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Self-assembled micelles prepared from bio-based hydroxypropyl methyl cellulose and polylactide amphiphilic block copolymers for anti-tumor drug release

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Abstract:

Fully bio-based amphiphilic diblock copolymers were synthesized from hydroxypropyl methyl cellulose (HPMC) and amino-terminated poly(L-lactide) (PLLA) or poly(L-lactide-co-DL-lactide) (PLA) by reductive amination. The resulting HPMC-PLLA and HPMC-PLA copolymers with various hydrophobic block lengths were characterized by NMR, DOSY-NMR and FT-IR. Micelles were obtained by self-assembly of copolymers in aqueous medium. The micelles are spherical in shape, and the micelle size ranges from 150 to 180 nm with narrow distribution. The critical micelle concentration decreases with increasing PLA block length. Paclitaxel was loaded in micelles. Enhanced drug loading is obtained with increase of PLA block length. A biphasic release profile is observed with a burst of 40% followed by slower release up to 80%. MTT assay indicates the good cytocompatibility of HPMC-PLA micelles. SRB assay shows a significant cytotoxicity of paclitaxel-loaded micelles against SK-BR-3 cells. It is thus concluded that bio-based HPMC-PLA block copolymers could be promising nano-carrier of anti-tumor drugs.

Key words: hydroxypropyl methyl cellulose; polylactide; self-assembly; paclitaxel; micelle; drug release; cytotocompatibility

1. Introduction

Amphiphilic block copolymers are able to self-assemble in aqueous medium, yielding a variety of ordered structures, including micelles, cylinders, lamellae, vesicles, etc. These aggregates present great interests for applications such as drug and gene delivery, nanoreactor, diagnostic imaging, etc [1]. Among them, micelles with a core-shell structure have been largely studied as nano-carrier of hydrophobic drugs due to their outstanding properties, e.g. prolonged blood circulation, enhanced drug bioavailability and reduced side effects [2]. The hydrophobic core of micelles generally consists of a biodegradable polymer such as polylactide (PLA), polyglycolide (PGA), or poly(ε-caprolacone) (PCL), and serves as reservoir for poorly soluble drugs. On the other hand, the hydrophilic shell is usually composed of hydrophilic polymers such as poly(ethylene glycol) (PEG), poly(N-isopropyl acrylamide) (PNIPAAm), or poly(2-ethyl-2-oxazoline) (PEOz), which assures micelle stabilization and interactions with plasmatic proteins and cell membranes. The biodistribution of micelles is mainly dependent on the nature of the hydrophilic shell [3].

Aliphatic polyesters, and in particular PLA, PGA and PCL have been widely used in the biomedical field for drug delivery, tissue regeneration and tissue engineering in the form of scaffolds, sutures, drug carriers, and bone fracture internal fixation devices due to their biocompatibility, degradability and good processability [4]. PLA is generally obtained by ring-opening polymerization (ROP) of lactide, the cyclic dimer of lactic acid. As lactic acid is a chiral molecule which has two enantiomers, L- and D-lactic acids, various PLA polymers are available, including isotactic poly(L-lactide) (PLLA), poly(DL-lactide) (PDLLA), and poly(L-lactide-co-DL-lactide) (PLA) stereocopolymers with different L/DL ratios [5]. The stereochemistry of PLA is a most important parameter determining its physico-chemical properties such as morphology, mechanical strength, degradation rate, etc. Interestingly, Jelonek et al. reported that the PLA chain stereoregularity greatly affects the self-assembled architecture of PEG-PLA micelles [6]. Worm-like micelles are obtained for PEG-PLLA diblock copolymers, whereas PEG-PDLLA copolymers with similar compositions yield spherical micelles. PEG-PDLLA micelles entrapping an anti-tumor drug, paclitaxel (PTX), have been approved under the trademark of Genexol-PM in South Korea for treating breast cancer and non-small cell lung cancer [7].

Polysaccharides have attracted growing attention as a hydrophilic building block for construction of amphiphilic block copolymers because of their bio-based origin, biodegradability, biocompatibility, non-toxicity and inherent bioactivity which allows to improve drug bioavailability and to reduce inflammation [2, 8]. Various polysaccharides have been considered, including chitosan, dextran, starch, cyclodextrin and cellulose. Cellulose chains are composed of β (1 → 4) linked D-glucose units which endow cellulose with unique physical and

chemical properties [9]. Nevertheless, the poor solubility of cellulose greatly restricts its potential applications. Thus, chemical modification of cellulose is performed to obtain soluble derivatives for uses as a hydrophilic building block, such as ethyl cellulose [10], hydroxylpropyl cellulose [11], hydroxylpropylmethyl cellulose (HPMC) [12], etc. Actually, various cellulose-based amphiphilic copolymers have been designed via grafting hydrophilic polymers onto the main chain of cellulose [9]. However, such reactions are poorly controllable and may lead to complex structures. And it is difficult to achieve cellulose-based graft copolymers with high purity, low dispersity and good reproducibility which strongly influence their micellization characteristics [9]. In contrast, the reducing hemiacetal end group of cellulose which is in equilibrium with an aldehyde one allows to synthesize amphiphilic block copolymers via reductive amination [8, 12].

In a previous work, we reported for the first time the synthesis of linear HPMC-PLA and AB_2-type $(HPMC)_2$-PLA block copolymers by combination of ROP, amination reduction and UV initiated thiol-ene or thiol-yne click reaction. The copolymers exhibit interesting thermo-responsive and self-assembly properties, but the overall yield of the reaction is low [12, 13]. In this work, a more efficient and simpler procedure was proposed to synthesize HPMC-PLA block copolymers by reductive amination. Amino-terminated PLA (NH_2-PLA) was first synthesized by tert-butyl-N-(3-hydroxypropyl) carbamate initiated ROP of lactide, followed by deprotection of the N-tert-butoxycarbonyl (Boc) group. HPMC-PLA copolymers with various PLA chain lengths were then synthesized by reductive amination between NH_2-PLA's amino group and HPMC's hemiacetal end group. The self-assembly of copolymers including morphology, micelle size and size distribution, zeta potential and critical micelle concentration (CMC) was investigated. Drug loading and drug release, MTT and SRB assays were performed to evaluate the potential of HPMC-PLA copolymers as nano-carrier of hydrophobic drugs.

2. Materials and Methods

2.1 Materials

L-lactide and DL-lactide were purchased from Purac Biochem (Goerinchem, Netherlands) and Alfa-Aesar, respectively, and purified by recrystallization in ethyl acetate. HPMC was kindly supplied by Dow Colorcon Limited France. HPMC oligomers with Mw of 7000 were obtained by enzymatic depolymerization as previously reported [12, 13]. Tert-butyl-*N*-(3-hydroxypropyl) carbamate (3-(Boc-amino)-1-propanol), tin (II) 2-ethyl hexanoate ($Sn(Oct)_2$) and sodium triacetoxyborohydride ($NaBH(OAc)_3$) were obtained from Sigma-Aldrich and used as received. Trifluoroacetic acid (TFA) was purchased from Merck KGaA. Toluene and dichloromethane

were obtained from Sigma-Aldrich, dried over by calcium chloride ($CaCl_2$) and distilled prior to use.

SK-BR-3 cell line was provided by American Type Culture Collection (ATCC-LGC Standard). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific. All other organic solvents were of analytic grade from Sigma-Aldrich and used as received.

2.2 Synthesis of amino-terminated PLA

Amino-terminated PLA was synthesized by ROP of lactide using 3-(Boc-amino)-1-propanol as initiator and $Sn(Oct)_2$ as catalyst, followed by deprotection of the BOC group using TFA. Typically, L-lactide (27 mmol, 3.888g), DL-lactide (3 mmol, 0.432g), 3-(Boc-amino)-1-propanol (2 mmol, 0.35g) and $Sn(Oct)_2$ (2.3 mmol, 0.0932g) were added into a dried Schlenk tube. 20 mL anhydrous toluene was added under nitrogen atmosphere with stirring. The solution was degassed by performing five freeze–pump-thaw cycles. The reaction proceeded 24 h at 80℃. Finally, the crude product Boc-NH-PLA was purified by precipitation in ethanol, and drying in vacuo.

1.0 g Boc-NH-PLA was introduced in a nitrogen purged flask. 5 mL freshly distilled dichloromethane was then added, followed by addition of 5 mL anhydrous TFA. The reaction proceeded at room temperature for 1 h, and terminated by evaporating all the liquid. The crude product was dissolved in dichloromethane, successively washed with 5% $NaHCO_3$ aqueous solution and water, and finally dried over $MgSO_4$. After filtration, the solution was precipitated in cold ethanol, and the product dried in vacuum at room temperature up to constant weight.

2.3 Synthesis of HPMC-PLA block copolymer

HPMC-PLA copolymers were synthesized by reductive amination according to the method reported in literature [14, 15]. Typically, under nitrogen atmosphere, 1.0 g HPMC (1.0 g, 0.14 mmol), NH_2-PLA (0.154 mmol, 0.23g) and $NaBH(OAc)_3$ (0.042 g, 0.2 mmol) were dissolved in 20 mL anhydrous DMSO. The reaction was allowed to proceed under nitrogen atmosphere at 35 ℃ for 72 h. The reaction mixture was dialyzed against distilled water for 72 h, followed by 48 h freeze-drying to yield the final product.

2.4 Characterization

^1H NMR spectra were obtained using Bruker AVANCE 300 spectrometer with $CDCl_3$, DMSO-d_6 or D_2O as solvent. Data were analyzed by using MestRe Nova software. DOSY NMR was performed on Bruker Avance 400 spectrometer equipped with a Bruker multinuclear z-gradient which can produce gradients in the z-direction with a strength of 55 Gcm^{-1}. The spectra were processed using Bruker topspin software (version 3.5) with the ledbpgp2s pulse program.

GPC was performed on a Viscotek TDA 305 multidetector GPC/SEC system. All samples were prepared at a concentration of 10 g/L and filtered through 0.22 μm PTFE filter. THF was used as eluent at a flow rate of 1.0 mL/min. Calibration was realized by using polystyrene standards (Polysciences, Warrington, PA).

FT-IR spectra were recorded on a Nicolet NEXUS spectrometer with attenuated total reflectance (ATR) accessory in the frequency range from 400 to 4000 cm^{-1}. 32 scans were performed for each analysis at 4 cm^{-1} resolution.

DSC was performed on TA DSC Q20 system in the temperature range of 0 ℃-160 ℃ under dry nitrogen atmosphere. Samples of *c.a.* 5 mg were put into an aluminum pan and sealed. Measurements were made using the following procedure: 1) heating from 0°C to 160 ℃ at 10 ℃/min to erase the thermal history of samples, 2) cooling down to 0℃, 3) heating to 160 ℃ at 10 ℃/min. The glass transition temperature (Tg), melting temperature (Tm) and melting enthalpy (ΔHm) were determined from the second heating scan.

A Zetasizer Zano-ZS from Malvern Instrument equipped with a He–Ne laser (λ =632.8 nm) was used to determined the hydrodynamic size and zeta potential of copolymer micelles. Dynamic light scattering (DLS) was performed at 25°C at a scattering angle of 90°. Copolymer solutions at 1.0 mg mL^{-1} were filtered through a 0.45 μm cellulose acetate (CA) membrane filters before measurements.

The CMC of copolymers was determined by fluorescence spectroscopy using pyrene as fluorescent probe. The copolymers solutions were prepared with concentrations varying from 1.0×10^{-3} to 2.0 mg/mL, whereas the concentration of pyrene was fixed at 6×10^{-7}g/L. The excitation spectra of the above solutions were recorded on PerkinElmer LS55 fluorescence spectrometer at an excitation wavenumber of 395 nm. The CMC value was estimated from the intersection of regression lines of intensity ratios at 336 nm and 334 nm *versus* the logarithm of concentration plots.

TEM images were obtained by using JEOL 1400 plus instrument, operating at an acceleration voltage of 120 kV. Samples were prepared by dropping 1.0 mg mL^{-1} micelle solution onto a carbon coated copper grid and exposed to ruthenium oxide vapor for 7 min before measurements.

2.5 In vitro drug release

Drug-loaded micelles were prepared in two steps. 20 mg copolymer was first dissolved in distilled water to yield blank micelles. 2 mg paclitaxel dissolved in 50 μL ethanol 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 CA filter. Finally the solution was lyophilized and stored at 4 ℃.

HPLC-MS measurements were performed to determine the concentration of PTX in solution. The mobile

phase was a 50/50 (v/v) mixture of acetonitrile and pH 7.4 phosphate buffered saline (PBS). A calibration curve was previously established from standard PTX solutions at concentrations ranging from 0.128 to 5 ppm (R^2 =0.999). The following equations [16] were used to determine the drug loading content (DLC) and drug loading efficiency (DLE):

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

$$\mathrm{DLE} = \frac{\text{weight of drug in micelles}}{\text{therotical drug loading}} \times 100\% \quad (2)$$

In vitro drug release from PTX-loaded micelles was carried out using dialysis method. Lyophilized drug-loaded micelles were dispersed in pH 7.4 PBS at 2.0 mg/mL. 1 mL micelle solution was introduced into a Float-A-Lyzer G2 dialysis device with a MWCO of 3 500 Da which was immerged in 40 mL PBS. Drug release experiments were realized at 37°C under constant shaking. At preset time intervals up to 120 h, an aliquot of 25 μL solution was withdrawn from each device and replaced by the same volume of fresh PBS. The whole release medium was removed and replaced regularly to keep sink conditions. The amount of residual PTX in collected samples was measured by HPLC.

2.6 MTT assay

L-929 cells in logarithmic growth phase were added in 96-well plates (Corning Costar, U.S.A.) at 1×10^4 cells/mL in DMEM medium (10% calf serum, 100 μg/mL Penicillin, 100 μg/mL streptomycin), 100 μL per well. The plates were placed in an NU-4850 incubator (NuAire, U.S.A.) at 37 °C under humidified atmosphere with 5% CO_2. After 24 h, the medium was removed and replaced with 100 μL fresh medium or HPMC-PLA micellar solution at concentrations from 0.25 to 5.0 mg/mL containing 10.0 % calf serum. 100 μL fresh medium was used as the negative control, and 100 μL phenol solution as the positive control [17]. After 24, 48 and 72 h, 20 μL MTT solution at 5 mg/mL was added. The medium was removed after 6 h incubation. 150 μL DMSO was added, and the plates were shaken for 10 min. The optical density (OD) was measured by using a microplate reader (Elx800; BioTek, U.S.A.) at 570 nm. All experiments were carried out in triplicate. The cell viability was calculated from the OD values of the test sample and negative control using the following equation:

$$\text{Relative activity (\%)} = (OD_{\text{test sample}} / OD_{\text{negative control}}) \times 100 \quad (3)$$

2.7 Sulforhodamine B (SRB) assay

SK-BR-3cells in growth phase were suspended in 96-well plates at a density of 3×10^4 cells, 100 μL per well in McCoy's 5A (Modified) Medium supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated 24 h at 37 °C in a humidified atmosphere containing 5 % CO_2 to allow

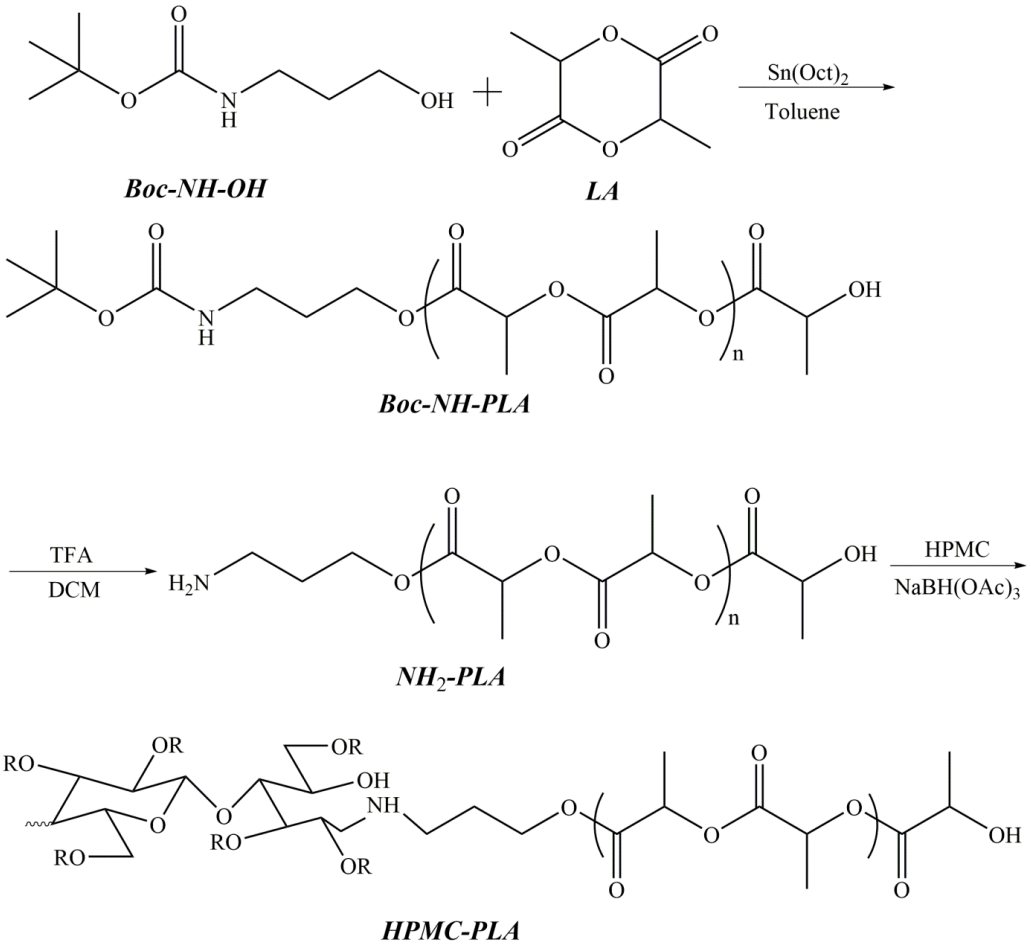
cell adhesion. Subsequently, the medium was replaced by blank micelles, drug-loaded micelles, or free drug solution. Blank micelles and drug-loaded micelles were dissolved directly in culture medium. Free PTX was dissolved in 0.125 % DMSO/medium. Untreated cells were used as negative control. PTX concentration of both free drug and drug-loaded micelles ranged from 0.025 to 25 μg/mL. Blank micelles were prepared at concentrations used for drug-loaded micelles (18-48 μg/mL). Each concentration was carried out in quadruplicate, and each experiment repeated three times. After 72 h incubation, the cells were fixed with 100 μL 10 % trichloroacetic acid for 1 h at 4 ℃, and washed twice with deionized water. Then the cells were stained with 0.4 % SRB dissolved in 1.0 % acetic acid for 30 min at room temperature and rinsed three times using 1.0 % acetic acid. Finally, 200 μL of 10 mM Tris base solution were used to dissolve the incorporated dye. Absorbance at 570 and 690 nm was measured using MRX Revelation plate reader (Dynex Technologies). One-way ANOVA and Tukey post-hoc test in SPSS software were used for statistical analysis. The relative activity of cells was calculated from the following equation:

$$\text{Relative activity (\%)} = (OD_{\text{test sample}} / OD_{\text{negative control}}) \times 100 \qquad (4)$$

3. Results and discussion

3.1 Synthesis of amphiphilic block copolymers

The synthesis of HPMC-PLA copolymers is realized in 3 steps as illustrated in Scheme 1. At first, 3-(Boc-amino)-1-propanol was used to initiate the ROP of lactide, yielding Boc-NH-PLA. Then, the protective Boc group at the chain end was removed by treatment using trifluoroacetic acid to obtain amino terminated PLA. Finally, the hemiacetal end group of hydrophilic HPMC block and the amino group of hydrophobic PLA block were coupled by reductive amination in the presence of $NaBH(OAc)_3$, yielding amphiphilic HPMC-PLA block copolymers. To the best of our knowledge, this is the first time that such copolymers are synthesized by reductive amination.



Scheme 1. Synthesis route of HPMC-PLA block copolymers by reductive amination.

Amino terminated NH_2-PLA was synthesized according to the method reported by Gotsche et al. [18] and Ju et al. [19]. Boc group was used as the protective group since it is stable during lactide polymerization and could be easily removed. Boc-NH-PLA was synthesized by ROP of L-lactide or a mixture of L- and DL-lactides using 3-(Boc-amino)-1-propanol as initiator with various monomer/initiator molar ratios ranging from 10/1 to 40/1. In fact, PLLA with a degree of polymerization (DP) above 20 is poorly soluble in DMSO, solvent used for reductive amination in the last step. A mixture of L- and DL-lactides with L/DL ratio of 90/10 was thus used for the synthesis of PLA with higher DP to ensure full solubility in DMSO. For the sake of clarity, PLLA and PLA refer to poly(L-lactide) and poly(L-lactide-co-DL-lactide), respectively. Five polymers were synthesized in this work, namely PLLA10, PLLA20, PLA20, PLA30 and PLA40. In these abbreviations, the number refers to the DP of PLLA or PLA.

Fig. 1A shows the ^{1}H NMR spectrum of Boc-NH-PLA. The characteristic signals of Boc are detected at 1.42 ((C*H*$_3$)$_3$-O-, **a**), 4.18 (-CH$_2$-C*H*$_2$-O-, **e**), 1.84 (-NH-CH$_2$-C*H*$_2$-, **d**) and 3.20 (-NH-C*H*$_2$-CH$_2$-,**c**) ppm. The

signals at 1.62 (-CO-CH (CH_3)-O-,-**f**) and 5.25 (-CO-C*H* (CH_3)-O,-**g**) ppm are attributed to protons of PLA main chain, and those at 1.50 (-CO-CH (CH_3)-OH, **j**) and 4.42 (-CO-C*H* (CH_3)-OH **h**) ppm corresponding to the hydroxyl terminated unit. The DP of PLA is determined from the integration ratio of signals **e** to **g**. As summarized in Table S1, the DP determined by NMR is very close to the feed ratio, in agreement with the successful ring opening polymerization of lactide initiated by Boc-NH-OH. The Mn calculated from the DP of PLA and the molar mass of the protecting group varies from 950 to 3040 (Table S1).

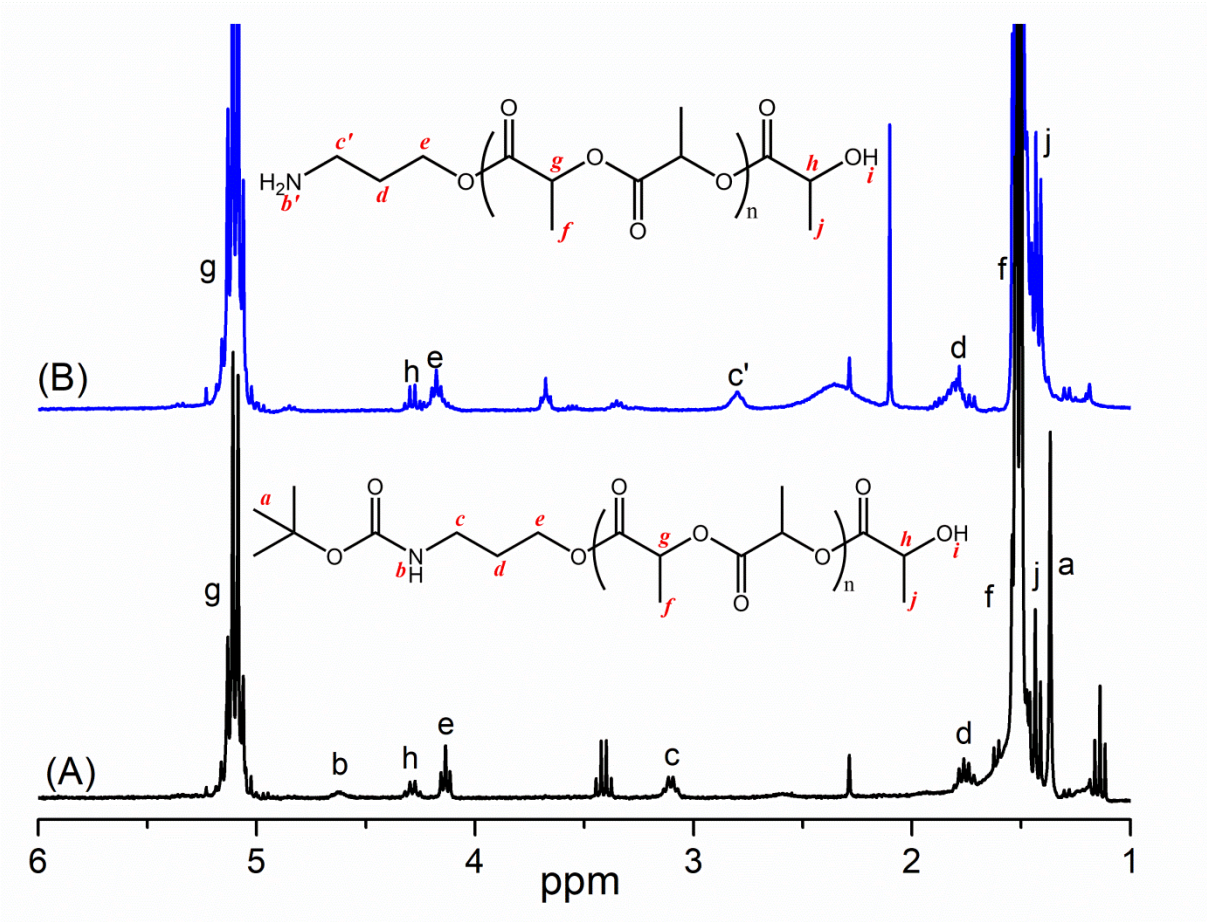


Fig. 1. NMR spectra of Boc-NH-PLA (A) and NH_2-PLA (B) in $CDCl_3$.

The molar masses of the various Boc-NH-PLA polymers are also measured by GPC. In all cases, the GPC curve exhibits a symmetric molar mass distribution, as shown in Fig. S1. The Mn obtained by GPC varies from 1670 to 3130, which is slightly higher than that calculated from NMR. The dispersity of polymers ranges from 1.29 to 1.59, indicating narrow distribution of molar mass.

The thermal properties of Boc-NH-PLA polymers were determined by DSC as shown in Table S1. $PLLA_{10}$ and $PLLA_{20}$ exhibit a Tg at 23.8 and 43.7℃, respectively, indicating that the Tg of PLLA oligomers increases

with increasing molar mass or DP according to the free volume theory of glass transition. In fact, the chain ends present higher mobility than the main chain. In a given polymeric matrix, the free volume increases with increase of the number of chain ends or with decrease of DP, thus leading to lower Tg. Similarly, the Tg of PLA_{20}, PLA_{30} and PLA_{40} is detected at 38.5, 39.7 and 43.6°C, respectively. It is of interest to note that $PLLA_{20}$ exhibits higher Tg than PLA_{20} because the former presents higher chain stereoregularity. On the other hand, $PLLA_{10}$ and $PLLA_{20}$ exhibit a Tm at 112.4 and 136.5°C with corresponding ΔHm of 25.6 and 62.2 J/g, respectively, suggesting higher crystallinity with increasing DP of PLLA oligomers. The same trend is observed for PLA_{20}, PLA_{30} and PLA_{40}. It is also noteworthy that $PLLA_{20}$ exhibits higher Tm and ΔHm than PLA_{30} and PLA_{40} in spite of its lower DP, which means that the chain stereoregularity has stronger influence on the crystallinity than the DP of PLA oligomers.

Amino terminated PLA was obtained by removal of the Boc group from Boc-NH-PLA in the presence of TFA. As shown in Fig. 1B, the characteristic signal ((C*H*$_3$)$_3$-O-, **a**) at 1.42 ppm attributed to the terminal Boc group totally disappears, whereas the signal **c** at 3.20 ppm shifts to 2.75 ppm (**c'**) since the adjacent amide group is converted to amino group. These findings indicate full deprotection of all primary amino groups. The integration ratio between signal **c'** and signal **g** or **f** of lactyl units remains constant after removal of the Boc group, indicating that deprotection did not provoke PLA degradation. This is proved by GPC analysis (Fig. S1). The GPC curves of Boc-NH-PLA_{20} and NH-PLA_{20} almost overlap, in agreement with the same molar mass.

Finally, reductive amination was carried out between the hemiacetal group of HPMC and the amine group of PLA using $NaBH(OAc)_3$ as reducing agent, yielding HPMC-PLA block copolymers. The ^{1}H NMR spectra of HPMC-PLA in DMSO-d_6 and D_2O are shown in Fig. 2. The characteristics signals of HMPC are observed on the spectrum in DMSO-d_6 (Fig. 2A), including signals at 1.05 ppm (-CH_2-CHOHC*H*$_3$ of substituent groups, **n**), 2.75–4.75 ppm (C-H of the glucose units and substituent groups, **m**) and those of PLA at 1.49 (-CO-CH (C*H*$_3$)-O-, **f**) and 5.24 (-CO-C*H* (CH_3)-O, **g**). These results suggest the successful synthesis of HPMC-PLA copolymers by reductive amination between HPMC and amino terminated PLA. Compared to the synthesis of HPMC-PLA and $(HPMC)_2$-PLA copolymers by thiol-ene or thiol-yne click reaction in our previous work [12,13], the synthesis of HPMC-PLA by reductive amination is simpler and more efficient as it allows to shorten the synthesis route. In fact, the previous method involves a three step procedure: ring-opening polymerization of L-lactide initiated by allyl alcohol or propynol, amination reduction of the aldehyde endgroup of HPMC, and UV-initiated thiol-click reaction. Reaction of cysteamine with the aldehyde endgroup of HPMC yields a mixture of HPMC-S-S-HPMC together with small amounts of unreacted HPMC, HPMC-SH, and

HPMC-S-S-$(CH_2)_6$-NH_2. A supplementary step was needed to reduce HPMC-S-S-HPMC to HPMC-SH using 1,4-dithiothreitol (DTT) [12,13].

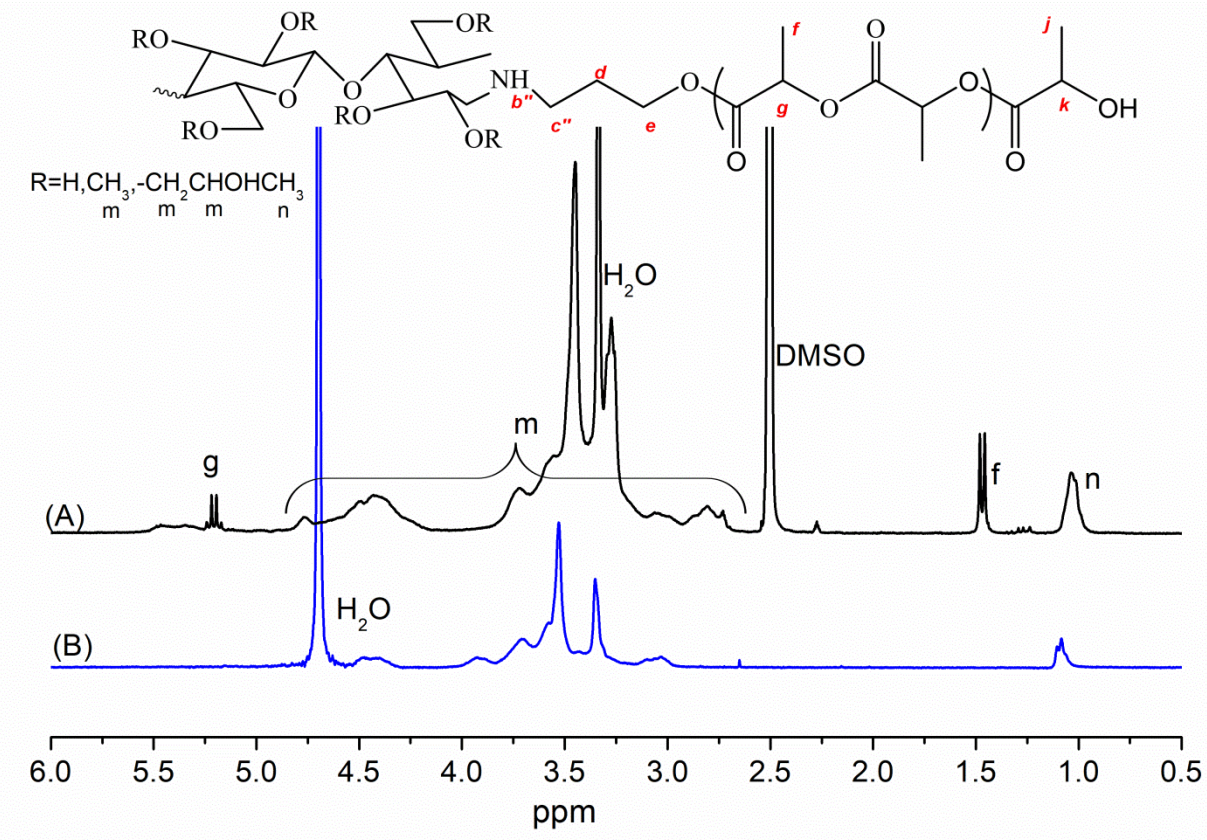


Fig. 2. ^{1}H NMR spectra of HPMC-PLA in DMSO-d_6 (A) and D_2O (B) .

NMR is also a means to evidence the core-shell structure of micelles self-assembled from amphiphilic block copolymers. In selective solvents, only the characteristic signals of soluble block can be detected, while those of insoluble block are invisible because of the limited movement. Thus, on the spectrum in D_2O as solvent, only the signals corresponding to hydrophilic HPMC block are detected as shown in Fig. 2(B).

DOSY-NMR is a technique of choice to characterize block copolymers, which allows the separation of NMR signals of different species according to their diffusion coefficients related to the hydrodynamic radius of molecules [20]. As shown in Fig. 3, the signals corresponding to both PLA_{10} and HPMC blocks present the same diffusion coefficient of 3.16×10^{-11} m^2/s. It also should be noted that the diffusion coefficient of PLA_{10} and HPMC homopolymers is 1.0×10^{-10} and 5.62×10^{-11} m^2/s (Fig. S2), respectively. Thus, DOSY-NMR clearly indicates the effective combination of the two blocks in a copolymer without residual homopolymers.

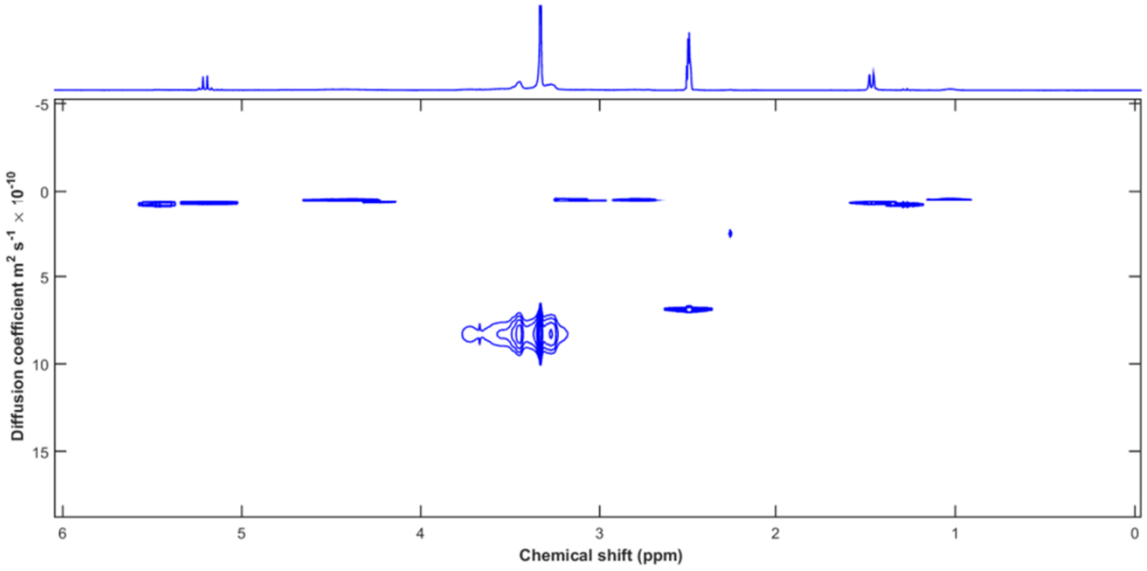


Fig. 3. DOSY- NMR spectrum of HPMC-PLA$_{10}$ in DMSO-d_6.

FT-IR spectra confirmed the chemical structure of copolymers. Fig. 4 shows the FT-IR spectra of Boc-OH, Boc-NH-PLA, NH_2-PLA, HPMC-PLA and HPMC. Boc-OH presents a large band around 3260 cm^{-1} of O-H bond, and characteristic bands at 1689, 1527, 1366 and 1392 cm^{-1} assigned to CO-NH, C=O and C-H (methyl) bonds, respectively. These characteristic bands become much weaker in Boc-PLA, and aren't visible in NH_2-PLA due to removal of the Boc group. On the spectrum of HPMC-PLA, characteristic bands at 3480, 2890, and 1047 cm^{-1} corresponding to O-H, C-H and C-O from HPMC and at 1760 cm^{-1} of PLA are detected, thus confirming the presence of both blocks.

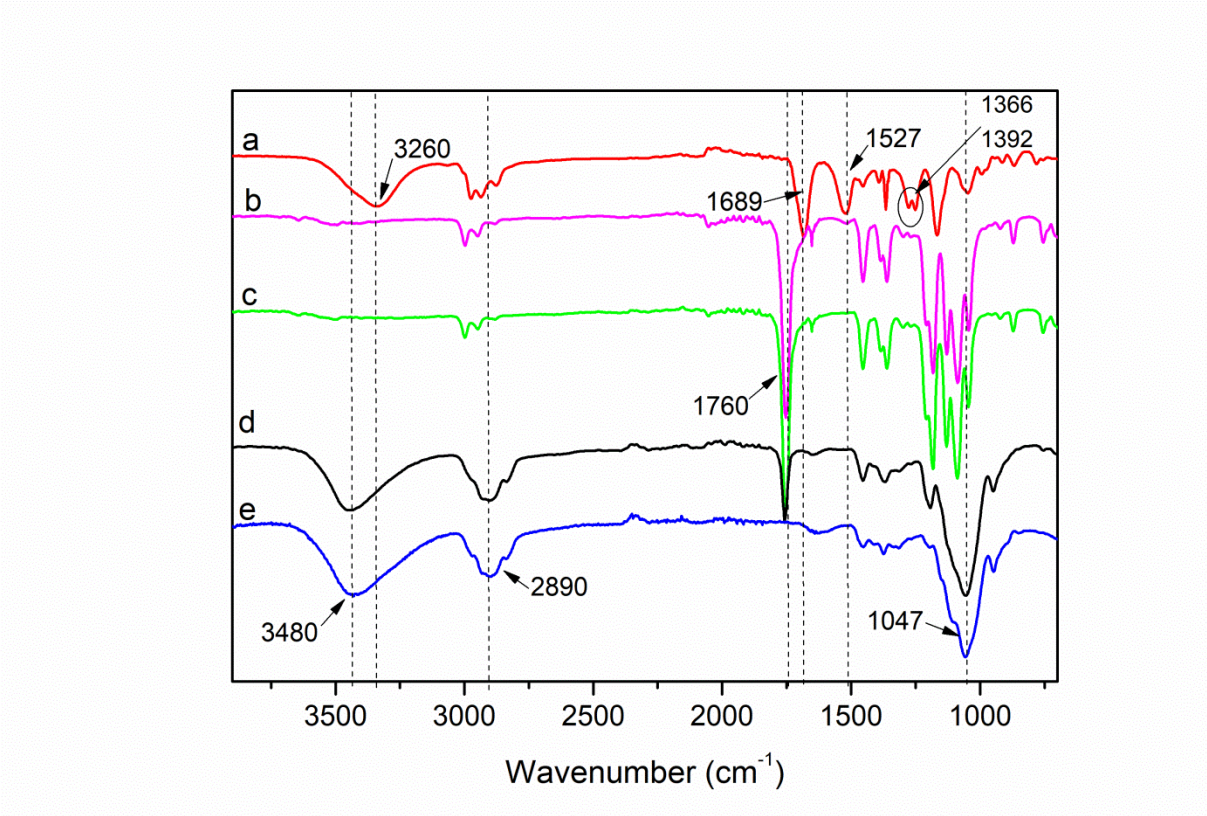


Fig. 4. FT-IR spectra of Boc-OH (a), Boc-NH-PLA (b), NH_2-PLA (c), HPMC-PLA (d) and HPMC (e)

3.2 Self-assembly of copolymers

The self-assembly of water soluble HPMC-PLA copolymers is achieved by dissolution in Milli-Q water. The CMC of block copolymers was determined by fluorescence spectroscopy using pyrene as probe due to its characteristic dependence of the vibrational fine structure [21]. As shown in Fig. 5A, the absorption at 334 nm of the fluorescence excitation spectra corresponds to a polar environment of pyrene, and the absorption at 336 nm corresponds to a nonpolar environment of pyrene because of the migration of pyrene into the hydrophobic core of micelles. Thus, the onset of micellization is evidenced by a red shift of the fluorescence excitation spectra due to the variation of polar environment of pyrene. The intensity ratio I_{336}/I_{334} *versus* copolymer concentration is plotted as shown in Fig. 5B, and the CMC is obtained from the intersection point of regression lines.

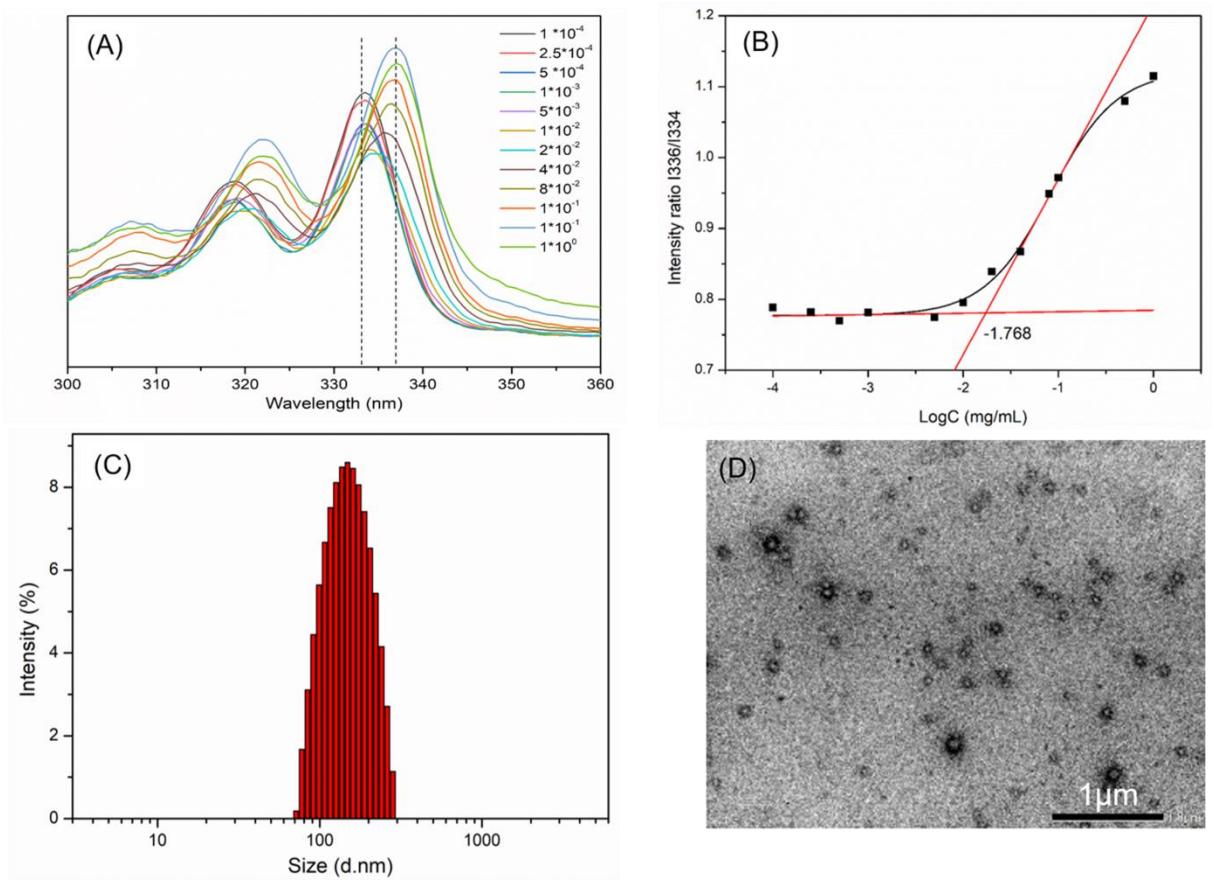


Fig. 5. Fluorescence excitation spectra (A), and I_{336}/I_{334} *vs.* LogC plot (B) of HPMC-PLA_{20} solutions at different concentrations, size distribution (C) and TEM image (D) of HPMC-PLA_{20} micelles.

In Table 1 are listed the CMC values of HPMC-PLA copolymers. HPMC-$PLLA_{10}$ exhibits a CMC of 0.15 g/L, which is the highest among the 5 copolymers. The CMC of HPMC-$PLLA_{20}$ and HPMC-PLA_{20} is 0.017 and 0.016 g/L, respectively. With longer PLA block length, HPMC-PLA_{30} and HPMC-PLA_{40} exhibit a CMC of 0.011 and 0.009 g/L, respectively. Therefore, longer hydrophobic block length facilitates micelle formation, thus leading to lower CMC [22, 23]. On the other hand, the stereochemistry of PLA block seems not to affect the CMC of HPMC-PLA copolymers.

Table 1. Self-assembly and drug loading properties of HPMC-PLA block copolymers.

Sample	Blank micelles				PTX-loaded micelles			
	Size (nm)	PDI	Zeta (mV)	CMC (mg/mL)	Size (nm)	PDI	DLC (%)	DLE (%)

HPMC-PLLA$_{10}$	159.3±1.2	0.25	-3.3	0.15	184.9±1.2	0.23	4.8	45.6
HPMC-PLLA$_{20}$	153.8±3.0	0.15	-2.1	0.017	186.6±1.2	0.20	3.6	26.3
HPMC-PLA$_{20}$	169.7±1.6	0.16	-1.9	0.016	175.2±1.2	0.15	5.3	50.2
HPMC-PLA$_{30}$	171.5±1.2	0.23	-2.0	0.011	180.4±1.2	0.27	6.2	58.2
HPMC-PLA$_{40}$	178.3±2.1	0.28	-6.9	0.009	216.3±1.2	0.16	6.4	59.8

The size and polydispersity index (PDI) of micelles were determined by DLS, and the morphology visualized by TEM as shown in Fig. 5(C, D). The average micelle size of copolymers ranges from 153.8 to 178.3 nm, and the PDI from 0.15 to 0.28 (Table 1). These findings suggest that the micelle size of HPMC-PLA copolymers is hardly affected by the PLA block length. On the other hand, Fig. 5(D) shows that micelles are uniformly distributed with well-defined spherical structure. The zeta potential of the micelles is close to zero, which corroborates with the fact that the micelle surface is almost neutral.

3.3 In vitro drug release

The hydrophobic core of micelles can encapsulate poorly soluble drugs. Many factors affect the encapsulation capacity of micelles, such as compatibility between drugs and core polymers, core block length and crystallinity, *etc.* PLA has good compatibility with PTX since PTX contains OH and NH groups which can interact with carbonyl groups of PLA [24]. Thus PTX was selected to investigate the drug encapsulation and release properties of HPMC-PLA copolymer micelles.

PTX-loaded micelles were prepared by adding a PTX solution in ethanol to pre-formed micellar solution under vigorous stirring. The solution of HPMC-PLLA$_{10}$ appears transparent, whereas that of other copolymers are whitish. This difference could be assigned to the fact that HPMC-PLLA$_{10}$ presents the highest hydrophilic/hydrophobic ratio. The size of PTX-loaded micelles is slightly larger than that of blank micelles, as shown in Table 1. This increase of micelles size could be caused by PTX encapsulation in the micelle core [25]. The DLC and DLE data are also summarized in Table 1. HPMC-PLLA$_{10}$ presents a DLE of 45.6% and a DLC of 4.8%. Higher DLE and DLC are obtained for HPMC-PLA$_{20}$, HPMC-PLA$_{30}$ and HPMC-PLA$_{40}$. HPMC-PLA$_{40}$ presents the highest DLE and DLC of 59.8% and 6.4%, respectively. Surprisingly, lower DLE and DLC are obtained for HPMC-PLLA$_{20}$. This discrepancy could be attributed to the higher crystallinity of PLLA$_{20}$ as mentioned above since higher crystallinity disfavors drug loading [26]. Increasing DLC and DLE are obtained with increase of PLA block length from HPMC-PLA$_{20}$ to HPMC-PLA$_{20}$, in agreement with literature. It has been

reported that the DLC of PCL-PEG-PCL micelles encapsulating nimodipine increases from 3.03 to 4.96% with increasing PCL block length [23]. Similar results are obtained for PEG-PCL micelles encapsulating amiodarone [27]. It is also noteworthy that the drug loading capacity of HPMC-PLA is comparable with those of PEG-PLA copolymer micelles which present a DLC from 4.7% to 6.5% [6].

HPMC-PLA micelles were selected for drug release studies as they exhibit better drug loading capacity as compared to HPMC-PLLA ones. Fig. 6 shows the release profiles of PTX from copolymers micelles. A biphasic pattern is observed. All micelles exhibit a large burst release of *c.a.* 40% at 6 h, followed by a slower release to reach 80.4%, 73.8%, and 66.7% at 120 h for HPMC-PLA$_{20}$, HPMC-PLA$_{30}$ and HPMC-PLA$_{40}$, respectively. The burst release could be assigned to the fact that drug is partly located at the core-shell interface [26]. HPMC-PLA$_{40}$ exhibits the lowest release ratio at the later stages. Nevertheless, the amount of cumulative released drug is very close for the 3 copolymers. Similar results has been reported for PCL-PEG-PCL micelles which exhibit a decrease of drug release rate with increase of PCL block length [23].

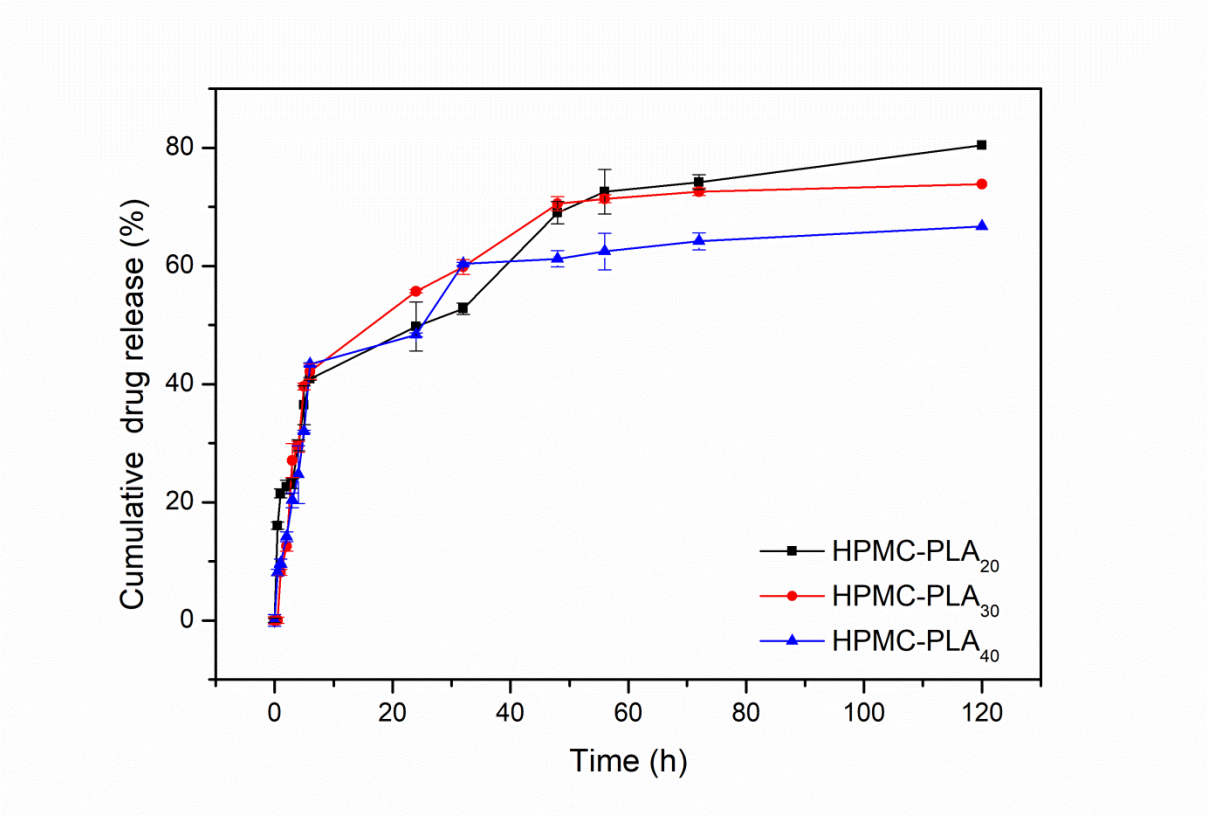


Fig. 6. In vitro release curves of PTX from HPMC-PLA copolymer micelles. Each point represents mean ± SD for three samples. Error bars represent the standard deviation (n = 3)

3.4 Biocompatibility of HPMC-PLA copolymer micelles

In vitro cytotoxicity of blank micelles is evaluated by MTT assay using murine fibroblasts L-929 cells [28]. The effect of HPMC-PLLA$_{10}$ and HPMC-PLLA$_{20}$ micelle solutions on the growth of L-929 cells is shown in Fig. 7A and 7B. The cell viability of cells remains above 95% when the micelle concentration increases from 0.25 to 5.0 mg/mL, and after incubation up to 72 h. In contrast to the positive control, HPMC-PLLA micelles are not toxic to L-929 cells according to the US Pharmacopeia Standards.

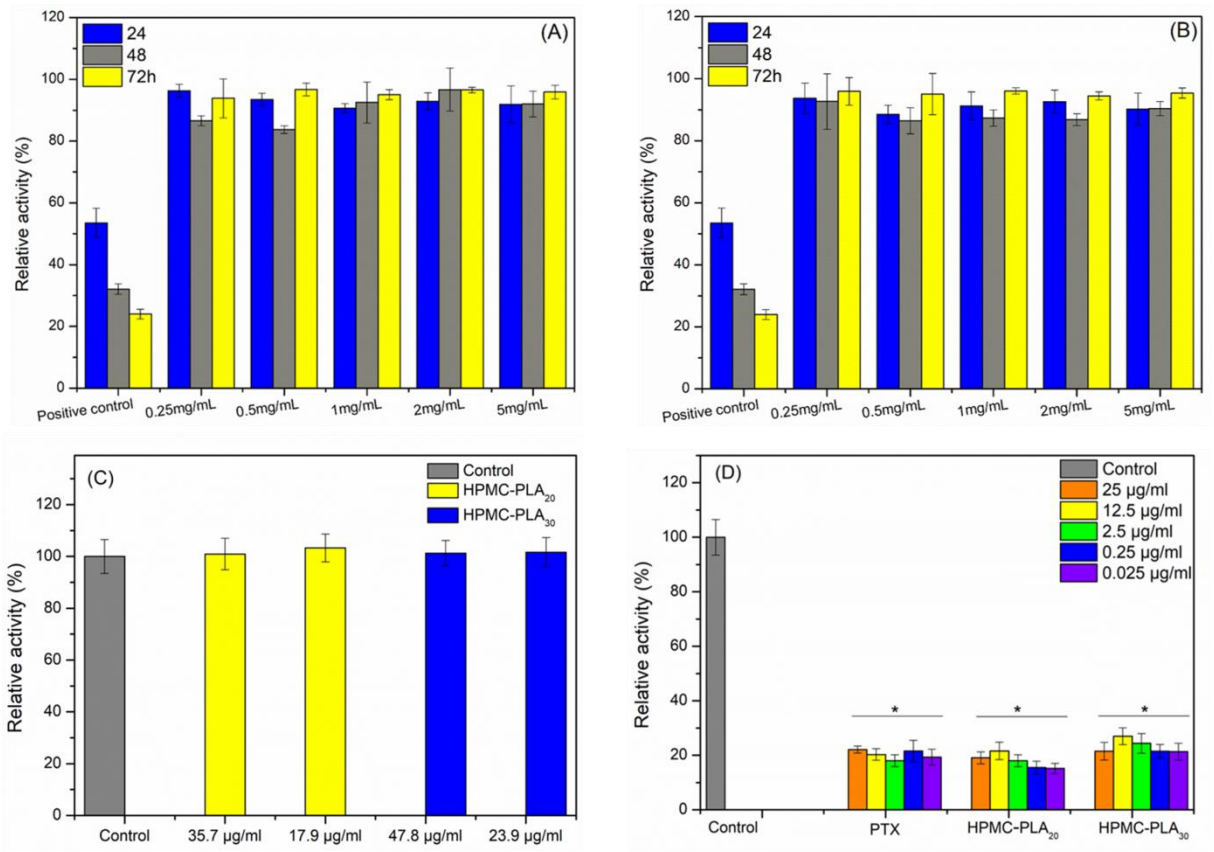


Fig. 7. Relative activity of L-929 cells after 24, 48 and 72 h culture with HPMC-PLLA$_{10}$ (A) and HPMC-PLLA$_{20}$ (B) micelle solutions at different concentrations compared to the positive control. Data are presented as the mean ± sd (n = 3). Relative activity of SK-BR-3cells after 72 h culture with blank micelles of HPMC-PLA$_{20}$ and HPMC-PLA$_{30}$ at different concentrations (C), free PTX and drug-loaded micelles at different PTX concentrations compared to the positive control (D). Data are presented as the mean ± sd (n = 12) (*P < 0.05 *versus* the control)

3.5 Cytotoxic activity of drug-loaded micelles

In vitro cytotoxicity of drug-loaded micelles was evaluated by SRB assay using a human breast cancer cell line, SK-BR-3 cells. SRB assay was first used by Skehan et al. to determine cell density by measuring cellular

protein content [29]. SRB is a water-soluble protein dye that can bind with residues basic amino acids of protein in cells under mild acidic conditions, and can be dissolved under mild basic conditions. The amount of SRB bound in cells reflects the total protein amount and thus the number of cells. A linearity is observed over a 20-fold range with high reproducibility [30].

The effect of HPMC-PLA_{20} and HPMC-PLA_{30} blank micelles on the growth of SK-BR-3 cells is shown in Fig. 7C. Blank micelles were tested at concentrations corresponding to those of drug-loaded micelles (18 – 48 μg/mL). The viability of cells cultured with blank micelles is comparable to that of the control. Even the highest micelle concentration did not affect the cell viability.

The effect of free PTX and PTX-loaded micelles on the proliferation of SK-BR-3 cells was examined in the PTX concentration range of 0.025–25 μg/mL as shown in Fig. 7D. For both free PTX and drug-loaded micelles, even the lowest concentration (0.025 μg/mL) significantly reduced cell proliferation. The cytotoxic effect was comparable in the whole PTX concentration range. Similar cytotoxicity on SKBR3 cells was observed after treatment with 0.3-30 nM PTX or Tripalm-NPs-PTX [31].

4 Conclusion

Amphiphilic HPMC-PLLA and HPMC-PLA diblock copolymers with different hydrophobic block lengths were synthesized by using combination of ROP and reductive amination. NMR, DOSY-NMR, and FT-IR analyses showed that block copolymers were successfully obtained. All copolymers can self-assemble in aqueous medium into spherical micelles with narrow distribution. The CMC decreases with increase of hydrophobic block length. The DLC and DLE of copolymers increase with increasing PLA block length. HPMC-PLA micelles exhibit biphasic drug release. Both MTT and SRB assays demonstrate the outstanding cytocompatibility of HPMC-PLA copolymers. Last but not least, PTX-loaded micelles exhibit significant cytotoxicity to SK-BR-3cells, thus showing that HPMC-PLA copolymer micelles could be most promising as nano-carrier of antitumor drugs.

Acknowledgements

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