Nanoscale Res Lett (2009) 4:1502–1511
DOI 10.1007/s11671-009-9427-2

NANO EXPRESS

Self-Assembled Polymeric Micellar Nanoparticles as Nanocarriers for Poorly Soluble Anticancer Drug Ethaselen

Xinru Li · Zhuoli Yang · Kewei Yang · Yanxia Zhou · Xingwei Chen · Yanhui Zhang · Fei Wang · Yan Liu · Lijun Ren

Received: 10 June 2009 / Accepted: 19 August 2009 / Published online: 16 September 2009

Abstract A series of monomethoxy poly(ethylene glycol)-poly(lactide) (mPEG-PLA) diblock copolymers were synthesized, and mPEG-PLA micelle was fabricated and used as a nanocarrier for solubilization and delivery of a promising anticancer drug ethaselen. Ethaselen was efficiently encapsulated into the micelles by the dialysis method, and the solubility of ethaselen in water was remarkably increased up to 82 μg/mL before freeze-drying. The mean diameter of ethaselen-loaded micelles ranged from 51 to 98 nm with a narrow size distribution and depended on the length of PLA block. In vitro hemolysis study indicated that mPEG-PLA copolymers and ethaselen-loaded polymeric micelles had no hemolytic effect on the erythrocyte. The enhanced antitumor efficacy and reduced toxic effect of ethaselen-loaded polymeric micelle when compared with ethaselen-HP-β-CD inclusion were observed at the same dose in H_{22} human liver cancer cell bearing mouse models. These suggested that mPEG-PLA polymeric micelle nanoparticles had great potential as nanocarriers for effective solubilization of poorly soluble ethaselen and further reducing side effects and toxicities of the drug.

Keywords Monomethoxy poly(ethylene glycol)-poly (lactide) · Polymeric micelles · Hemolysis · Ethaselen · Antitumor efficacy

X. Li · Y. Zhou · X. Chen · Y. Zhang · F. Wang · Y. Liu (✉)
Department of Pharmaceutics, School of Pharmaceutical Sciences, Peking University, Xueyuan Road 38, 100191 Beijing, Haidian, People's Republic of China
e-mail: yanliu@bjmu.edu.cn

Z. Yang
TEAM Academy of Pharmaceutical Sciences, Beijing, People's Republic of China

K. Yang
Department of Pharmaceutical Technology, Institute of Pharmacy, University Jena, Jena, Germany

L. Ren
Department of Three, Institute of Chemical Defence, Beijing, People's Republic of China

Introduction

BBSKE (Fig. 1), chemically named (1,2-[bis(1,2-benzisoselenazol-3(2H)-one)]ethane), is a novel organic selenium compound, which is one of the derivatives of ethaselen (For convenience, ethaselen is referred to BBSKE in this study). It showed a positive antitumor activity and became a potential anticancer agent with lower toxicity and side effects [1, 2]. Unfortunately, ethaselen is poorly soluble in water (2.57 μg/mL) and in commonly used organic solvents such as methanol, ethanol, ether and chloroform. Its bioavailability by oral administration is also considerably low. Poor solubility creates major obstacles for formulations and successful chemotherapy with ethaselen. Although several methods including drug delivery systems were investigated [3], developing a ethaselen delivery system for higher selectivity, efficient solubilization and delivery of ethaselen to the intended site without provoking any adverse reactions is still a challenge.

Recently, polymeric micelles as a means to solubilize poorly water-soluble drugs have attracted much attention [4–6]. Generally, block copolymers with concentration above the critical association concentration (CAC) self-assemble into spherical polymeric micelles with a core–shell structure in water: the hydrophobic segments aggregate to form an inner core being able to accommodate hydrophobic drugs with improved solubility by hydrophobic interactions;

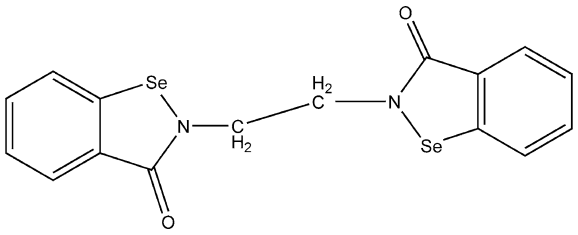

Fig. 1 Chemical structure of BBSKE

the hydrophilic shell consists of a brush-like protective corona that stabilizes the micelles in aqueous solution [7–9]. Polymeric micelles as novel drug vehicles present numerous advantages, such as reduced side effects of anticancer drugs, selective targeting, stable storage and prolonged blood circulation time [9, 10]. Furthermore, polymeric micelles possess a nanoscale size range with a narrow distribution, and they can achieve higher accumulation at the target site through an enhanced permeation retention effect (EPR effect) [11]. They can protect drugs against premature degradation in vivo owing to their core–shell architecture [12, 13]. More importantly, polymeric micelles are fabricated according to the physicochemical properties of drugs and the compatibility between the core of micelles and drug molecules [9, 14].

Biodegradable polymers, especially aliphatic polyesters such as polylactide (PLA), poly(DL-lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) have attracted much attention as biomaterials due to their biocompatibility, degradability and nontoxicity. They have been applied in a wide range of biological systems ranging from drug delivery to tissue engineering. PLA is the most attractive candidate. A number of PLA-based amphiphilic block copolymer micelles have been commonly used for solubilization of hydrophobic drugs [15–17]. In our research, PLA was also chosen as hydrophobic segment of a block copolymer due to the higher compatibility between the micelle core-forming PLA and the ethaselen molecules. In previous work, our group has prepared micellar ethaselen formulation employing PLA-based copolymers and characterized its drug loading contents and stability (data not shown), which have demonstrated the compatibility between the core of micelles and the ethaselen molecules. Polyethylene glycol (PEG) is frequently chosen as a hydrophilic segment to complement a hydrophobic segment due to the outstanding physicochemical and biological properties including solubility in water and in organic solvents, nontoxicity and filterability through kidney when the molar mass is below 30,000 [18]. In addition, PEG is able to form a palisade avoiding protein adsorption and subsequent nonspecific uptake by the reticuloendothelial system (RES) after intravenous injection.

In the present work, amphiphilic diblock copolymers monomethoxy poly(ethylene glycol)-poly(lactide) (mPEG-PLA) with molecular weight of 2,500, 5,000, 10,000 and 15,000 for PLA block were strategically designed and synthesized by ring-opening polymerization of D,L-dilactide (D,L-LA) in the presence of mPEG with molecular weight of 5,000, respectively. The micelles preparation, ethaselen solubilization and micelles properties were investigated by the UV–vis assay, size measurement and TEM in the micellar solution or in the state of lyophilized powder. The CAC of mPEG-PLA was measured by using the standard fluorescence substance of pyrene. Ethaselen-loaded polymeric micelle was evaluated with respect to its hemolytic toxicity and antitumor efficacy.

Materials and Methods

Materials

D,L-Dilactide was obtained from Fluka. Stannous 2-ethylhexanoate and monomethoxy poly(ethylene glycol) with molecular weight of 5,000 were purchased from Sigma–Aldrich. All other chemicals and reagents were of analytical grade or better and used without further purification.

Animals

Male Kunming mice were obtained from Experimental Animal Center of Peking University and acclimatized for 7 days after arrival. New Zealand White rabbits were purchased from the same supplier and acclimatized for 2 days after arrival. All animals were provided with standard food and water ad libitum and were exposed to alternating 12-h periods of light and darkness. Temperature and relative humidity were maintained at 25 °C and 50%, respectively. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

Synthesis and Characterization of mPEG-PLA

mPEG-PLA diblock copolymers were synthesized from D,L-dilactide and mPEG using stannous 2-ethylhexanoateas as a catalyst as described previously [19] with modification. Briefly, D,L-dilactide was recrystallized in ethyl acetate at room temperature until the racemic mixture melting point was attained (124–126 °C), and mPEG was used without further purification. The synthesized diblock copolymers were referred to as mPEG*x*-PLA*y*. *x* and *y* represented the weight-averaged molecular weight of the mPEG and PLA block in kDa. mPEG5-PLA2.5, for

example, consisted of a 5 kDa mPEG block connected to a 2.5 kDa PLA block.

mPEG*x*-PLA*y* was obtained by modulating the feed ratio of D,L-dilactide and mPEG. The molecular weight of mPEG block was 5,000 (mPEG5). The molecular weights of PLA block were 2,500 (PLA2.5), 5,000 (PLA5), 10,000 (PLA10) and 15,000 (PLA15), respectively. As a typical example, the synthesis of mPEG5-PLA15 was carried out as follows: to remove any trace of water, the starting materials (5 g of mPEG5 and 15 g of D,L-dilactide) were each dissolved in 100 mL toluene in a round-bottomed flask. About 40 mL of toluene was distilled off using a water separator. The water-free solutions were united in a three-neck flask, a precisely weighed amount of 25 mg stannous 2-ethylhexanoate was added, and the mixture was refluxed for 24 h at 120 °C under nitrogen atmosphere. After toluene was distilled off with a rotary evaporator, the residue was redissolved by the addition of appropriate amount of chloroform, and vigorously stirred and precipitated in diethyl ether at 0 °C, and then filtered. The precipitated polymer was dried in a desiccator at room temperature under high vacuum. After drying for 2 days, white solid powder of the copolymer was obtained. The resulting copolymers were dissolved in $CDCl_3$, and ^{1}H-NMR spectra were taken at 300 MHz with trimethylsilane (TMS) as internal reference standard using a Bruker MSL2300 spectrometer (Bruker, Germany). The copolymer molecular weights were determined by gel permeation chromatography (GPC).

Determination of Critical Association Concentration (CAC)

The CAC values of mPEG-PLA diblock copolymers were determined by fluorescence spectroscopy using pyrene (Fluka, >99%) as a hydrophobic probe [12]. Briefly, a known amount of pyrene in acetone was added to each of a series of 50 mL vials, and the acetone was evaporated, then a known amount of various concentrations of mPEG-PLA solutions in acetone were added to each vial, and the acetone was evaporated. The appropriate amount of distilled water was then added to each vial to obtain polymeric aqueous solutions with final concentration of 2.24×10^{-4}–224 mg/L. The final concentration of pyrene was 6.0×10^{-7} mol/L. The sample solutions were kept in a constant temperature shaking water bath at 37 °C for 24 h to equilibrate the pyrene and the micelles, and cooled overnight at room temperature. The solutions were filtered with a 0.22 μm pore-sized filtration membrane (Millex-GV, Millipore, USA). Fluorescence spectra of pyrene were recorded with a Shimadzu RF-5301 PC fluorescence spectrometer. The excitation wavelength used was 333 and 335 nm, and the emission spectra were recorded at 390 nm. The peak height intensity ratio (I_{335}/I_{333}) of the peak of 335 nm to the peak of 333 nm was plotted against the logarithm of polymer concentration. Two tangents were then drawn, one to the curve at high concentrations and another through the points at low concentrations. The CAC value was taken from the intersection between the two tangents.

Preparation of Ethaselen-Loaded Polymeric Micelles

Ethaselen-loaded polymeric micelle (ethaselen-PM) was prepared by the dialysis method [20]. Briefly, 100 mg of mPEG-PLA and 20 mg of ethaselen were dissolved in 45 mL dimethyl sulfoxide (DMSO). The mixture was introduced into a dialysis bag (Spectrapor, MWCO = 3,500 g/mol), and then dialyzed against 4 L of physiological saline, which was replaced every 12 h in the course over 48 h. The suspension in the dialysis bag was then filtered through a 0.22 μm filter to remove aggregates. To determine drug loading content (LC, w/w %) and entrapment efficiency (EE, w/w %) of micelles, the ethaselen-loaded micelle solution was lyophilized using PEG6000 as a lyoprotectant, and then dissolved in DMSO by ultrasonication for 15 min, and ethaselen content was measured with ultraviolet–visible spectrophotometer (Agilent 8453, Agilent Technologies, UK) at 320 nm. The LC and EE of the micelles were then calculated based on the following formula:

$$\mathrm{LC}(\%) = \frac{\text{mass of ethaselen extracted from freeze-dried micelles}}{\text{total mass of freeze-dried micelle}} \times 100\%$$

$$\mathrm{EE}(\%) = \frac{\text{mass of ethaselen extracted from freeze-dried micelles}}{\text{total mass of Eb loaded micelle initially used}} \times 100\%.$$

Particle Size and Morphology Analysis

The average hydrodynamic radius of the micelles was determined by dynamic light scattering (DLS) (Zetasizer ZEN 3500, Malvern, UK). All DLS measurements were done with an angle detection of 173° at 25 °C after diluting the dispersion to an appropriate volume with water. The results were the mean values of three experiments for the same sample. The morphology of micellar nanoparticles was also observed by transmission electron microscopy (TEM) (Hitachi-500, Hitachi, Japan). To improve the contrast, the samples were treated with a 1 wt% phosphotungstic acid solution for 2 h, deposited on copper grids, and allowed to dry for 48 h before TEM examination.

Physical Stability of Ethaselen-Loaded Micelles

The lyophilized powder and the polymeric micelle solution were stored at room temperature. Their physical stability was monitored over time by dynamic light scattering and visually for signs of opalescence and precipitation. The leakage percent was also measured by using the same method as the determination of LC and EE described earlier. In addition, the concentrated magnitude of micelle solution by freeze-drying compared with initially prepared micelle solution was evaluated by comparing the volume of the reconstituted micelle solution of lyophilized powder with that of initially prepared micelle solution. It should be noted that the volume of the reconstituted micelle solution of lyophilized powder was the smallest volume of physiological saline in which the obtained lyophilized powder was redissolved to produce a clear micelle solution.

Hemolysis Assay

The effect of the copolymers on the integrity of erythrocyte membranes was investigated by in vitro hemolysis assay [21]. The release of hemoglobin from the erythrocytes (RBC) was used as a measure of toxicity of these copolymers. Briefly, rabbit RBC was separated from 20 mL fresh rabbit blood by centrifugation at 1,500 rpm for 15 min and then washed three times with 20 mL of normal saline. The purified RBC was resuspended in normal saline to obtain 2% (v/v) of RBC suspension. Then 2 mL of the RBC suspension was incubated with 3 mL of the drug-free mPEG-PLA micelle solution (copolymer concentration: 0.5 and 1 mg/mL) or ethaselen-loaded mPEG-PLA micelle solution (ethaselen concentration: 0.1 and 0.2 mg/mL) at 37 °C for 1 h in an incubator shaker and then centrifuged at 5,000 rpm for 10 min. The percentage of hemolysis was measured by UV–vis analysis of the supernatant at 576 nm absorbance. Normal saline was used as the negative control with 0% hemolysis, and distilled water was used as the positive control with 100% hemolysis. All hemolysis data points were presented as the percentage of the complete hemolysis. Hemolysis percent (HP%) was calculated according to the following equation:

$$\mathrm{HP\%} = \frac{\mathrm{ABS_{sample}} - \mathrm{ABS_{saline}}}{\mathrm{ABS_{distilled\ water}} - \mathrm{ABS_{saline}}} \times 100.$$

Assay for Antitumor Efficacy

Sixty of male Kunming mice (body weight = 18–22 g, Peking University Experimental Animal Center, SPF-level, Quality certificated Number: SCXK 2007-0008) were randomly divided into six groups. And seven-day-old liver cancer H_{22} ascites (0.2 mL, 2×10^6 cells) were transplanted subcutaneously into the right axilla of each mouse of the groups. The mice were treated as follows: negative control group (normal saline); ethaselen-HP-β-CD group (1 mg/kg body weight); ethaselen-loaded mPEG5-PLA2.5 micelle group (1 mg/kg body weight, low dose); ethaselen-loaded mPEG5-PLA2.5 micelle group (2 mg/kg body weight, middle dose); ethaselen-loaded mPEG5-PLA2.5 micelle group (4 mg/kg body weight, high dose). All the groups were administered through the tail vein of animals once daily for 10 days, starting 24 h after tumor implantation. At day 11, all the mice were killed by cervical dislocation following by separation and measurement of the tumor block. The antitumor efficacies of each formulation were evaluated by tumor inhibition rate, which was calculated by the following formula: inhibition rate = (tumor weight of test group − tumor weight of negative control group)/tumor weight of negative control group × 100%.

Statistical Analysis

All data were expressed as mean ± SD (standard deviation). Comparisons between the group means were evaluated by the unpaired t test. The statistical significance of differences among more than two groups was determined by one-way ANOVA. A value of $p < 0.05$ was regarded as significant.

Results and Discussion

Synthesis and Characterization of mPEG-PLA

mPEG-PLA copolymers were synthesized by ring-opening polymerization of D,L-dilactide by using mPEG as initiator. Various chain lengths of PLA in the copolymers were obtained by modulating the feed ratio of mPEG and D,L-dilactide. Figure 2 showed the ^{1}H-NMR spectrum of mPEG5-PLA15, which was representative for all synthesized mPEG-PLAs: the peaks at 3.65 and 3.36 ppm corresponded to methylene units and CH_3O- in the mPEG blocks, signals at 1.58 and 5.18 ppm could be attributed to the hydrogen atoms of CH_3- and CH-groups for PLA segments, respectively. From the peak integrity ratio of their methylene and methyl groups, the mass ratio of repeating units in mPEG and PLA blocks could be calculated in each polymer. The results of this analysis were summarized in Table 1. The values were very close to those of feed compositions. Furthermore, the molecular weight data (M_w, M_n) and polydispersity indexes (PI = M_w/M_n) resulting from GPC analysis of the copolymers after synthesis were also listed in Table 1. The number-averaged molecular weight (M_n) of the mPEG and PLA blocks of each polymer was calculated from ^{1}H-NMR data. The last column showed the actual weight ratio of mPEG/PLA of the polymers (as

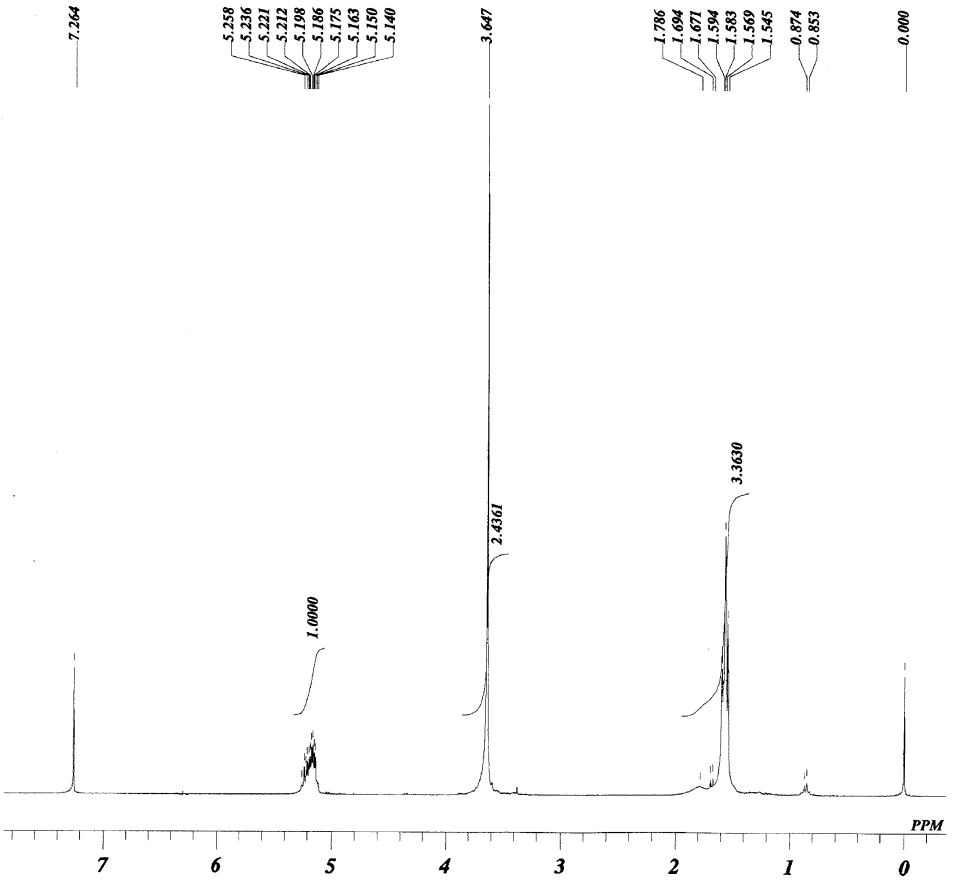

Fig. 2 ^{1}H NMR spectrum of mPEG5-PLA15 copolymer in $CDCl_3$

Table 1 Survey on the composition of mPEG-PLAs

Feed ratio mPEG/DL-LA	mPEG*x*-PLA*y*	M_w^a	M_n^b	PI[c]	M_n (mPEG)	M_n (PLA)	Found ratio mPEG/PLA
2:1	mPEG5-PLA2.5	9,300	7,500	1.16	5,000	2,450	67:33
1:1	mPEG5-PLA5	13,500	11,500	1.26	5,000	4,560	52:48
1:2	mPEG5-PLA10	19,600	13,800	1.54	5,000	9,650	34:66
1:3	mPEG5-PLA15	25,300	16,200	1.66	5,000	15,060	25:75

[a] Number-average molecular weight of mPEG*x*-PLA*y*

[b] Weight-average molecular weight of mPEG*x*-PLA*y*

[c] Polydispersity index

determined from ^{1}H-NMR data). The PI of the mPEG-PLAs indicated a narrow molecular weight distribution for all polymers. Altogether the results confirmed that the copolymers could be synthesized reliably. The yield of the reactions ranged from 89 to 94%.

Polymeric micelles can be formed only when the block copolymer concentration is higher than CAC, which characterizes the micelle stability [6]. Compared with low molar mass surfactant micelles, polymeric micelles are generally more stable, exhibiting a remarkably lower CAC [22]. They are liable to retain thermodynamic stability even after intravenous injection, which induces severe dilution [7, 23]. Table 2 summarized the CAC values of the various synthesized mPEG-PLA diblock copolymers ranging from 0.96 to 2.31 $\times$ 10^{-7} mol/L or 1.71 to 2.12 mg/L. These values appeared much lower than those of low molar mass surfactants, indicating that micelles formed from mPEG-PLA copolymers as drug carriers could preserve stability without dissociation after dilution, which was of major interest for intravenous injection. Moreover, the hydrophilicity of mPEG-PLA copolymers mainly depending on the mass ratio of mPEG/PLA or mPEG content had much

Table 2 Characterization of micelles prepared from a series of mPEG-PLA copolymers

Copolymers	Drug-free		Drug-loaded		CAC[c] (10^{-7} mol/L)	LC[d] (%)	EE[e] (%)
	d[a] (nm)	PI[b]	d (nm)	PI			
mPEG5-PLA2.5	44.33	0.137	51.57	0.122	2.31	16.43	24.22
mPEG5-PLA5	61.80	0.164	68.41	0.154	1.87	14.21	20.29
mPEG5-PLA10	79.36	0.127	84.25	0.106	1.45	15.52	19.89
mPEG5-PLA15	84.55	0.091	97.54	0.118	0.96	14.67	20.32

[a] Diameter of micelles

[b] Polydispersity index

[c] Critical association concentration

[d] Drug loading content

[e] Entrapment efficiency

influence on the CAC value [24]. As shown in Table 2, the CAC values of copolymers appeared to decrease with increasing in PLA block length. mPEG5-PLA15 had longer hydrophobic PLA blocks and thus, could self-assemble more easily to form micelles, leading to lower CAC values. This was consistent with previous reports [25, 26].

Characterization of mPEG-PLA Micelles

Core–shell type polymeric micelles were prepared by dialysis method. Size and size distribution of micelles were measured by DLS. The mean diameter ranged from 44 to 85 nm for drug-free micelles and from 51 to 98 nm for drug-loaded micelles (Table 2). It appeared that the micelle size gradually increased with increasing in the length of PLA chains. This result was in agreement with the characteristic of amphiphilic copolymer micelles, i.e., the shorter the hydrophobic block length, the smaller the micelles. It might be attributed to the fact that it is difficult to form compact polymeric micelles for amphiphilic copolymers with longer hydrophobic chain length. In addition, similar to drug-loaded micelles reported earlier [15, 27], the size of drug-loaded micelles was about 10 nm bigger than that of drug-free micelles, suggesting that ethaselen molecules were trapped in the hydrophobic inner cores and that these entrapped ethaselen molecules increased the average size of ethaselen-loaded polymeric micelles. Importantly, the PI values of ethaselen-loaded polymeric micelles were fairly low, indicating a monodisperse distribution [28]. Among four polymeric micelles, the size of mPEG5-PLA2.5 micelles was smallest.

The morphology of the ethaselen-loaded micelles was examined by using TEM. As shown in Fig. 3, these micelles were clearly distinguished as bright and discrete spots with nearly spherical shape and equal granule. The diameter derived from TEM was lower than that from DLS, which could be assigned to the dehydration and shrinkage of the micelles during drying. These results indicated that

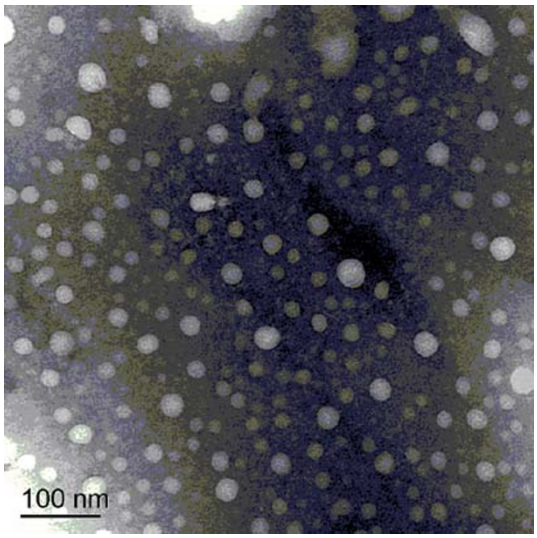

Fig. 3 TEM photography of ethaselen-loaded mPEG5-PLA2.5 micelles (×100,000)

the ethaselen-loaded polymeric micelles were well dispersed in aqueous media and formed homogeneous nano-sized micelle structures.

Drug loading content (LC, w/w%) and entrapment efficiency (EE, w/w%) of polymeric micelles were calculated by absorbance of ethaselen at 320 nm from UV. The results were also summarized in Table 2. It could be found that mPEG5-PLA2.5 micelles exhibited the highest LC and EE, about 16 and 24%, respectively, while only 1.61% of LC for hydroxylpropyl beta cyclodextrin (HP-β-CD) inclusion [3]. The solubility of ethaselen in water was improved to be about 82 μg/mL before freeze-drying, which was about 32 times higher than that of ethaselen in water. Notably, the results presented in Table 2 were not the general trend that LC and EE of polymeric micelles increased with increasing hydrophobic chain length. This finding may be assigned to the factors contributing to LC and EE. In general, LC and EE depend on the composition

of the copolymers, initial diblock copolymeric concentration or the feed weight ratio of the drug to the copolymer, solvent used in formulation process and micelle preparation method and so on. Therefore, the length of hydrophobic chains was not the only one factor influencing LC and EE. When the factors mentioned earlier except the composition of the mPEG-PLA copolymers are optimal for all copolymers, the length of hydrophobic chains is a vital one. In case of our experiment, all micelles were prepared in the same conditions, which may not be optimal for all copolymers, thereby the result was not the general trend. The other possible reason might be filtration step removed larger particles. This phenomenon was also seen in previous report [29]. Nevertheless, the result would not have influence on the subsequent experiments.

The physical stabilities of both the lyophilized powder and micelle solution of ethaselen were evaluated by determination of leakage percent and mean size during storage. Figure 4 showed the representative change profiles of leakage percent and mean size. It could be seen that the lyophilized powder exhibited far improved physical stability compared to micelle solution. It could be stored at room temperature for at least 2 months without significant changes of entrapment efficiency and micelle size ($p > 0.05$). In addition, the micelle solution could be concentrated by reconstitution of lyophilized samples in physiological saline at least one time when compared with the micelle solution before freeze-drying, hence, the solubility of the drug in water would be further increased. However, the physical stability of mPEG-PLA micelle solutions was poor at room temperature and retained only for 3 days. After storage for 5 days the drug started to leak from micelles (Fig. 4a), the leakage percent ranged from 1.37 to 2.49%. And the initial transparent micelle solution became translucent or turbid, thereby the size could not be determined by DLS. The leakage percent markedly increased to 9.1–17.4% and sedimentation appeared after 15 days. The similar results were reported previously [30, 31], where mPEG-PLA micelles maintained their stability after drug loading only for several hours or days. It was attributed to the lost of a hydrophilic and hydrophobic balance, which was the critical influence factor for micelle stability owing to the encapsulation of hydrophobic drugs, and drug-loaded PLA-PEG polymer micelles broke up to result in drug precipitation. For this reason, it was suggested that the obtained polymeric micelles might be freeze-dried for a longer storage and reconstituted in aqueous solutions prior to use.

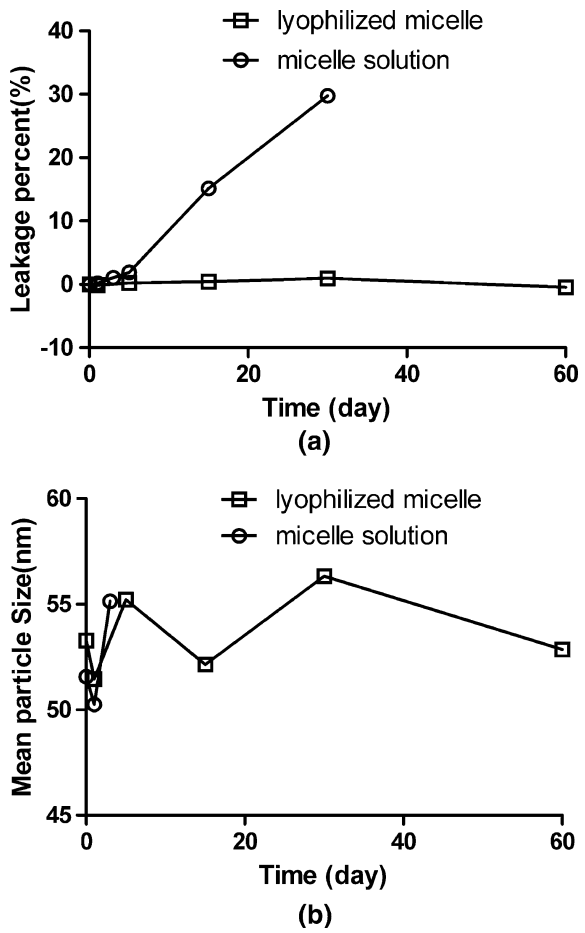

Fig. 4 Leakage percent (**a**) and mean size (**b**) changes of ethaselen-loaded mPEG5-PLA2.5 micelles in 2 months at 25°

Hemolytic Toxicity of Micelles

The block copolymers in this study were amphiphilic and could solubilize lipids or insert into phospholipid membranes to destabilize them [32–34]. When the micelles are injected into the blood for drug delivery or drug detoxification, detrimental interaction of these particles with blood constituents must be avoided. Therefore, the hemolysis assay would give additional information about the biocompatibility in the case of an in vivo application. Although the concentrations of the copolymers were very high, micelles did not show any observational hemolytic activities in the RBC in the experimental range. Figure 5 showed the hemolytic activities of drug-free micelle solutions and ethaselen-PM with different PLA block lengths. It was observed that the hemolytic percentage of mPEG-PLA diblock copolymers seemed not to depend on their concentrations. The hemolytic percentage was always lower than 5% in the whole tested concentration range. According to the Guiding Principles of Hemolysis Test [H]GPT4-1, the samples were considered as hemolytic if the hemolytic percentage was above 5%. Consequently, the mPEG-PLA diblock copolymers had no hemolytic effect on the RBC. The results also suggested that the mPEG-PLA micelles were suitable for intravenous administration. In addition, the hemolytic activity of ethaselen-loaded

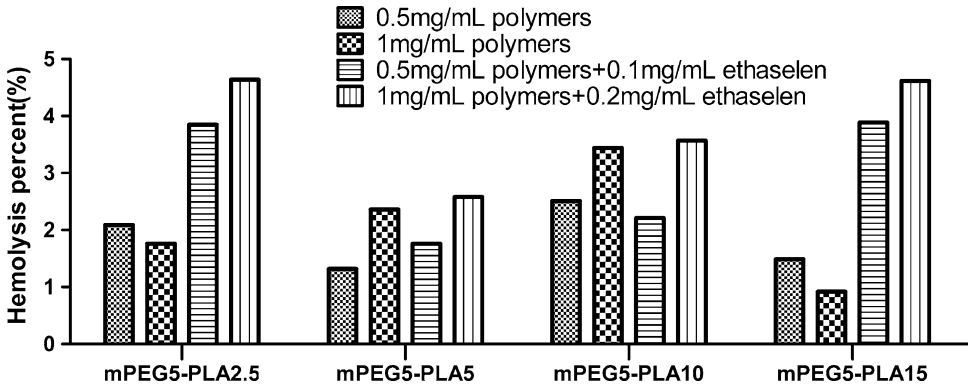

Fig. 5 Hemolysis activity of copolymers and ethaselen-loaded mPEG-PLA micelles

Table 3 In vivo antitumor effect of ethaselen-loaded mPEG5-PLA2.5 micelle and ethaselen-HP-β-CD in H_{22} human liver cancer cell bearing mice model ($\bar{x} \pm s$, $n = 10$)

Formulation	Dose (mg/kg)	Body weight (g)		Tumor weight (g)	Inhibition rate (%)
		Before administration	After administration		
Physiological saline	0	20.38 ± 0.94	28.01 ± 2.24	1.29 ± 0.26	–
Ethaselen-HP-β-CD	1	19.94 ± 0.86	26.94 ± 2.11*	0.81 ± 0.13*	36.93
Ethaselen-micelle (L)	1	20.52 ± 0.66	28.10 ± 1.65	0.71 ± 0.12*,**	45.10
Ethaselen-micelle (M)	2	20.92 ± 0.58	28.75 ± 1.76	0.57 ± 0.17*,**,***	55.60
Ethaselen-micelle (H)	4	20.20 ± 0.42	28.42 ± 1.81	0.53 ± 0.11*,**,***,****	58.47

* $p < 0.05$, versus physiological saline; ** $p < 0.05$, versus ethaselen-HP-β-CD; *** $p < 0.05$, versus ethaselen-micelle (L); **** $p > 0.05$, versus ethaselen-micelle (M)

polymeric micelles slightly increased, and it appeared to ethaselen-loaded polymeric micelles that the hemolysis was ethaselen concentration-dependent to a certain degree. The dilution stability and hemolytic potential of micelle formulation indicated that ethaselen-loaded polymeric micelles could be administered intravenously at a wide range of drug concentrations so that a precise dilution is not required.

In vivo Antitumor Efficacy

According to the results earlier mentioned and favorable pharmacokinetics characteristic (dada not shown) ethaselen-loaded mPEG5-PLA2.5 micelle was chosen to evaluate the in vivo antitumor efficacy with the animal tumor models set up by inoculation of H_{22} human liver cancer cell by measuring tumor weight or relative tumor inhibition rate after tumor implantation. None of the animals treated with tested formulations died during the experimental period. As shown in Table 3, ethaselen-HP-β-CD and ethaselen-loaded mPEG5-PLA2.5 micelle significantly inhibited the growth of tumor compared with the control group ($p < 0.05$). After 11 days, ethaselen-HP-β-CD at 1 mg/kg suppressed tumor growth by 36.93%, and ethaselen-loaded mPEG5-PLA2.5 micelle at 1 mg/kg, 2 and 4 mg/kg suppressed tumor growth by 45.10, 55.60 and 58.47%, respectively, compared with the control group, indicating that ethaselen-loaded mPEG5-PLA2.5 micelle inhibited tumor growth in a dose-dependent manner to some extent. More importantly, ethaselen-loaded mPEG5-PLA2.5 micelle had significantly stronger inhibitory effect on the tumor growth compared with ethaselen-HP-β-CD at the same dose of ethaselen ($p < 0.05$), reinforcing that ethaselen-loaded polymeric micelles had more effective antitumor activity than ethaselen-HP-β-CD. This could be attributed to the "enhanced permeation and retention" (EPR) effect of nano-sized micellar delivery systems [35–37]. Fast growing tumor tissues need a tremendous amount of oxygen and nutrients supplied by blood vessels. They release special growth factors including vascular endothelial cell growth factor (VEGF) to facilitate neovascularization. As a result, many new vessels are formed, but their cell junctions are not as tight as those of normal tissues. Ethaselen-loaded mPEG5-PLA2.5 micelle with a size of about 51 nm was likely to freely pass through the endothelial junctions of the capillaries in tumor tissue. In addition, the prevention of hydrophobic interactions between vascular endothelial cell in tumor tissues and the drug by hydrophilic and flexile PEG shell of polymeric micelle made the drug enter into the tumor tissue successfully [38]. On the other hand, it has been reported that CD

couldn't influence the pharmacokinetics of drugs [39, 40], while much more drug would be accumulated in the solid tumor region due to delay of the circulation time of drugs for polymeric micelles [22, 27, 39, 40]. A combined effect of improved pharmacokinetics and enhanced cellular uptake would be the main reason for the suppression of tumor growth. It could also be seen that the middle and high dose groups suppressed the tumor growth more significantly than the low one ($p < 0.05$), but there was no significant difference of the tumor inhibition rate for the middle and high dose group ($p > 0.05$). Taken together, the finding that ethaselen-loaded mPEG5-PLA2.5 micelle at 2 mg/kg significantly improved antitumor efficacy could have important clinical implications.

The body weight of mice treated with physiological saline without drug continuously increased due to probably its nontoxic effect as well as the rapid growth of tumor (Table 3). In ethaselen-loaded polymeric micelle treated groups at all doses, the body weight of mice did not significantly increase. This might be due to their antitumor efficacies thereby the slower growth of tumor. On the other hand, ethaselen-HP-β-CD group appeared to have significant weight loss, resulting from the toxic effect of the drug, indicating that this micelle-based drug delivery system could reduce unwanted side effects of anticancer drugs during cancer therapy.

Overall, ethaselen-loaded polymeric micelle possessed improved antitumor activity and reduced toxic side effects of anticancer drug than ethaselen-HP-β-CD mainly due to the enhanced vascular permeability and EPR effect, and passive targeting function although they do not have active targeting function [41–43]. Furthermore, tumor tissues are characterized with leaky blood vessels and the premature lymphatic drainage [44]. Resultantly, we speculated that ethaselen-loaded polymeric micelles would also be a superior formulation for other tumor models.

Nevertheless, ethaselen, as a poorly water-soluble drug, might be physically incorporated into the inner core of the polymeric micelles by hydrophobic interactions. Further, it may avoid RES recognition due to a size smaller than 200 nm. Therefore, it is advantageous for ethaselen to be efficiently encapsulated in micelles. In case of our experiment, the EE was unfavorable, thereby the enhancing of hydrophobic interaction between ethaselen and the inner core of the polymeric micelles would be vital by chemical modification the hydrophobic block of mPEG-PLA.

Conclusions

We have successfully synthesized a series of mPEG-PLA copolymers. It was found that monodispersed micelles self-assembled from mPEG-PLA could effectively solubilize the anticancer drug ethaselen when compared with HP-β-CD inclusion. The hemolysis assay indicated that ethaselen-loaded mPEG-PLA micelles could be administered intravenously at a wide range of drug concentrations. In mice, ethaselen-loaded polymeric micelles showed noticeable antitumor efficacy, and reduced the toxic effect of the drug, compared with ethaselen-HP-β-CD inclusion. These results suggested that polymeric micelles might be an effective drug delivery system for ethaselen for cancer chemotherapy. Nevertheless, the micelles could still be improved, especially with respect to enhancing their entrapment efficiency by modification of inner core of micelles, which are in progress. Better drug retention in the micelle core is a key to ensure prolonged circulation time and eventually maximize drug accumulation at the tumor site via the enhanced permeation and retention effect.

Acknowledgments The authors wish to thank Prof. Huihui Zeng from Department of Chemical Biology, Peking University for friendly providing ethaselen.

References

1. C.J. Shi, L.Z. Yu, F.G. Yang, H.H. Zeng, Biochem. Biophy. Res. Commun. **309**, 578 (2003)
2. J. Yan, S.J. Deng, H.H. Zeng, J. Chin. Pharm. Sci. **13**, 199 (2004)
3. H.M. Cui, C.G. Zhang, F.L. Wu, Chin. Pharm. Sci. **42**, 765 (2007)
4. V.P. Torchilin, Adv. Drug Deliv. Rev. **54**, 235 (2002)
5. A. Rösler, G.W. Vandermeulen, H.A. Klok, Adv. Drug Deliv. Rev. **53**, 95 (2001)
6. M. Jones, J. Leroux, Eur. J. Pharm. Biopharm. **48**, 101 (1999)
7. X. Shuai, T. Merdan, A.K. Schaper, F. Xi, T. Kissel, Bioconjug. Chem. **15**, 441 (2004)
8. T. Riley, C.R. Heald, S. Stolnik, M.C. Garnett, L. Illum, S.S. Davis, Langmuir **19**, 8428 (2003)
9. L. Liu, C. Li, X. Li, Z. Yuan, Y. An, B. He, J. Appl. Polym. Sci. **80**, 1976 (2001)
10. E. Pierri, K. Avgoustakis, J. Biomed. Mater. Res. A **75**, 639 (2005)
11. K. Kataoka, A. Harada, Y. Nagasaki, Adv. Drug Deliv. Rev. **47**, 113 (2001)
12. M. Wilhelm, C.L. Zhao, Y.C. Wang, R.L. Xu, M.A. Winnik, J.L. Mura et al., Macromolecules **24**, 1033 (1991)
13. R. Barreiro-Iglesias, L. Bromberg, M. Temchenko, T.A. Hatton, A. Concheiro, C. Alvarez-Lorenzo, J. Control. Release **97**, 537 (2004)
14. P.Z. Li, X.R. Li, H.X. Zhou, Y.H. Zhang, F. Wang, Y. Liu, Chin. J. New Drug **18**, 262 (2009)
15. Y. Zhang, T. Jin, R.X. Zhuo, Colloids Surf. B: Biointerfaces **44**, 104 (2005)
16. E.S. Lee, K. Na, Y.H. Bae, J. Control. Release **91**, 103 (2003)
17. D. Le Garrec, S. Gori, L. Luo, D. Lessard, D.C. Smith, M.A. Yessine et al., J. Control. Release **99**, 83 (2004)
18. Y. Hu, X. Jiang, Y. Ding, L. Zhang, C. Yang, J. Zhang et al., Biomaterials **24**, 2395 (2003)
19. A. Lucke, J. Tessmar, E. Schnell, G. Schmeer, A. Göpferich, Biomaterials **21**, 2361 (2000)
20. B.G. Yu, T. Okano, K. Kataoka, G. Kwon, J. Control. Release **53**, 131 (1998)

21. S. Zhu, F. Qian, Y. Zhang, C. Tang, C. Yin, Eur. Polym. J. **43**, 2244 (2007)
22. G.S. Kwon, K. Kataoka, Adv. Drug Deliv. Rev. **16**, 295 (1995)
23. S.Y. Kim, I.G. Shin, Y.M. Lee, J. Control. Release **56**, 197 (1998)
24. S. Li, M. Vert, Macromolecules **36**, 8008 (2003)
25. X. Zhang, J.K. Jackson, H.M. Burt, Inter. J. Pharm. **132**, 195 (1996)
26. B. Jeong, Y. Han Bae, S. Wan Kim, Colloids Surf. B: Biointerfaces **16**, 185 (1999)
27. K.W. Yang, X.R. Li, Z.L. Yang, P.Z. Li, F. Wang, Y. Liu, J. Biomed. Mater. Res. Part A **87A**, 140 (2009)
28. A. Harada, K. Kataoka, Macromolecules **28**, 5294 (1995)
29. Y.C. Chang, I.M. Chu, Eur. Polym. J. **44**, 3922 (2008)
30. K.M. Huh, S.C. Lee, Y.W. Cho, J. Lee, J.H. Jeong, K. Park, J. Control. Release **101**, 59 (2005)
31. H.M. Burt, X. Zhang, P. Toleikis, L. Embree, W.L. Hunter, Colloids Surf. B: Biointerfaces **16**, 161 (1999)
32. J. Zastre, J. Jackson, M. Bajwa, R. Liggins, F. Iqbal, H. Burt, Eur. J. Pharm. Biopharm. **54**, 299 (2002)
33. R. Savic, L. Luo, A. Eisenberg, D. Maysinger, Science **300**, 615 (2003)
34. M.K. Pratten, J.B. Lloyd, G. Hörpel, H. Ringsdorf, Die Makromol. Chem. **186**, 725 (1985)
35. J.G. Shiah, M. Dvorák, P. Kopecková, Y. Sun, C.M. Peterson, J. Kopecek, Eur. J. Cancer **37**, 131 (2001)
36. J.J. Shiah, Y. Sun, C.M. Peterson, J. Kopecek, J. Control. Release **61**, 145 (1999)
37. M. Yokoyama, S. Fukushima, R. Uehara, K. Okamoto, K. Kataoka, Y. Sakurai et al., J. Control. Release **50**, 79 (1998)
38. Z. Zuo, Y.K. Tam, J. Diakur, L.I. Wiebe, J. Pharm. Pharm. Sci. **5**, 292 (2002)
39. H.W. Frijlink, E.J. Franssen, A.C. Eissens, R. Oosting, C.F. Lerk, D.K. Meijer, Pharm. Res. **8**, 380 (1991)
40. G. Piel, B. Evrard, T. Van Hees, L. Delattre, Int. J. Pharm. **180**, 41 (1999)
41. Y. Jeong, H.S. Na, K.O. Cho, H.C. Lee, J.W. Nah, C.S. Cho, Int. J. Pharm. **365**, 150 (2009)
42. K. Greish, A. Nagamitsu, J. Fang, H. Maeda, Bioconjug. Chem. **16**, 230 (2005)
43. H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, J. Control. Release **65**, 271 (2000)
44. Y. Bae, K. Kataoka, Adv. Drug Deliv. Rev. **61**, 768 (2009)