Original article

Self-assembled filomicelles prepared from polylactide-poly(ethylene glycol) diblock copolymers for sustained delivery of cycloprotoberberine derivatives

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ABSTRACT

Polylactide-poly(ethylene glycol) (PLA-PEG) block copolymers were synthesized by ring opening polymerization of l-lactide using a monomethoxy PEG (mPEG) as macromonomer and zinc lactate as catalyst. The resulting diblock copolymers were characterized by 1H NMR and GPC. Polymeric micelles were prepared by self-assembly of copolymers in distilled water using co-solvent evaporation or membrane hydration methods. The resulting micelles are worm-like in shape as shown by TEM measurements. A hydrophobic anticancer drug, cycloprotoberberine derivative A35, was successfully loaded in PLA-PEG filomicelles with high encapsulation efficiency (above 88%). Berberine (BBR) was studied for comparison. In both methods, PLA-PEG filomicelles were prepared with a theoretical loading of 5%, 10% and 20%. Physical stability studies indicated that BBR/A35-loaded filomicelles were more stable when stored at 4°C than at 25°C. Compared with BBR-loaded filomicelles, A35-loaded filomicelles exhibited higher anti-tumor activity. Importantly, the in vitro cytotoxicity and stability of A35-loaded filomicelles evidenced the potential of drug-loaded filomicelles in the development of drug delivery systems.

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1. Introduction

Berberine (BBR, 1, Fig. 1) is one of the most frequently used herbal medications in East Asia. It’s an isoquinoline alkaloid isolated from the rhizome, roots and stem bark of a number of Chinese herbs of Berberis species (Godugu et al., 2014; Ning et al., 2009). BBR presents various pharmacological activities such as antibacterial, antioxidant, cholesterol-lowering, and anti-inflammatory activities (Liang et al., 2014; Riccardi et al., 2001; Li et al., 2014; Kong et al., 2004; Choi et al., 2006). It also exhibits anticancer effects in a variety of cancer cells including glioblastoma, hepatoma, melanoma, colon, breast, prostate, oral, colorectal, lung, leukemia, and osteosarcoma (OGuaman Ortiz et al., 2014). However, BBR presents some side effects as it can lower blood sugar and blood pressure. Various derivatives of BBR have been obtained in the past years as a novel class of anticancer drugs. A cycloprotoberberine derivative, namely A35 (2, Fig. 1) was accidentally obtained and structurally identified (Li et al., 2013). A35 exhibits higher antiproliferative activity as compared to BBR (Zou et al., 2017). Nevertheless, both BBR and A35 are poorly water soluble, and present poor bioavailability. Thus it is of crucial importance to improve the bioavailability of BBR and A35 by using a drug carrier.

Nowadays, amphiphilic block copolymers composed of a hydrophilic block such as poly(ethylene glycol) (PEG) and a degradable
and hydrophobic polyester block such as polylactide (PLA) or poly(ε-caprolactone) (PCL) have been widely studied as drug carrier in sustained drug delivery systems (Ahmed and Discher, 2004). They can self-assemble in aqueous media to form micelles with a core-shell structure (Letchford and Burt, 2007). The hydrophobic blocks aggregate to form an inner core able to encapsulate hydrophobic drugs, while the hydrophilic ones constitute the shell ensuring micelles' stability and long circulation in the bloodstream (Chen et al., 2008). Hydrophobic drugs can be loaded at much higher concentrations than their inherent water solubility in the micelle core by chemical, physical or electrostatic interactions (Kataoka et al., 2001). Various micelle structures have been obtained from PLA-PEG or PCL-PEG block copolymers such as spherical micelles, rod-like micelles, worm-like or filomicelles, polymersomes and nanotubes, mainly depending on the hydrophilic/hydrophobic balance of copolymers (Blanazs et al., 2012). Among them, filomicelles have been attracting increasing attention as hydrophobic drug carrier since they can achieve long circulation time and high drug loading as compared to spherical micelles (Liu et al., 2012; Daum et al., 2012; Christian et al., 2009). Moreover, better accumulation of filomicelles at tumor sites has been evidenced by several studies showing low phagocytic uptake, deep penetration, enhanced vascular targeting and tumor homing, and rapid diffusion into tissue (Geng et al., 2007; Kim et al., 2005; Lee et al., 2013).

In this work, two PLA-PEG diblock copolymers were synthesized by ring opening polymerization of ε-lactide using monomethoxy PEG2000 as macro-initiator and zinc lactate as catalyst. Polymeric micelles were prepared by self-assembly of the resulting copolymers using co-solvent evaporation and membrane hydration methods (Jelonek et al., 2015; Zhang et al., 2012a,b). A35 or BBR were encapsulated in the core of micelles with a theoretical loading of 5%, 10% or 20%. The in vitro cytotoxicity and stability of drug loaded micelles were determined in order to evaluate the potential of PLA-PEG filomicelles as carrier of A35 and BBR for prolonged release.

2. Materials and methods

2.1. Materials

Monomethoxy PEG (mPEG) with molar mass of 2000, ε-lactide and zinc lactate were obtained from Sigma-Aldrich (St-Quentin Fallavier, France). ε-lactide was purified by crystallization from ethyl acetate before use. A35 and BBR were provided by Chinese Academy of Medical Science & Peking Union Medical College. All other organic solvents were of analytic grade from SCRC (China) and used without further purification.

2.2. Synthesis of PLA-PEG diblock copolymers

Two PLA-PEG diblock copolymers were synthesized by ring opening polymerization as described previously (Li and Vert, 2003). In brief, predetermined amounts of ε-lactide and mPEG were introduced into polymerization ampoule. The initial molar ratio of ethylene oxide to lactate repeat units (EO/LA) was 1.5/1 or 3/1. Zinc lactate (0.1 wt%) was then added. After degassing, the ampoule was sealed under vacuum, and polymerization proceeded for 72 h at 140 °C. The product was recovered by dissolution in dichloromethane and precipitation in diethyl ether. The product was finally dried under vacuum to constant weight.

2.3. Preparation of drug-free micelles

Drug-free micelles were prepared by using co-solvent evaporation method (Jelonek et al., 2015). Typically, 10 mg of copolymer were dissolved in 200 μL chloroform and the solution was added in 10 mL of distilled water. The mixture was vigorously stirred at room temperature for 3.5 h, and left for solvent evaporation for 24 h, yielding a micelle solution at a concentration of 1 mg/mL. Finally, the micellar solution was filtered through 0.85 μm filter to remove large aggregates.

Membrane hydration method was also used to prepare drug-free micelles (Zhang et al., 2012a,b). The copolymer was dissolved in methanol, and then the solvent was evaporated in rotary evaporator at 40 °C to yield a membrane at the wall of the round flask. Distilled water was added to the flask under stirring at room temperature, yielding micelles with a final concentration of 1 mg/mL.

2.4. Preparation of drug-loaded micelles

Two preparation methods (co-solvent evaporation and membrane hydration) were applied to encapsulate A35 or BBR in PLA-PEG micelles (Jelonek et al., 2015; Zhang et al., 2012a,b). In method 1, the drug was dissolved in methanol, and the solution was added to a previously prepared micelle solution. Drug encapsulation was achieved by stirring for 4.5 h. In method 2, both the drug and copolymer were dissolved in methanol. And then membrane hydration method was applied to obtain drug-loaded micelles. In both methods, PLA-PEG micelles were prepared with a theoretical drug loading of 5, 10 and 20%.

The resulting micellar solution was then centrifuged at 3000 rpm for 5 min to remove unloaded A35 or BBR, lyophilized and stored at 4 °C. The lyophilized micelles in the form of powder were dispersed in deionized water to yield a drug loaded micellar solution at a concentration of 1 mg/mL for physio-chemical characterization and physical stability studies.

The encapsulation efficiency (EE) and the loading content (LC) of micelles were determined as follows (Zhang et al., 2012a,b). 100 μL of drug loaded micelle solution (1 mg/mL) was added in 2.5 mL methanol. The drug concentration was determined 3 times by UV-vis spectroscopy. A calibration curve was previously established from a series of standard solutions with concentrations from 0.0025 to 0.025 mg/mL and 0.005 to 0.05 mg/mL for BBR and A35, respectively. The EE and LC were calculated from following formulae:

\[
EE = \frac{\text{weight of loaded drug}}{\text{theoretical drug loading}} \times 100\%
\]

\[
LC = \frac{\text{weight of loaded drug}}{\text{weight of drug loaded micelles}} \times 100\%
\]

2.5. Physical stability study

The physical stability of drug loaded micelles in solution was assessed by analyzing the drug content after dispersion of lyophilized micelles in distilled water. A35 or BBR-loaded micelles were stored at either 4 °C or room temperature (RT) for 6 weeks. The
drug content changes were monitored by UV–vis spectroscopy. The appearance of micelle solution was also examined during the 6 weeks period.

2.6. In vitro cytotoxicity measurement

In vitro cytotoxicity of drug-loaded micelles with a theoretical loading of 10% was evaluated by using MTT method (Min et al., 2008).

Human lung carcinoma (A549) cells were cultured in 96-well tissue culture plates at a seeding density of $1 \times 10^4$ cells per well in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, and incubated at 37 °C in 5% CO₂ for 12 h. Then, the culture medium was replaced by 100 µL of drug loaded micellar solution in DMEM at drug concentrations ranging from 0.025 to 50 µg/mL. Free drug solutions at the same concentrations were studied for the sake of comparison. The cell viability cultured with DMEM medium was used as control. After 72 h incubation, the medium was removed and replaced by 100 µL of drug loaded micellar solution in DMEM at drug concentrations ranging from 0.025 to 50 µg/mL. Free drug solutions at the same concentrations were studied for the sake of comparison. The cell viability cultured with DMEM medium was used as control. After 72 h incubation, the medium was removed and replaced by 100 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL) solution, and further incubated for 4 h. Then, the medium was removed and replaced by 150 µL of dimethyl sulfoxide (DMSO). After gentle shaking for 10 min, the optical density (OD) was determined with an enzyme-linked immuno-assay (ELISA) reader at a wavelength of 570 nm (Bhandari et al., 2012). The cell viability was calculated from the OD data of the test group and the negative control using the following equation:

$$\text{Cell viability} \left(\%ight) = \frac{\text{OD of the test group}}{\text{OD of the negative control}} \times 100\% \quad (3)$$

All experiments were carried out in triplicate. The data were expressed as the mean ± SD. P value of <0.05 is considered statistically significant.

2.7. Characterization

The composition of copolymers was determined by means of proton nuclear magnetic resonance ($^1$H NMR). The spectra were recorded at room temperature with Bruker spectrometer operating at 250 MHz, using CDCl₃ as solvent. Chemical shifts ($\delta$) were given in ppm using tetramethylsilane as an internal reference.

The molar mass and molar mass distribution of diblock copolymers were measured by gel permeation chromatography (GPC) performed on Agilent-1260 with an RI detector. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1.0 mL/min. 20 mL of solution at a concentration of 10 mg/mL were injected for analysis.

The morphology of micelles was observed by using transmission electron microscopy (TEM) performed on a JEOL JEM-2100 microscope. One drop of micelle solution at 1 mg/mL was placed on a copper grid covered with a nitrocellulose membrane, stained negatively with 2% phosphotungstic acid (PTA) and air dried before measurements.

3. Results

3.1. Characterization of PLA-PEG copolymers

Fig. 2 shows the $^1$H NMR spectrum of PLA-PEG diblock copolymers. Signals at 5.2 ppm (peak a) and 1.6 ppm (peak e) are attributed to the CH and CH₃ protons of lactyl moieties, and signals at 3.6 ppm (peak c) and 3.4 ppm (peak d) to the CH₂ main chain and CH₃ endgroup protons of PEG block. Besides, a multiplet is detected at 4.2–4.4 ppm (peak b), which is assigned to the methylene group of PEG linking to PLA block and methine group of PLA end unit. The composition or EO/LA ratio of the copolymers was determined from the integrations of NMR signals a and c belonging to lactyl moieties of PLA and ethylene oxide units of PEG, respectively. The DP of the two components and the number average molecular weight (Mn) of the copolymers were calculated according to the following equations:

$$\text{DP}_{\text{PEG}} = \frac{M_{\text{PEG}}}{44} \quad (4)$$

$$\text{DP}_{\text{PLA}} = \frac{\text{DP}_{\text{PEG}}}{[\text{EO/LA}]} \quad (5)$$

$$\text{Mn} = M_{\text{PEG}} + \text{DP}_{\text{PLA}} \times 72 \quad (6)$$

Table 1 presents the molecular characteristics of the two block copolymers. For the sake of clarity, the diblock copolymers are named as EOₘₙ. In these acronyms, L and EO represent L-PLA
and PEG blocks, respectively, and \( m \) and \( n \) represent the number-average degree of polymerization of the corresponding blocks. The DP_{PEG} is 45 for mPEG2000, and DP_{PLA} is 24 or 12 for EO_{45}LA_{24} and EO_{45}LA_{12}, respectively. Concerning the Mn, it varies from 3730 for EO_{45}LA_{24} to 2860 for EO_{45}LA_{12}. The molecular weights of the copolymers were also determined by GPC (Table 1). The Mn is 5380 and 3090 for EO_{45}LA_{24} and EO_{45}LA_{12}, respectively, which is higher than the Mn values obtained by NMR. The dispersity (D) is inferior to 1.3, in agreement with narrow molar mass distributions. The weight fraction of EO units (\( f_{EO} \)) is 0.52 and 0.73 for EO_{45}L_{24} and EO_{45}L_{12}, respectively.

3.2. Characterization of drug-loaded micelles and drug-free micelles

In this work, A35 and BBR are loaded in the core of micelles to improve the bioavailability. Two preparation methods, namely co-solvent evaporation and membrane hydration, were applied for drug encapsulation with theoretical drug loading of 5%, 10% and 20%.

The morphology of drug-loaded and drug-free micelles was examined by TEM measurements. As shown in Fig. 3, worm-like or filomicelles were observed in all cases with a diameter of about 20 nm. Drug-free EO_{45}L_{24} filomicelles are apparently shorter than that obtained by EO_{45}L_{12} (Fig. 3(a) and Fig. 3(c)). The architecture of the filomicelles seemed unchanged after encapsulation of A35 (Fig. 3(b) and Fig. 3(d)).

BBR and A35 were physically incorporated in the hydrophobic core of EO_{45}L_{24} and EO_{45}L_{12} micelles using co-solvent evaporation or membrane hydration methods. The drug loading content and encapsulation efficiency of filomicelles were obtained by using UV-vis spectroscopy (Depan et al., 2011). As shown in Table 2, when the theoretical loading of A35 increases from 5% to 20%, the loading content increases from 4.6% to 15.1% in EO_{45}L_{24} micelles prepared by using co-solvent evaporation (method 1), and the encapsulation efficiency slightly decreases from 96.0 to

| Copolymer  | \( M_n, \text{PEG} \) | EO/LA | DP_{PEG} | DP_{PLA} | Mn_{NMR} | Mn_{GPC} | D   | \( f_{EO} \) |
|------------|---------------------|------|----------|----------|-----------|-----------|-----|----------|
| EO_{45}L_{24} | 2000               | 1.86 | 45       | 24        | 3730      | 5380      | 1.3 | 0.52     |
| EO_{45}L_{12}  | 2000               | 3.88 | 45       | 12        | 2860      | 3090      | 1.1 | 0.73     |

\( a \) Date in parentheses represent the EO/LA feed ratios.

Fig. 3. TEM images of drug-free EO_{45}L_{24} micelles (a), A35-loaded EO_{45}L_{24} micelles with a theoretical loading of 10% (b), drug-free EO_{45}L_{12} micelles (c) and A35-loaded EO_{45}L_{12} micelles with a theoretical loading of 10% (d).
90.7%. Using the same method, the drug-loading content increases from 4.0% to 14.4% in EO 45L12 micelles, and the encapsulation efficiency slightly decreases from 90.8 to 86.5% with the theoretical loading of A35 increasing from 5% to 20%.

It is also of interest to compare the drug loading properties of filomicelles prepared by using co-solvent evaporation or membrane hydration methods. As shown in Table 2, when the theoretical loading of A35 increases from 5% to 20%, the drug loading content increases from 4.3% to 14.6% in EO 45L12 micelles prepared by using membrane hydration (method 2), and the encapsulation efficiency slightly decreases from 92.6 to 88.1%. These data are very close to those obtained with method 1. In the case of BBR, similar high drug loading was also obtained.

The images of A35-loaded filomicelles in distilled water at 1 mg/mL are shown in Fig. 4. In distilled water, A35 is hardly soluble and remains as sediments at the bottom. In contrast, a homogeneous and transparent brown solution is obtained in the case of A35-loaded micelles with theoretical loading of 5%, 10% and 20% (Fig. 4(A), 4(B) and 4(C)), showing that hydrophobic A35 was successfully loaded inside EO 45L12 filomicelles.

3.3. Physical stability study

The physical stability of drug-loaded micelles is crucial to their clinical performance (Chen et al., 2011). The appearance of BBR or A35-loaded filomicelle solutions remained almost unchanged when stored at 25 °C within the 6 weeks. The drug content changes in drug-loaded filomicelles during storage are shown in Fig. 5 and Fig. 6. No significant changes were observed in the content of BBR or A35 of drug-loaded filomicelles stored at 4 °C during the follow-up period of 6 weeks (Fig. 6). In the case of storage at 25 °C, it seems that drug content slightly decreased beyond 5

![Fig. 4. Images of A35 solution: (A) Sample 5 (LC = 4.0%); (B) Sample 6 (LC = 7.9%); (C) Sample 8 (LC = 14.4%).](image)

![Fig. 5. BBR or A35 content changes in drug-loaded EO45L24 filomicelles during storage at 4 °C and 25 °C. Data are represented as the mean ± SD (n = 3).](image)

![Fig. 6. BBR or A35 content changes in drug-loaded EO45L12 filomicelles during storage at 4 °C. Data are represented as the mean ± SD (n = 3).](image)
weeks (P > 0.05). Nevertheless, the content of A35 or BBR is still above 90%, which does not affect the clinical performance in all groups (P > 0.05).

3.4. In vitro cytotoxicity measurement

The in vitro cytotoxicity of drug-loaded filomicelles and free drugs was evaluated with A549 cells at drug concentrations between 0.025 and 50 µg/mL. In the meantime, blank filomicelles with the same concentrations were studied for comparison. All the 3 formulations showed dose-dependent cytotoxicity against A549 cells (Fig. 7).

As shown in Fig. 7(A), the cell viability of EO45L24 and EO45L12 blank filomicelles slightly decreased with increasing micelle concentration from 0.25 to 500 µg/mL (P > 0.05). It was 87.7% and 85.9% at the highest tested concentration (500 µg/mL) for EO45L24 and EO45L12, respectively. In the case of free drugs, A35 exhibited greater antiproliferative activity than BBR in the whole concentration range as shown in Fig. 7(B). At 50 µg/mL, for example, the cell viability was 47.0% and 38.6% for BBR and A35, respectively. In the case of drug-loaded filomicelles, the cell viability decreased with increasing drug concentration (Fig. 7(C)). At 50 µg/mL, the cell viability was 54.2%, 60.7%, 52.3%, 57.6%, 50.2%, 55.8% for sample 2, 3, 6, 7, 10 and 11, respectively.

4. Discussion

The architecture of self-assembled aggregates depends not only on the hydrophilic/hydrophobic balance of the amphiphilic copolymers (Discher and Eisenberg, 2002; Jelonek et al., 2015), but also on the chain regularity of the hydrophobic block and the preparation method. Discher reported that with increasing the PEG fraction of PCL-PEG diblock copolymers, the corresponding architecture changes from solid-like particles, fluid-like vesicles, worm-like or filomicelles to spherical micelles (Discher and Eisenberg, 2002). In the present work, filomicelles were obtained by self-assembly of EO45L24 and EO45L12 copolymers using co-solvent evaporation or membrane hydration methods. It is of interest to note that in our previous work, needle-like micelles were obtained from a similar EO45L12 copolymer by using direct dissolution method (Wu et al., 2011). On the other hand, Cai et al. showed that worm-like and spherical micelles could be prepared from the same PEG-PCL copolymer (Cai et al., 2007). The authors first obtained filomicelles by using co-solvent evaporation. And then spherical micelles were obtained by sonication of the filomicelles. The chain structure regularity of the hydrophobic block also plays a major role in the self-assembly process. Recently, we have shown that poly(L-lactide-co-glycolide)-PEG, poly(DL-lactide)-PEG and ploy(L-caprolactone-co-glycolide)-PEG copolymers lead to spherical micelles, whereas poly(L-lactide)-PEG and PCL-PEG copolymers with similar PEG fraction lead to filomicelles (Liu et al., 2017; Jelonek et al., 2015; Sun et al., 2017).

As a cycloprotoberberine derivative, A35 exhibits outstanding antiproliferative activity. But its application is limited by the poor water solubility (Zou et al., 2017). In this work, BBR and A35 were physically incorporated in the hydrophobic core of micelles to improve the bioavailability, using co-solvent evaporation and membrane hydration methods. EO45L24 filomicelles with longer hydrophobic blocks showed slightly better drug loading properties than EO45L12 ones, in agreement with literature (Huh et al., 2005; Xiao et al., 2011). With theoretical drug loading content of 5, 10, or 20%, very high encapsulation efficiency ranging from 86.5% to 96.0% was obtained. No significant difference in drug loading properties of the two methods was observed, indicating that both are very efficient drug loading methods.

The stability of drug formulations is of crucial importance for clinical applications. The drug contents of BBR or A35-loaded filomicelles remained unchanged during 4 weeks storage at 25 °C or 6 weeks storage at 4 °C. These findings suggest that the drug-loaded filomicelles could be safely used for clinical applications.

In vitro cytotoxicity of drug-loaded micelles with a theoretical loading of 10% was evaluated with A549 cells by using MTT method. Samples 6 and 7 prepared from EO45L12 present lower cell viability than samples 2 and 3 prepared from EO45L24 at concentrations up to 50 µg/mL (P < 0.05). This finding suggests that EO45L12 drug-loaded filomicelles showed better antitumor effect than EO45L24 probably because of the faster drug release of EO45L12. In fact, it has been
shown that drug release rate decreases with increasing PLA block length for PEG–PLA filomicelles (Jelonek et al., 2015). On the other hand, comparison between A35 loaded samples 2, 6 and 10 with BBR loaded samples 3, 7 and 11 shows that A35 exhibits higher antitumor activity than BBR. This is in agreement with the data obtained for free drug solutions. At the same drug concentrations, free drug solutions exhibit higher antitumor activity than drug loaded micelle solutions, which could be assigned to the slow drug release process for the latter. Last but not least, blank filomicelles prepared from PEG–PLA copolymers have insignificant effect on release process for the latter. Last but not least, blank filomicelles free drug solutions exhibit higher antitumor activity than drug loaded samples 3, 7 and 11 shows that A35 exhibits higher antitumor activity than BBR. It is thus concluded that A35-loaded filomicelles prepared from PEG–PLA copolymers are promising for anti-cancer applications. The drug release behaviors, pharmacokinetics, biodistribution and efficacy of A35-loaded filomicelles will be further studied.

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