

It is known that there exists an equilibrium between ring-closed and ring-opened forms in the polysaccharide chain end which is called hemiacetal reducing end, creating an aldehyde group (Scheme 1C). Thus the hemiacetal end group of the hydrophilic DEX block can be conjugated to the amino group of the hydrophobic PLA block in the presence of NaBH $(OAc)_3$, yielding amphiphilic DEX-PLA block copolymer (Scheme 1C).

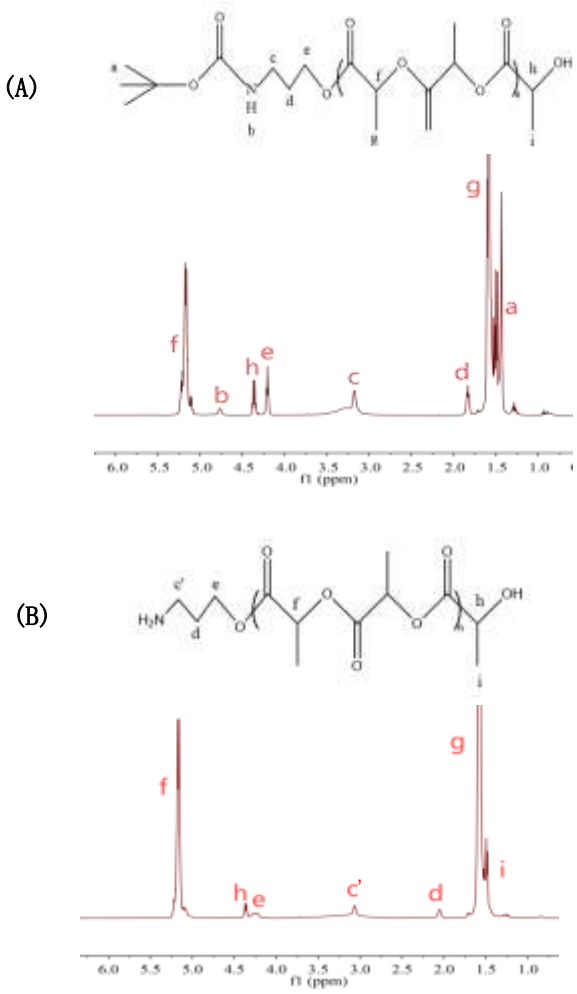


Figure 1. ^{1}H NMR spectra of Boc-NH-PLA (A) and NH_2-PLA (B) in $CDCl_3$.

Three PLA polymers, namely PLA_{20}, PLA_{30} and PLA_{40} were synthesized in this work using lactides/3-(Boc-amino)-1-propanol molar ratio of 10/1, 15/1, and 20/1, respectively. In these abbreviations, the subscript refers to the theoretical DP of PLA. **Figure 1A** shows the ^{1}H NMR spectrum of Boc-NH-PLA. The signals at 1.60 (g) and 5.19 (f) belong to the methyl and methine groups in the PLA main chain, and the signals at 1.50 (i) and 4.38 (h) to the methyl group and methine groups of the hydroxyl end of PLA, respectively. The characteristic methyl signal of Boc group is detected at 1.45 (a), the NH connected to the Boc group is detected at 4.75 (b), and the three methylene groups of 3-(Boc-amino)-1-propanol are detected at 3.20 (c), 1.85 (d) and 4.23 (e), respectively.

The DP of Boc-NH-PLA was determined from the integral ratio of signal f and

signal h. As shown in **Table 1**, the DP obtained from NMR are close to the theoretical values. The number average molecular weight (Mn) of the three Boc-NH-PLA samples ranges between 1820 and 2900, as calculated from the DP of PLA and the molecular weight of the protective group.

GPC was used to determine the molecular weight and dispersity (Đ) of the polymers, and the results are shown in **Table 1**. The Mn values obtained from GPC range from 1884 to 3160, which are slightly larger than those obtained from NMR. The dispersity of the polymers is between 1.22 and 1.37, in agreement with narrow molecular weight distribution of the polymers.

Table 1. Characterization of Boc-NH-PLA polymers

Sample	DP_{NMR}	Mn_{NMR}	Mn_{GPC}	PD_{GPC}
Boc-NH-PLA_{20}	23	1820	1880	1.23
Boc-NH-PLA_{30}	28	2180	2230	1.37
Boc-NH-PLA_{40}	38	2900	3160	1.22

Amino terminated PLA was obtained by removing the Boc group from BoC-NH-PLA under the action of TFA, as shown in **Figure 1B**. The characteristic methyl signal of the Boc group disappears, and the methylene signal at 3.20 (c) shifts to 3.10 (c') due to the conversion of amide group to amino group. These findings indicate that the deprotection was completed and amino PLA was successfully synthesized.

Finally, DEX-PLA block copolymer was obtained by reducing amination of DEX and amino PLA under the action of $NaBH(OAc)_3$ as reducing agent. The NMR spectra of DEX-PLA polymer in DMSO-d_6 and D_2O are shown in **Figure 2**. The spectrum in DMSO-d_6 shows all the characteristic signals of DEX and PLA, i.e. signals at 1.46 (h) and 5.19 (g) corresponding to the methine and methyl groups of main chain PLA, signals at 1.28 (j) and 4.22 (i) corresponding to the methine and methyl groups of the chain end of PLA, and signals corresponding to the various protons on the glucan ring of DEX (a, b, c, d, e, and f).[40] Signals belonging to different OH protons are also

observed (k, l, m). In contrast, the signals of PLA are not detected on the NMR spectrum of DEX-PLA in D_2O because amphiphilic DEX-PLA block copolymer tends to form micelles in aqueous medium and, in consequence, PLA signals are invisible in NMR as PLA blocks are embedded in the core of micelles. It is also noticed that the signals of OH protons disappear on the NMR spectrum of DEX-PLA in D_2O. On the other hand, the signals of protons on the glucan ring of DEX shifted downfield in D_2O as compared to those in DMSO-d_6. These results indicate that DEX-PLA polymer has been successfully synthesized by reducing amination.

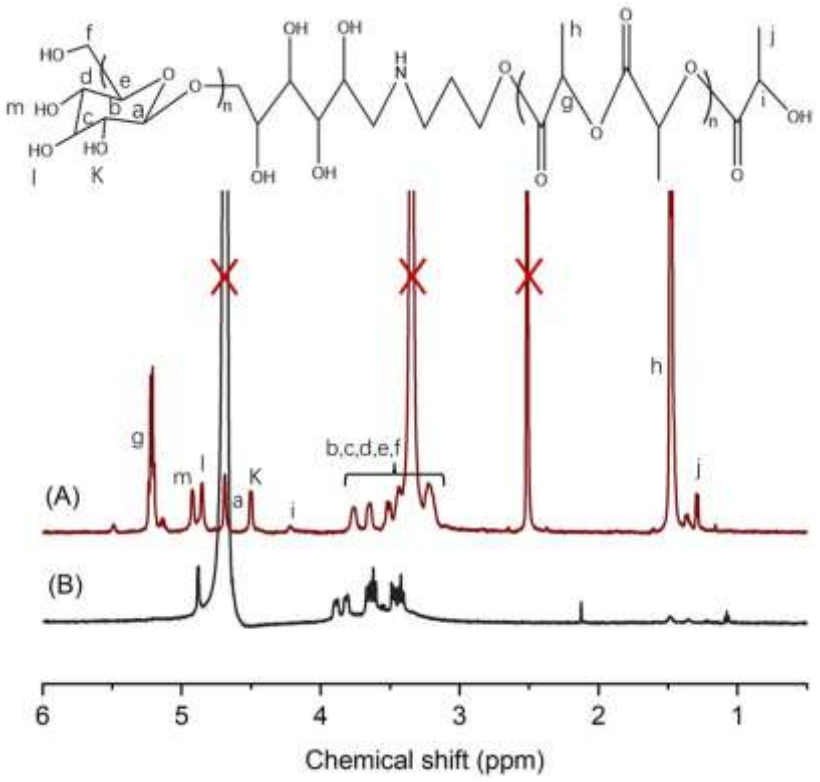


Figure 2. NMR spectra of DEX-PLA in DMSO-d_6 (A) and D_2O (B). The cross symbols indicate peaks of water (3.3 ppm) in Fig. 2A, and the isotopomer with one less deuterium than the perdeuterated molecule, *i.e.* DMSO-d_5 (2.5 ppm) in Fig. 2A, and HDO (4.8 ppm) in Fig. 2B.

FT-IR was also used to analyze the chemical structure of DEX-PLA and its precursors. **Figure 3** shows the FT-IR spectra of 3-(Boc-amino)-1-propanol, Boc-NH-PLA, NH_2-PLA, DEX, and DEX-PLA. 3-(Boc-amino)-1-propanol has a large band at

3340 cm^{-1} attributed to hydroxyl groups, and several bands around 3000 cm^{-1} belonging to various methyl and methylene groups. The bands at 1690 and 1528 cm^{-1} are attributed to the carbonyl NH bonds. The bands at 1252 and 1278 cm^{-1} are attributed to the C=O and C-H bonds. On the spectrum of Boc-NH-PLA, an intense band is detected at 1756 cm^{-1}, which is assigned to the carbonyl bond of PLA ester groups. After removal of the Boc group, the intensity of several bands near 3000 cm^{-1} is weakened. The spectrum of DEX presents three major bands at 3450, 2920, and 1638 cm^{-1}, which are assigned to hydroxyl groups, C-H bonds, and carbonyl group of the sugar hydrate, respectively. On the spectrum of DEX-PLA, the characteristic bands of both DEX and PLA are observed, especially the hydroxyl band and carboxyl band of DEX at 3450 and 1638 cm^{-1}, and the carbonyl band of PLA at 1756 cm^{-1}, which further proved the successful synthesis of DEX-PLA polymer.

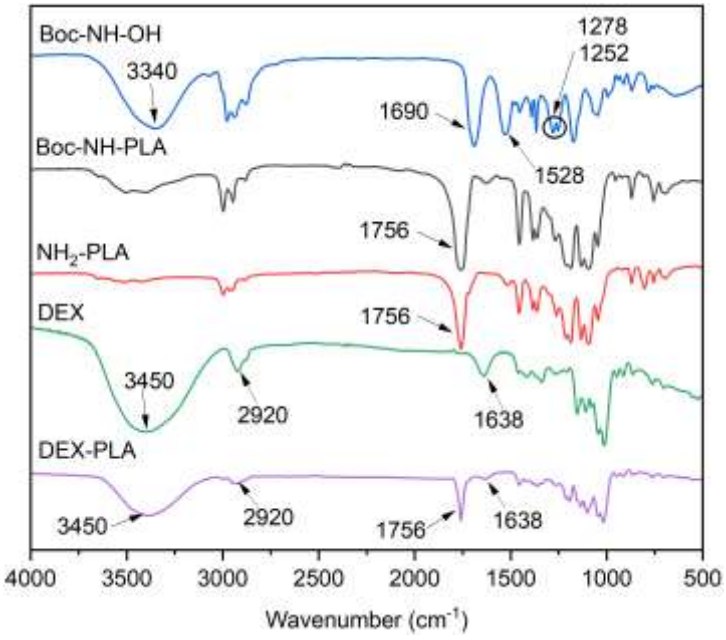


Figure 3 FT-IR spectra of 3-(Boc-amino)-1-propanol, Boc-NH-PLA, NH_2-PLA, DEX, and DEX-PLA.

Kaiser test is commonly used to verify the presence of primary amines since the action of ninhydrin with an amino group will produce a unique bluish purple color. As the main component of the kit, ninhydrin is first dehydrated and condensates with an

amino group (ammonia or primary amine) to obtain imines. Then the imines are isomerized into another form of imines after decarboxylation, and hydrolyzed to yield a primary amine and an aldehyde. The primary amine is further condensed with ninhydrin to produce a dimerized imine derivative that appears bluish purple. As shown in **Figure 4**, the color of Boc-NH-PLA and DEX-PLA is yellow, which is the same as that of the control, whereas the color of NH_2-PLA is blue-purple. This finding indicates that the Boc group has been successfully removed and the final polymer has been successfully synthesized, in agreement with NMR data in **Figure 1**.

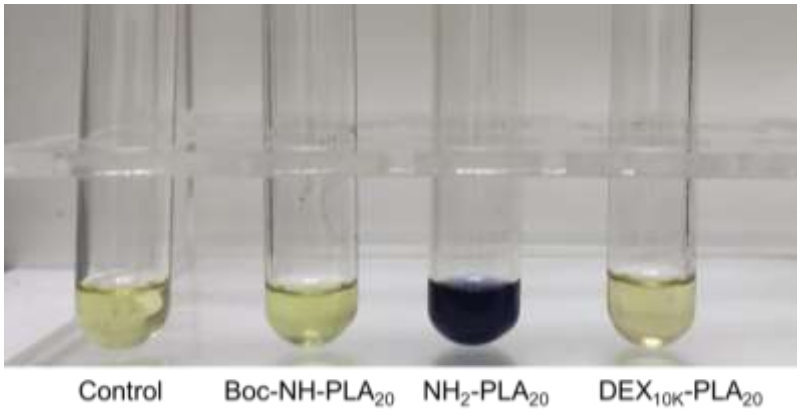


Figure 4. Kaiser test of Boc-NH-PLA_{20}, NH_2-PLA_{20} and DEX_{5K}-PLA_{20}

3.2. Self-assembly of copolymers

DEX-PLA copolymer micelles were prepared by direct dissolution method. The copolymer was dissolved in ultra-pure water. The solution was stirred overnight to achieve self-assembly of the copolymer. The self-assembly properties of copolymers were studied by using fluorescence spectroscopy DLS, and TEM.

The CMC is an important parameter of micelles as it reflects the micelle stability.[41-43] The lower the CMC, the better the micelle stability. Fluorescence spectroscopy was used to determine the CMC of block copolymers using pyrene as a fluorescence probe. As shown in **Figure 5A**, with the increase of polymer concentration, the fluorescence intensity of pyrene molecules at the same concentration continuously increases, and its maximum excitation wavelength red-shifts from 336.0 nm to 337.6 nm. The finding implies that pyrene molecules are transferred from aqueous

environment to hydrophobic environment, in agreement with formation of micelles with a hydrophobic core. The fluorescence intensity ratio at 337.6 and 336.0 nm was plotted with the logarithm of copolymer concentration as shown in **Figure 5B**. The CMC was obtained from the intersection point of the regression line.

Table 2 summarizes the CMC values of the six DEX-PLA copolymers. The CMC decreases from 0.0629 mg/mL for DEX_{5K}-PLA_{20} to 0.0092 mg/mL for DEX_{5K}-PLA_{40}, and from 0.0597 mg/mL for DEX_{10K}-PLA_{20} to 0.0084 mg/mL for DEX_{10K}-PLA_{40}. Therefore, the longer the hydrophobic block length, the lower the CMC of copolymers. The DEX block seems to have little influence on the CMC.[44,45]

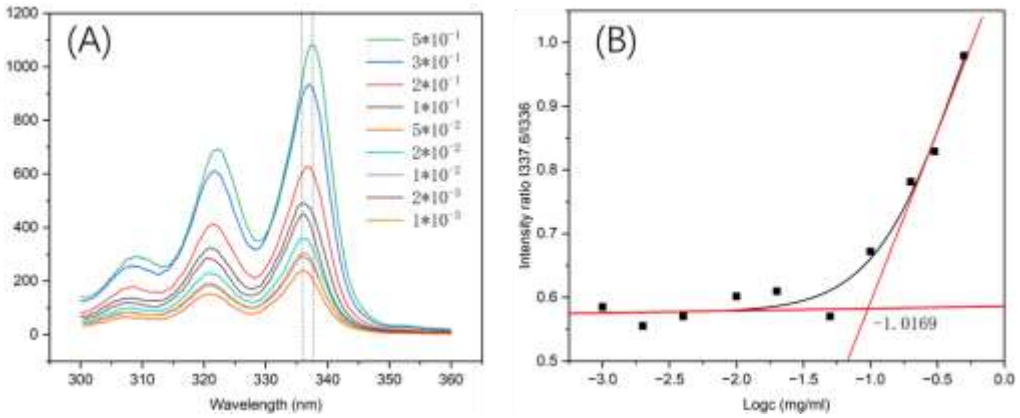


Figure 5. Fluorescence excitation spectra of DEX_{5K}-PLA_{20} solutions with concentrations varying from 1.0×10^{-3} to 5.0 mg/mL (A), and fluorescence intensity ratio at 337.6 and 336.0 nm ($I_{337.6}/I_{336}$) plotted *versus* the logarithm of copolymer concentration (logC) (B).

The particle size and polydispersity index (PDI) of micelles were determined by DLS (**Figure 6A**), and the results are summarized in **Table 2**. The micelle size ranges from 208.5 nm of DEX_{5K}-PLA_{20} to 241.9 nm of DEX_{5K}-PLA_{40}, and from 243.5 nm of DEX_{10K}-PLA_{20} to 267.5 nm of DEX_{10K}-PLA_{40}. The PDI ranges from 0.104 to 0.174, suggesting a narrow micelle size distribution. It appears that the micelle size increases with increase of both the hydrophilic and hydrophobic block length.[46,47] The morphology of micelles was observed by TEM. As shown in **Figure 6B**, the micelles are spherical in shape and relatively uniform in size, ranging from 20 to 50 nm. The

micelle size obtained from TEM is much smaller than that obtained from DLS because of the shrinkage of micelles during air drying before observation by TEM.[48,49] The zeta potential of the blank micelle was also determined. It ranges from -6.92 for DEX_{10K}-PLA_{40} to -4.67 for DEX_{5K}-PLA_{20}, which is close to zero as the copolymers have no positive or negative charges.

Table 2. Size, polydispersity index (PDI), zeta potential and critical micelle concentration (CMC) date of DEX-PLA block copolymers.

Sample	Blank micelles			
	Size (nm)	PDI	Zeta (mV)	CMC (mg/mL)
DEX_{5K}-PLA_{20}	208.5±2.9	0.146	-4.67±0.32	0.096
DEX_{5K}-PLA_{30}	222.9±6.9	0.109	-5.09±0.25	0.0629
DEX_{5K}-PLA_{40}	241.9±8.8	0.121	-5.86±0.21	0.0092
DEX_{10K}-PLA_{20}	243.5±0.6	0.104	-5.03±0.15	0.0984
DEX_{10K}-PLA_{30}	257.8±4.3	0.125	-5.53±0.13	0.0696
DEX_{10K}-PLA_{40}	265.3±9.9	0.135	-6.92±0.2	0.0103

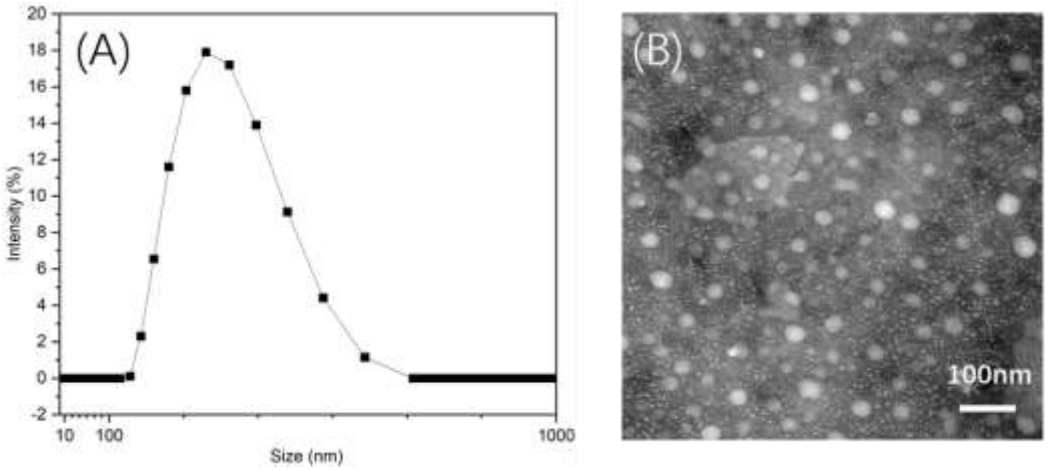


Figure 6. DLS pattern (A) and TEM image (B) of DEX_{10K}-PLA_{40} micelles.

3.3. Drug loading and in vitro drug release

Curcumin is a natural polyphenol derived from curcuma longa. It exhibits a wide

417 spectrum of pharmacological activities including anti-oxidant, anti-inflammatory, anti-
418 microbial, anti-amyloid and anti-tumor properties, and is used for treating inflammation,
419 cystic fibrosis, Alzheimer's and malarial diseases, and cancer.[50] However, the
420 therapeutic efficacy of curcumin is limited by its low water solubility of *c.a.* 11 ng mL^{-1}
421 .[51] In this work, curcumin was used as a model drug to evaluate the drug loading and
422 release properties of DEX-PLA micelles.
423
424 Table 3. Size, polydispersity index (PDI), zeta potential, DLC and DLE data of drug loaded
425 micelles prepared from DEX-PLA block copolymers.

Sample	Drug- loading micelles				
	Size (nm)	PDI	Zeta (mV)	DLC (%)	DLE (%)
DEX_{5K}-PLA_{20}	239.1±5.2	0.111	-2.47±0.27	4.03±0.08	38.7±0.69
DEX_{5K}-PLA_{30}	240.5±5.5	0.115	-2.94±0.12	4.78±0.4	45.6±3.6
DEX_{5K}-PLA_{40}	267.3±3.3	0.12	-3.87±0.3	5.44±0.42	51.6±3.73
DEX_{10K}-PLA_{20}	255.9±2.4	0.104	-2.14±0.21	3.96±0.12	38.1±1.14
DEX_{10K}-PLA_{30}	272.6±7.6	0.131	-1.81±0.18	4.76±0.39	45.4±3.51
DEX_{10K}-PLA_{40}	298.8±4.6	0.181	2.17±0.09	5.36±0.1	50.9±3.4

426
427 Drug loaded micelles were prepared by using a two-step method: blank micelles
428 were first obtained by dissolving DEX-PLA copolymer in distilled water, and a
429 curcumin solution in acetone was added to the micelle solution under stirring to yield
430 drug loaded micelles. The particle size, size distribution and zeta potential of micelles
431 were determined by DLS, as shown in Table 3. The average diameter of drug loaded
432 micelles ranges from 239.1 nm for DEX_{5K}-PLA_{20} to 267.3 nm for DEX_{5K}-PLA_{40}, and
433 from 255.9 nm for DEX_{10K}-PLA_{20} to 298.8 nm for DEX_{10K}-PLA_{40}. All copolymer
434 micelles exhibit a low polydispersity (PDI≤0.181). Therefore, the micelle size increases
435 with increase of DEX and PLA block length as in the case of blank micelles, and the
436 size of drug loaded micelles is larger than that of blank micelles. On the other hand, the
437 zeta potential of drug loaded micelles is in the range from -1.81 to 2.17, which is slightly

higher than that of blank micelles. This increase could be assigned to the fact that curcumin has a positive charge, in agreement with the successfully drug loading in micelles.

The performance of drug loading was determined by HPLC. A calibration curve was previously established using a series of curcumin solutions at various concentrations, as shown in **Figure S1**. **Table 3** shows the DLC and DLE data of all drug loaded micelles. The DLC increases from 4.03% for DEX_{5K}-PLA_{20} to 5.44% for DEX_{5K}-PLA_{40}, and from 3.96% for DEX_{10K}-PLA_{20} to 5.36% for DEX_{10K}-PLA_{40}. The same trend is observed for DLE data. Therefore, the drug loading capacity of micelles increases with the increase of hydrophobic block length .[52] In contrast, the hydrophilic block seems to have little effect on drug loading as drug is loaded in the hydrophobic core of micelles.

In vitro drug release was performed under in vitro conditions in pH 7.4 PBS at 37 ℃.[53] **Figure 7** shows the drug release profiles on the six drug loaded micelle systems. All micelles present similar drug release characteristics in two stages, namely a burst release in the first 8 h and a slow release up to 120 h. The rapid release in the early stage may be attributed to the release of drug located at the shell-core interface of micelles, whereas the slow release in the late stage results from drug diffusion inside the core of micelles.[54] At 120 h, the cumulative drug release rate reaches 81.34%, 77.5% and 65.33% for DEX_{5K}-PLA_{20}, DEX_{5K}-PLA_{30} and DEX_{5K}-PLA_{40}, respectively; and it reaches 84.17%, 79.52% and 67.72% for DEX_{10K}-PLA_{20}, DEX_{10K}-PLA_{30} and DEX_{10K}-PLA_{40}, respectively.

It is widely admitted that drug release from biodegradable systems is diffusion and/or degradation controlled.[55] In the present work, drug release was studied in pH 7.4 PBS in a short period of 120 h. The release rate was mainly dependent on drug diffusion as no degradation occurs under these conditions.[55] Shorter hydrophobic block or higher hydrophilic/hydrophobic ratio led to higher drug release rate as drug diffusion was facilitated. Total drug release was not observed probably because of the slow degradation of the hydrophobic core composed of PLA blocks, in agreement with literature.[56-58] Ge et al. reported that Nimodipine-loaded PCL-PEG-PCL micelles

present a decrease of drug release rate with increase of PCL block length.[56] Similarly, Manjili et al. reported that curcumin release from PCL-PEG micelles decreases from 80.2 to 57.1% with CL/EG ratio increase from 2 to 5.[57] On the other hand, micelles with longer hydrophobic block have higher drug load (Table 3). Hu et al. reported that curcumin loaded P(NIPAAm-co-DMAAm)-b-PLLA copolymer micelles with higher drug load present slower release rate compared to those with lower drug load.[10] The authors suggested that curcumin release rate could also depend on the drug loading as the solubility of curcumin in the release medium could be a limiting factor in cases where drug release experiments are performed under non sink conditions.

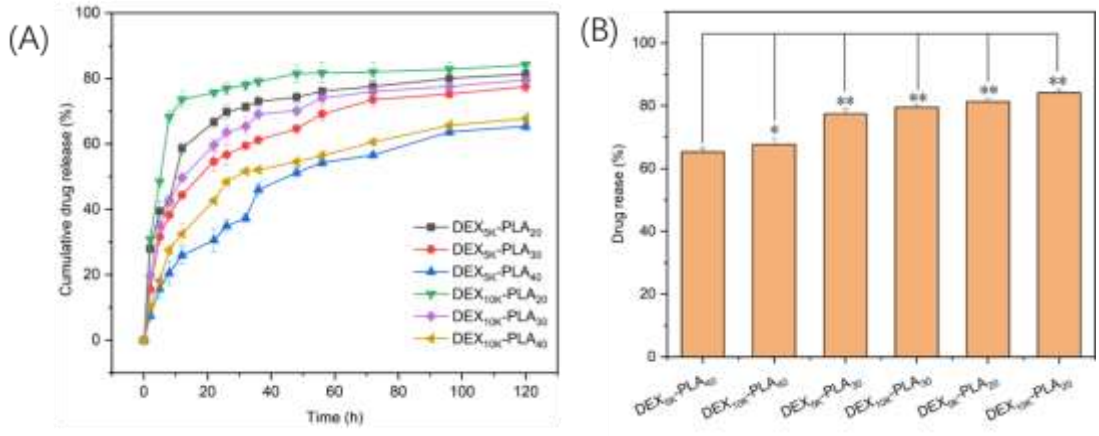


Figure 7. In vitro drug release of curcumin from DEX-PLA copolymer micelles. Data are presented as mean±sd (n = 3, *p < 0.05, **p < 0.01).

3.4. Assay of cytotoxicity of DEX-PLA micelles

The cytotoxicity of DEX-PLA micelles was evaluated by using MTT method. **Figure 8** shows the relative activity of L-929 cells after co-culture with solutions of DEX_{5K}-PLA_{20} and DEX_{10K}-PLA_{20} at concentrations from 0.2 to 1.0 mg/mL for 24, 48 and 72 h. The relative activity is above 90% in all cases, even for samples at 1.0 mg/mL after 72 h culture. Compared with the positive control which has a relative activity of 18% after 72 h culture, the micelles present no cytotoxicity to L-929 cells, indicating the outstanding biocompatibility of DEX_{5K}-PLA_{20} and DEX_{10K}-PLA_{20} micelles. Similar results were obtained for the 4 other micellar samples.

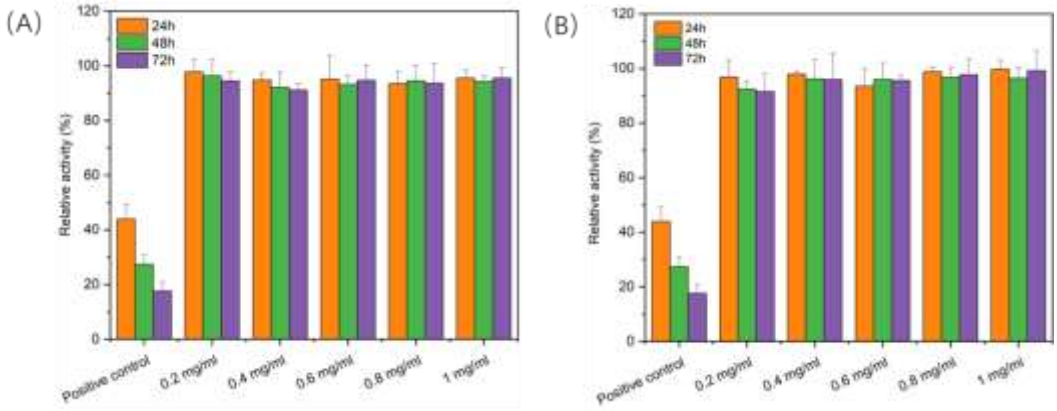


Figure 8. Relative activity of L-929 cells after 24, 48 and 72 h culture with DEX_{5K}-PLA_{20} (A) and DEX_{10K}-PLA_{20} (B) micelle solutions at different concentrations in comparison with the positive control. Data are presented as mean±sd ($n = 3$, $p < 0.01$).

3.5. In vitro antitumor activity

The effect of free curcumin and curcumin-loaded micelles on the proliferation of HeLa cells was examined in the curcumin concentration range of 0.25 to 25 μg/mL. **Figure 9A** shows the cell inhibition rate changes as a function of concentration. Free curcumin and curcumin loaded micelles are highly cytotoxic to HeLa cells, the cell inhibition rate increasing almost linearly with concentration. The half maximum inhibitory concentration (IC_{50}) was obtained from data in **Figure 9A**. The IC_{50} of drug loaded DEX_{5K}-PLA_{20} and DEX_{10K}-PLA_{20} micelles is 10.16 and 9.12 μg/mL, respectively, and the IC_{50} value of free curcumin is 11.9 μg/mL (**Figure 9B)**. Although these IC 50 values are rather close, statistical analysis showed that drug loaded DEX_{10K}-PLA_{20} micelles exhibit stronger anticancer effect than free curcumin, which may be attributed to the more stable nanostructure of micelles and easier endocytosis.[59,60] Moreover, drug-loaded micelles can not only increase the drug solubility, but also reduce the toxic side effects of curcumin on normal tissues.

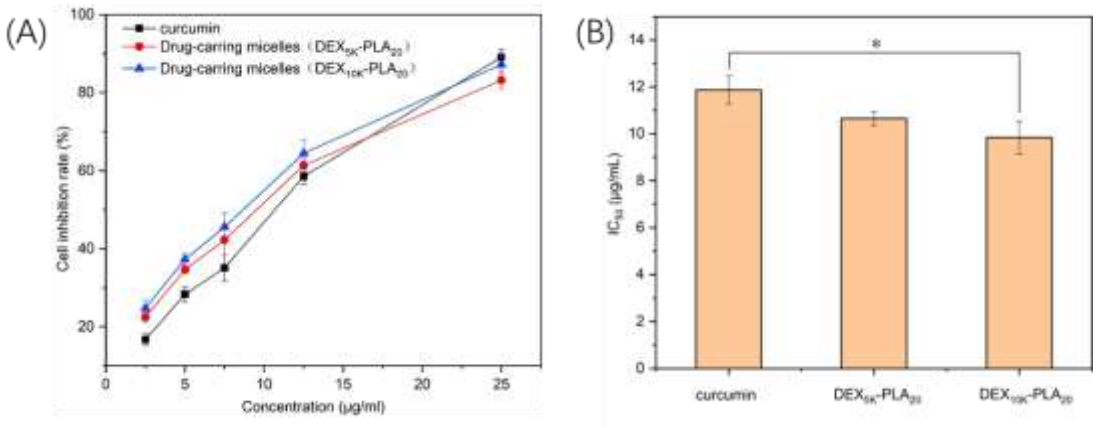


Figure 9. Inhibition rates (A) and IC_{50} values (B) of DEX_{5K}-PLA_{20} and DEX_{10K}-PLA_{20} drug-loaded micelles at different concentrations on HeLa cells after 48 h culture, in comparison with free curcumin. Data are presented as mean±sd (n = 3, *p < 0.05).

4. Conclusion

Amphiphilic DEX-PLA block copolymers with different DEX and PLA block lengths were synthesized by reduction amination between DEX and amino terminated PLA. All copolymers can self-assemble into spherical micelles with narrow distribution in aqueous medium. The CMC of copolymer micelles decreases, and the DLC and DLE increase with increasing PLA block length. DEX-PLA micelles exhibit biphasic drug release profiles. The drug release rate depends on the length of the hydrophobic block. The longer the hydrophobic block, the slower the drug release rate. MTT experiments showed that DEX-PLA copolymers present good cytocompatibility. Last but not least, curcumin-loaded micelles exhibit significant cytotoxicity to HeLa cells, suggesting that DEX-PLA copolymer micelles could be most promising as nanocarrier of hydrophobic antitumor drugs in cancer treatment.

CRediT authorship contribution statement

Haozhi Sun and Shuxin Wang: Methodology, Investigation, Validation, Formal analysis, Visualization, Writing – original draft; **Yuandou Wang**: Conceptualization, Methodology; **Linlin Lu**: Funding acquisition, Validation, Supervision; **Feng Su**: Conceptualization, Supervision, Funding acquisition, Writing -

review & editing; **Suming Li**: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Acknowledgements

The work was financially supported by the Shandong Province Medical and Health Technology Development Plan Project (202203100062) and National Natural Science Foundation of China (32471310), and the Science and technology department of Qingdao Municipality (project number: 24-1-4-xxgg-11-nsh).

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Declaration of competing interest

All cited authors certify that they have sufficiently participated in the work to assume public responsibility for the content. The authors declare that there is no conflict of interest regarding the publication of this article.

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