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Preparation of tri-block copolymer micelles loading novel organoselenium anticancer drug BBSKE and study of tissue distribution of copolymer micelles by imaging in vivo method

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ABSTRACT

BBSKE (1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)] ethane, PCT: CN02/00412) is a novel organoselenium anticancer drug that plays a role in anticancer through inhibiting TrxR (thioredoxin reductase). In this study, we prepared a tri-block copolymer micelles loading BBSKE utilizing the amphiphilic tri-block copolymers (PEG6000-PLA6000) which we synthesized. And then the characters of the copolymer micelles were investigated. When packaged in polymeric micelles, the water solubility of BBSKE was improved to 0.21 mg/ml. The IC_{50} were 7.14 μM, 5.05 μM and 4.23 μM when MCF-7 breast cancer cells were treated with BBSKE after 24 h, 48 h and 72 h. The inhibition effect of polymeric micelles to MCF-7 tumor cells was bettered when folate, whose receptor was highly expressed in various tumors, was coated on the surface of these nanoparticles. Finally, by adopting a new way of imaging in vivo, we studied the distribution of micelles in nude mice with and without MCF-7 tumor. The results demonstrated that this copolymer micelles loading BBSKE can accumulate into tumor efficiently.

1. Introduction

Thioredoxin reductase (TrxR) is a NADPH-dependent selenocysteine-containing flavoenzyme, it can catalyze the reduction of oxidized Trx (Holmgren, 1985). TrxR offers a target for the development of drugs to treat and prevent cancer (Gasdaska et al., 1995) due to its capability of stimulating cancer cell growth and inhibiting apoptosis. Recently, several clinical anti-tumor compounds have been reported to have anti-tumor activities through inactivating TrxR (Xiong et al., 1993; Harper et al., 1993; Deiry et al., 1993; Sabine and Katja, 2006).

Our lab has successfully synthesized a novel organoselenium compound 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)] ethane (BBSKE, Supplemental Fig. 1) targeting thioredoxin reductase, which has been shown to inhibit thioredoxin reductase activity in vitro (Shi et al., 2003; Zhao et al., 2006), and to repress the growth of a variety of human cancer cells from various organs, including lung, stomach, liver, cervix, prostate, blood, etc. (Deng et al., 2003; Shi et al., 2003; Yan et al., 2004; Zhao et al., 2006). As a new type of anticancer medicine, BBSKE is now in Phase I of the clinical trial. As far as we know, most of the anticancer drugs could not dissolve in water. BBSKE also has the characteristics of the anticancer drugs. In common solvents such as water, ethanol, chloroform, ethyl ether, ethyl acetate, etc., BBSKE is not easily dissolved, and only in dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF), it is dissolved easily. So we applied polymeric micelles to improve solubility of BBSKE. Li et al. have prepared polymeric micelles loading BBSKE with some bi-block copolymers such as MPEG5000-PLA2500, MPEG5000-PLA5000, MPEG5000-PLA10000, and investigated some characters of these polymeric micelles (Xinru et al., 2009). However, in our study, we prepared tri-block polymeric micelles loading BBSKE with another material PLA3000-PEG6000-PLA3000 that we synthesized. Besides, a targeting motif folate has been designed to coat the surface of the polymeric micelle by covalent connection, providing the nanoparticles with active targeting ability. Furthermore, we applied a novel method imaging in vivo to study the tissue distribution character of BBSKE polymeric micelles.

Current anticancer drug therapy results in systemic side effects due to non-specific uptake by normal healthy noncancerous tissues. Many anticancer drugs have marginal selectivity for malignant cells because they target the replicative apparatus in cells with high proliferation rates. Therefore, anticancer drugs having this mechanism

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of action also have high toxicity against rapidly dividing normal cells. The side effects associated with chemotherapy limit the dose or cumulative doses that can be administered to patients, which can lead to relapse of the tumor and which often leads to the development of drug resistance. To resolve the problem of difficult to dissolve, many attempts have been devoted to the development of new delivery systems such as emulsions, liposomes, copolymer micelles. Among these delivery systems, polymeric micelles formed by self-assembling of amphiphilic block copolymers seem to be a promising selective delivery system for many hydrophobic drugs. Recently, because of a number of advantages, polymer micelles have attracted increasing attention from both the scientific community and industry due to their promising applications in high-quality drug-delivery vehicles to treat cancers. Polymeric micelles have a core–shell structure with the drug in the core, thus the drug is effectively protected. It has a low level of toxicity in the human body, and a prolonged circulation time in the blood owing to its high water solubility (avoiding phagocytic and renal clearance). In addition, the passive accumulation of the micelles in a solid tumor is achieved by the enhanced permeability and retention (EPR) effect of the vascular endothelia at the tumor. It is also worth mentioning that a micelle as an invasive body is generally taken up by the cell through endocytosis, translated into endosomes, and then fused with lysosomes in which the proton concentration is 100 times higher (pH 4.0–5.0) than the physiological condition (pH7.4) (Kwon et al., 1994). Polymeric micelles have been applied in delivering many anticancer drugs such as cisplatin (Valery et al., 1999; Uchino et al., 2005), paclitaxel (Sung et al., 2001; Hamaguchi et al., 2007), doxorubicin, etc. (Hyuk and Tae, 2004).

In order to further improve the therapeutic indices of copolymer micelles loading anticancer drugs, the strategy of ligand-mediated has been applied. In this strategy, some ligands, whose receptors expressed selectively or overexpressed on tumor cells, are connected to the surface of the copolymer micelles. Folic acid (folate) is an attractive candidate molecule for targeting cancer cells because it is an essential vitamin for the biosynthesis of nucleotide bases and is consumed in elevated quantities by proliferating cells. The receptor for folic acid is overexpressed in many human cancers, including malignancies of the ovary, brain, kidney, breast, myeloid cells, and lung (Cummings and McArdle, 1986; Weitman et al., 1992; Goren et al., 2000). Folate has been popularly employed as a targeting moiety of various anticancer agents to avoid their non-specific attacks on normal tissues as well as to increase their cellular uptake within target cells (Wang et al., 1995; Lu et al., 1999; Reddy and Low, 2000; Lu and Low, 2002; Hyuk and Tae, 2004; Guangya et al., 2008).

In this study, a kind of tri-block copolymer of polyethylene glycol-polylactic acid (PEG-PLA) was synthesized for the purpose of improving the targeting and increasing the water solubility. With the tri-block copolymers as the carrier materials, we prepared a tri-block copolymer micelle packaged BBSKE in the core. In order to further enhance the targeting of polymer micelles, folic acid was connected to the hydrophilic side of the copolymer. Thus, we obtained a targeting function nanoparticle, which enhanced the targeting of drugs and reduced the toxicity of drugs. The polymeric micelles loading anticancer drug were investigated from different aspects, such as particle size, shape, and the inhibition of tumor cell growth, etc. And then, we focused on the study of distribution of the polymeric micelles in the tissues after they were injected into the nude mice. In the study of the tissue distribution of the copolymer micelles in nude mice, we applied a new technology — imaging in vivo. There are many media in which in vivo imaging could be used, such as fluorescence, NMR, infrared, etc. (Frank et al., 2002). In our experiment, we applied the Maestro in vivo imaging device (The company's Cambridge Research Instruments) to detect fluorescence signals (Maha et al., 2008; Erina et al., 2009; Muraru et al., 2009; Tomoyuki et al., 2009).

2. Material and methods

2.1. Materials

BBSKE (purity > 98.5%) was synthesized by us (School of Pharmaceutical Science, Peking University, Beijing, China). Polyethylene glycol 6000 (PEG6000) and Polyethylene glycol 4000 (PEG4000) were purchased from Merck. D,L-Lactide (CAS. R.NO: 95-96-5), and p-nitrophenylchloroformate (PNP) were all obtained from SIGMA. Adriamycin (doxorubicin, DOX) was purchased from Beijing Hua Feng Technology Co., Ltd. The model of scanning electron microscope was HITACHI, S-4800.

Female nude mice were obtained from Experimental Animal Center of Peking University acclimatized for several days after arrival. All animals were provided with standard food, water 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.

2.2. Synthesis of tri-block copolymer PEG6000-PLA6000

The lactide was first recrystallized with ethyl acetate three times. It was then dried in vacuum at room temperature to the melting point 127 °C. Then PEG6000 (2 g) and toluene (100 ml) was added into the flask. Water separation device is used next to remove the water in toluene. The lactide (2 g) and stannous octoate (0.5% quality of lactide), which were used as a catalyst, were added to the flask. Thereafter, the polymerization reaction then undergoes for 24 h under the conditions of oil bath at 120 °C and nitrogen protection. The crude product, after the toluene was evaporated, was dissolved with chloroform. The chloroform solution was dropped into the ether at 0 °C. Then we obtained a kind of solid powder by filtering the ether solution. The powder was dried under vacuum at room temperature, and then it was placed in −20 °C refrigerator for storage (Scheme 1).

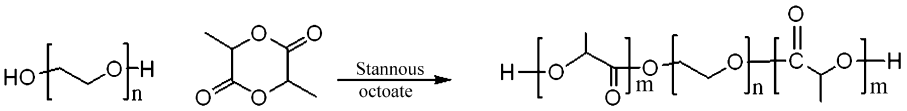
2.3. Synthesis of folic acid-polyethylene glycol-polylactic acid

2.3.1. Synthesis of single-amino-polyethylene glycol

First, PEG4000 (2 g, 0.5 mmol), methyl benzenesulfonyl chloride (0.09525 g, 5 mmol) and pyridine (5 ml) solution were dissolved in CH_2Cl_2 (10 ml). After a 24 h reaction was conducted at room temperature, 3 mol/L HCl (47 ml) was added into the solution. Then $NaHCO_3$ (0.5 g) was added into the organic layer following violent agitation. Crude PEG-p-toluene sulfonic acid ester was obtained after the filtrate was Rotary evaporated. The precipitate was obtained by dropping the tetrahydrofuran (0.87 ml) dissolved crude products to ether (43.5 ml). Subsequently through filtering and vacuum drying, the pure product was acquired. Then the pure product (1.61 g) was reacted with of 25% ammonia (16 ml) for 6 h at 140 °C in high-pressure reactor and 1 mol/L NaOH (14 ml) is added to the organic layer (approximately 14 ml, containing PEG-p-toluene sulfonic acid ammonium salt) attained by dichloromethane (16 ml) extraction, then the solution is stirred for 2 h. The product amino-polyethylene glycol separation was obtained, after some operations of separating organic layer, washing to neutral and drying. The Ninhydrin test was used to verify whether the amino-polyethylene glycol was obtained.

2.3.2. Synthesis of tertiary-butyloxycarbonyl amino-polyethylene glycol

Single-amino-polyethylene glycol (6 g, 0.01 mol) and $(Boc)_2O$ (0.22 g, 0.001 mol) were reacted in methanol at 50 °C for 10 h. Thereafter, the product of rotation evaporation was dissolved in



Scheme 1. Synthesis of PEG6000-PLA6000 (PEG: polyethylene glycol; PLA: polylactic acid).

CH_3Cl completely. A white powder could be obtained by adding the dissolution into cold ether. Finally, the solid precipitate was vacuum dried for 12 h.

2.3.3. Synthesis of Boc-PEG4000-PLA4000

Before the experiment lactide was recrystallized with ethyl acetate three times, and then it was vacuum dried for 24 h. Initially, Boc-PEG4000 (2 g) was placed in the flask, and then toluene (100 ml) was added. Water separation device was used next to remove the water in toluene. As described before, the lactide (2 g) and stannous octoate (0.5% quality of lactide) which were used as a catalyst were added to the flask. After that, the polymerization reaction was conducted for 24 h under the conditions of oil bath at 120 °C and nitrogen protection. The crude product, after the toluene was evaporated, was dissolved with chloroform. The chloroform solution was dropped into the ether at 0 °C. Then after the ether solution was filtered and the Boc-PEG4000-PLA4000 was obtained.

2.3.4. Synthesis of NH_2-PEG-PLA

Boc-PEG4000-PLA4000 and trifluoroacetic acid reacted for 3 h under the conditions of nitrogen protection. As mentioned earlier, the product of rotation evaporation was dissolved in CH_3Cl completely. We can get a white powder by adding the dissolution into cold ether. Thereafter, Ninhydrin reaction was used to test for the presence of the amino group. The results where the product becomes purple indicate the existence of free primary amino groups.

2.3.5. Synthesis of folate-NH-PEG-PLA

First, folic acid, N-hydroxy succinimide (NHS), and N,N-dicyclohexyl carbodiimide (DCC) (by the molar ratio of 1:1.5:1.5) reacted 6 h in DMSO, at room temperature. Then DCU was removed by filtering. And then The NH_2-PEG-PLA and 1.2 equivalent of DCC were added to the system, thereafter the system underwent the nitrogen protection for 12 h. When the reaction was completed, the solution was put for dialysis for 24 in the dialysis bag (3500). The dialysis solution was dried on the freeze-drying machine. When the dialysis was completed, a certain amount of folic acid-NH-PEG-PLA and pure folic acid were dissolved in DMSO, following UV scanning. The scanning results showed that the UV maps were exactly the same and thus confirmed that the folic acid was connected to the NH_2-PEG-PLA by chemical bond (Lee and Low, 1995; Guo et al., 2000; Hyuk and Tae, 2004; Guangya et al., 2008) (Scheme 2).

2.4. Synthesis of PEG-PLA-DOX

The reagent PNP, PLA-PEG-PLA and pyridine (PLA-PEG-PLA/PNP/pyridines molar ratio = 1/5/10) were reacted in anhydrous dichloromethane at 0 °C for 30 min under the conditions of nitrogen. The reaction was conducted for 4 h at room temperature under the conditions of nitrogen. After the reaction was completed, as depicted before, the product of rotation evaporation was dissolved in CH_3Cl completely. We can get a white powder by adding the dissolution into cold ether. The product of PNP-PLA-PEG-PLA-PNP was acquired. In our study, we adopted the method performed by Galdwell to test for the degree of substitution of the PNP. Next, the previous product PNP-PLA-PEG-PLA-PNP, DOX-HCL and Triethylamine (TEA) (PNP-PLA-PEG-PLA-PNP/DOX-HCL/TEA molar ratio = 1:2:8) dissolved in DMF and took a reaction for 24 h under the protection of nitrogen. After the reaction was accomplished, the solution was diluted five times with distilled water dialysis for 24 h in the dialysis bag (3500). Dialysis solution was dried on the freeze dryer. Then the freeze-dried powder was placed in a −20 °C refrigerator for storage (Kataoka et al., 2000; Yoo and Park, 2001; Hyuk and Tae, 2004) (Scheme 3).

2.5. Preparation of nano-micelle

We adopt the way of dialysis to the prepare the polymer micelles containing the drug.

2.5.1. Preparation of drug-loaded polymer micelles

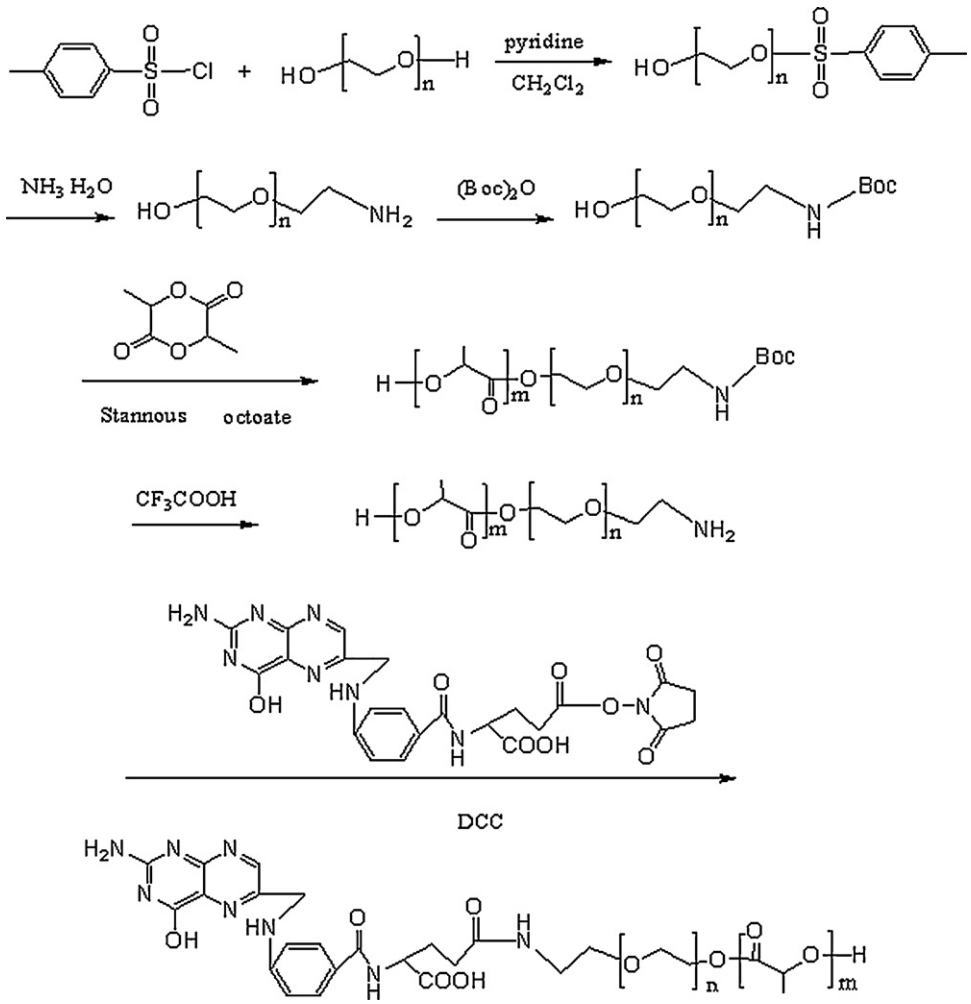
PEG6000-PLA 6000 (100 mg), folate-NH-PEG-PLA (12 mg) and BBSKE (10 mg) were dissolved in 50 ml DMSO and was put into dialysis bag (3500) for dialysis for 24 h in 3 L of distilled water. Upon completion of dialysis, the dialysis solution then underwent centrifuge at 3000 rpm for 30 min, and then it was filtered using 0.45 μm microporous membrane. The filtrate with PEG4000 as a protective agent was freeze-dried in the freeze-drying freeze dryer.

2.5.2. Preparation of polymeric micelles with fluorescent reagents

PEG6000-PLA6000-DOX (100 mg), folic acid-NH-PEG-PLA (12 mg) and BBSKE (10 mg) was dissolved in 50 ml DMSO and was put into a dialysis bag (3500) for dialysis for 24 h in 3 L of distilled water. Upon completion of dialysis, dialysis solution then underwent centrifuge at 3000 rpm for 30 min, and was filtered using 0.45 μm microporous membrane. The filtrate with PEG4000 as a protective agent was freeze-dried in the freeze-drying freeze dryer.

2.6. Analysis of critical micelle concentration (CMC)

In order to determine the critical micelle concentration of tri-block copolymers in distilled water, fluorescence measurements were carried out using pyrene (Sigma–Aldrich, Inc.) as probe (Kwon et al., 1993; Hagan et al., 1996; Yoo and Park, 2001; Jaeyoung et al., 2004; Xinru et al., 2009). Pyrene predissolved in acetone was added to the test tube, and the solvent was evaporated. Different amounts of nanoparticles solution and distilled water were added to this tube and different concentrations of tri-block copolymers ranging from 10^{-7} mg/ml to 10^{-1} mg/ml were made. The concentration of pyrene used was 6.0×10^{-7} mol/L. The solution was incubated at 37 ± 0.5 °C with mild stirring to ensure that the pyrene equilibrated between the nanoparticles and the aqueous phase completely. Fluorescence spectra of pyrene were recorded with a PC fluorescence spectrometer (Shimadzu RF-5301). The excitation wavelength used was 333 nm 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 CMC value was taken from the intersection between the two tangents.



Scheme 2. Synthesis of folate-PEG4000-PLA4000.

2.7. Drug content and solubility analysis

The method of liquid chromatography was applied to detect the content of the drug BBSKE loaded in polymeric micelles. First, we obtained the standard curve of BBSKE by HPLC. In drug content investigation, 2 mg lyophilized polymeric micelles loaded BBSKE were dissolved in 1 ml DMSO, then the solution was filtered by 0.45 μm pore-sized filtration membrane and the concentration of BBSKE in the solution was measured by HPLC (Agilent 1100, Agilent Technologies, Inc., USA). For solubility analysis, we put excessive BBSKE polymeric micelles lyophilized powder into 2 ml distilled water. Over a period of time, the solution was filtered through a 0.45 μm filter to remove aggregates. After that 2 ml DMSO was added to the solution and the content of BBSKE in the solution was detected by HPLC (Agilent 1100, Agilent Technologies, Inc., USA). The tri-block copolymer PEG6000-PLA6000 under the wavelength of 320 nm showed no UV absorption. The HPLC column was produced by Agilent Technologies, Inc., USA. The HPLC conditions are as follows: mobile phase – methanol:water (0.01%Phosphate) = 40:60; detection wavelength: 320 nm; temperature: 25 °C.

2.8. Particle size and morphology analysis

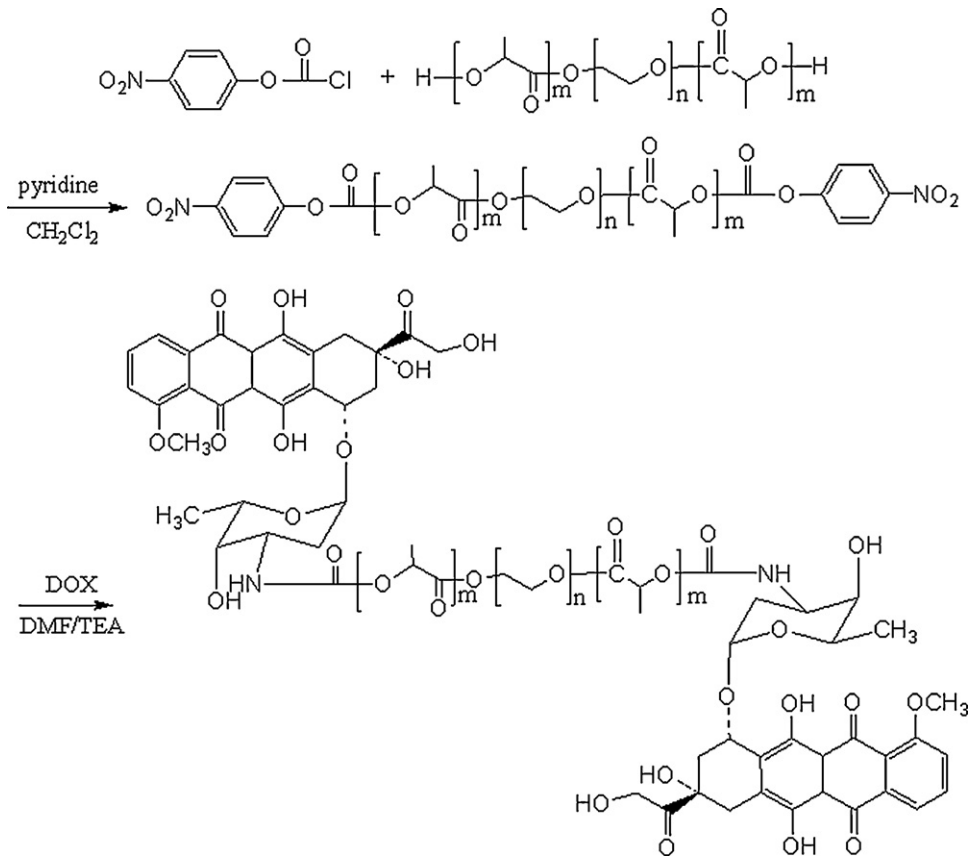
The size of the polymer micelles was determined using dynamic light scattering analysis. The machine utilized is Malvern Zetasizer Nano ZS particle size determination instrument. Wavelength of the laser beam equipment was set to 633 nm, the angle between the incident and scattered light was 90°. Before the examination, each sample was filtered by the utilization of the 0.45 μm membrane filter. Each sample was tested three times, we adopt the average of three times as the size of the sample.

First of all, drug-loaded micelles were plated, and then, we observed the micelles in the scanning electron microscopy. The model of scanning electron microscope is HITACHI, S-4800.

Precision micro-pipette was applied to check the volume of 2 ml of distilled water, the water was then added to the excessive drug polymer micelles. The solution underwent Ultrasonic for 5 min in order to make polymeric micelles dissolved fully in the water. 8 ml DMSO then was added to the system, following ultrasonic mixing. We measured the content of the drug through the liquid chromatography method.

2.9. In vitro test of BBSKE release from polymeric micelles

The BBSKE release from polymeric micelles in vitro was measured by a dialysis method. The dialysis was conducted in PBS media (pH 7.4) containing 50% fetal bovine serum (FBS) (Ishida et al., 2001; Jaeyoung et al., 2004; Xiong et al., 2005; Michael et al., 2006; Chen et al., 2009; Xue et al., 2010). The lyophilized polymeric micelles loaded BBSKE was dissolved in 10 ml release media, and then the media were placed into a dialysis bag (MW cutoff 3500) sealed at both ends with clips. The concentration of BBSKE was 150 μg/ml. The dialysis bag was then placed into a beaker containing 190 ml



Scheme 3. Synthesis of DOX-PLA3000-PEG6000-PLA3000-DOX.

of the media, and incubated with stirring for 24 h at 37 ± 0.5 °C. At various time points, 3 ml samples were withdrawn from the beaker and replaced with equal volume of the media. The concentrations of BBSKE in various samples were measured by HPLC (Agilent 1100, Agilent Technologies, Inc., USA). Liquid conditions are as follows: mobile phase – methanol:water (0.01%Phosphate) = 40:60; detection wavelength: 320 nm; temperature: 25 °C.

2.10. Cell growth inhibition assay

Stock solutions of BBSKE were made at 20 μM concentration in DMSO and freshly diluted in culture medium for all cell experiments. Stock solutions of drug-loaded polymer micelles without folic acid, drug-loaded polymer micelles with folic acid were dissolved in DMEM directly at concentration of 1 mM. and freshly diluted in culture medium for all cell experiments.

The effect of BBSKE and drug-loaded polymer micelles on growth of MCF-7 were determined using the procedure of MTT cell proliferation assay which was the first time for this novel drug to be used to inhibit these breast cancer cells. Cells were maintained as mono-layer cultures in DMEM with 10% FBS and antibiotics (100 U of penicillin/ml and 100 mg streptomycin/ml). The cells were inoculated in 5% CO_2 atmosphere. Exponentially growing cells were detached from the cell culture and a single cell suspension was produced. Then the cells were plated into 96-well plates with 5000 cells a well, treated with naked BBSKE, drug-loaded polymer micelles without folic acid, drug-loaded polymer micelles with folic acid, drug-loaded polymer micelles with folic acid containing free folic acid as a antagonist of desired concentration when cells began to grow exponentially. Free folic acid in drug-loaded polymer micelles with folic acid containing free folic acid was added before the polymer micelles were added. After incubation for 24 h, 48 h, or 72 h, 20 μl of 3-(4,5-diethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT 5 mg/ml) was added to each well, and the cells were further incubated at 37 °C for 4 h. The medium was then removed and 200 μl of HCl 0.04 M in isopropanol was added to dissolve the reduced formazan product. The plate was then read on a microplate reader (Bio-RAD, model 550) at 590 nm.

2.11. Confocal assay

BBSKE can reach a certain fluorescence signal, but the signal was not strong enough. As a result in Confocal trials, we connected doxorubicin to the polymer material of PEG6000-PLA6000 with covalent bond, which helped us to achieve DOX-PLA-PEG-PLA-DOX. And then, we utilized DOX-PLA-PEG-PLA-DOX as the polymer material to prepare polymer micelles, the doxorubicin (excitation wavelength 480 nm, detection wavelength 560 nm) was exploited as a fluorescent probe for detection. MCF-7 cells were inoculated into the Petri dish, and the percentage of cell aggregation was at least 50% before drug treatment. Subsequently, folic acid (100 μM of 100 μl) was added into the group of drug-loaded polymer micelles with folic acid containing free folic acid as a antagonist (free folic acid and folic acid of the micelle surface has molar ratio of 100:1). Each group was placed in the 37 °C incubator for 30 min, the groups were then placed in the 37 °C incubator for 3 h after drug-loaded polymer micelles was administrated. After these operations were performed, each group with polymer micelle added was rinsed with cold PBS three times and fixed 10 min with 4% paraformaldehyde. Next, we used hoechst33342 (excitation wavelength 350–363 nm, detection wavelength 450 nm) staining the nucleus for 5 min. Consequently, image analysis was conducted by Confocal laser

scanning microscope (LETCA TCS SP2) with 480 nm as excitation wavelength.

2.12. Flow cytometry assay

Adherent MCF-7 cells were digested with 0.25% trypsin, and then were harvested and resuspended into the single-cell suspension with fresh DMEM culture medium (without serum). In the group of polymer micelles with folic acid, the cells were pre-incubated for 30 min with free folic acid as an antagonist. Each group was incubated at 37 °C for 30 min. Subsequently, polymer micelles with fluorescence probe were added and incubated further for 3 h in 37 °C water bath. They were then rinsed three times with cold PBS. Finally the cell suspensions were transferred to the flow analysis tube and were detected by flow cytometer. A flow cytometric analysis was then carried out using CS Express V3 software (Beckman Coulter, Inc., Fullerton, CA, USA).

2.13. Study of tissue distribution by imaging in vivo method

As described before, the fluorescence signal of BBSKE was not strong enough, doxorubicin was connected to the tri-block copolymer materials PEG6000-PLA6000 with covalent bond and we obtained DOX-PLA-PEG-PLA-DOX. Then, we utilized DOX-PLA-PEG-PLA-DOX as the polymer material to prepare polymer micelles, thus we can exploit doxorubicin as a fluorescent probe for detection.

The tumor in armpit of nude mice was achieved through the way of embedding tumor blocks. The nude mice were fasted 12 h before the experiment. Before injection, the nano-micelles labeled with fluorescent were dissolved in 0.9% normal saline, and the nude mice were anesthetized by urethane. And then, the same amount of copolymer micelles were injected into the nude mice with tumor and nude mice without tumor through the access of tail vein injection. After 3 h and 24 h, the in vivo imaging device (Maestro, Cambridge Research Instruments) was used to observe the tissue distribution of the polymer micelles in the body. Subsequently, each vital organ was taken out to be detected on the in vivo imaging device for further observation.

The device of in vivo imaging belongs to Maestro (Cambridge Research Instruments). Parameters set are as follows: green light is applied as excitation; detection wavelength range from 550 nm to 700 nm; temperature was room temperature; light is set to 1 and station is set to 1B (Maha et al., 2008; Erina et al., 2009; Tomoyuki et al., 2009).

2.14. Statistical analysis

Results were analyzed using a two-tailed Student's *t*-test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Synthesis of block copolymer

We synthesized tri-block copolymer PEG6000-PLA6000, bi-block copolymer folate-PEG4000-PLA4000 and tri-block copolymer DOX-PLA3000-PEG6000-PLA3000-DOX. The methods of ^{1}H NMR and UV–vis were carried out to confirm the ratio of hydrophobic and hydrophilic chain in PEG6000-PLA6000 and folate, DOX were connected to NH_2-PEH-PLA.

The ^{1}H nuclear magnetic resonance (^{1}H NMR, $CDCl_3$, trimethylsilyl (TMS)) results of PEG6000-PLA6000 are shown in Fig. 1. From Fig. 1, we can witness the main peaks as follows: δ0.000 (TMS); δ1.554 (–CH_3, PLA); δ3.625 (–CH_2–, PEG); δ5.167 (–CH–, PLA); δ7.260 ($CDCl_3$). The ideal ratio of PEG6000-PLA6000 is 3:8:1 (δ1.567:δ3.617:δ5.167). ^{1}H NMR experiment indicated that the ratio of hydrophobic and hydrophilic chain segments in PEG6000-PLA6000 was 1:1. The analysis of DOX-PLA3000-PEG6000-PLA3000-DOX and folate-PEG4000-PLA4000 through the ^{1}H NMR also shows the same results as PEG6000-PLA6000.

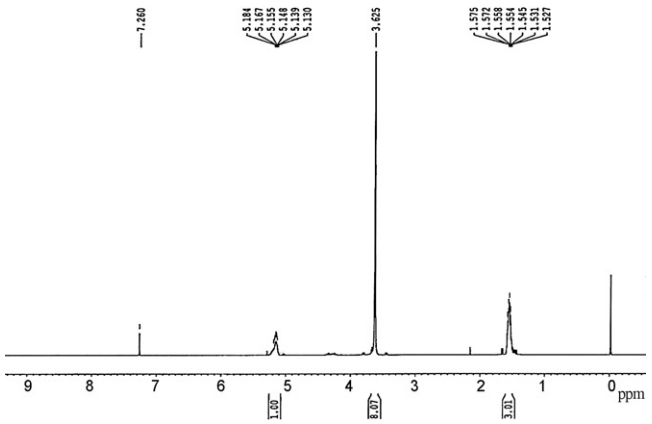


Fig. 1. ^{1}H NMR (H) for PEG-PLA.

Fig. 2B illustrated the UV–vis scanning result of PEG-PLA in the 200–600 nm patterns, which demonstrated that in this range the copolymer PEG-PLA had no UV–vis absorption. Fig. 2C and E shows the UV–vis absorption situation of DOX and folic acid in DMSO in the same pattern as PEG-PLA. The UV–vis scanning result of DOX showed that DOX had two main absorption peaks between 200 nm and 600 nm, one was at about 270 nm, another was at about 480 nm. Fig. 2E showed that folic acid has one main peak at about 290 nm. While, Fig. 2D and picture Fig. 2F, respectively, shows the UV–vis experiment results of DOX and folate in DMSO after they were connected to PEG-PLA. From Fig. 2D and F we could observe almost same results as those from Fig. 2C and E. Considering that PEG-PLA had no absorption in this range and before the measurement samples of PEG-PLA-DOX and folate-PEG-PLA were fully dialysed, to ensure the free DOX and folic acid, whose molecular weight were less than 3500, had been completely removed. According to all the evidence above we could safely draw a conclusion that DOX and folic acid had been connected to the PEG-PLA (Hyuk and Tae, 2004; Jae et al., 2004; Sabine and Katja, 2006; Zhigang et al., 2007).

3.2. Characters of drug-loaded polymeric micelles

Amphipathic tri-block copolymers self-assembled into polymeric micelles in an aqueous solution. The size of polymeric micelles is approximately less than 100 nm, which not only makes them ideal drug-delivery carriers for escaping from renal exclusion and the reticulo-endothelial system, but gives them an enhanced vascular permeability. Copolymeric micelles are composed of a hydrophilic outer shell exposed to the aqueous phase and a hydrophobic inner core encapsulating drug molecules. Fig. 3 depicts the structure of four different tri-block polymeric micelles employed in our study. Fig. 3A shows the structure of tri-block polymeric micelles loading BBSKE. The structure of copolymer micelles loading BBSKE, connected with DOX in the hydrophobic chain is displayed in Fig. 3C. While Fig. 3B and D are, respectively, the polymeric micelles, depicted in Fig. 3A and C, coated with folate. As shown in Fig. 3, the copolymer micelles usually are spherical, which have a hydrophobic core and a hydrophilic shell. BBSKE is packaged into the sphere through the hydrophobic interaction. It can be seen that the folate-PEG4000-PLA4000 is longer than folded PEG6000-PLA6000. This is because folate connected with a larger chain may

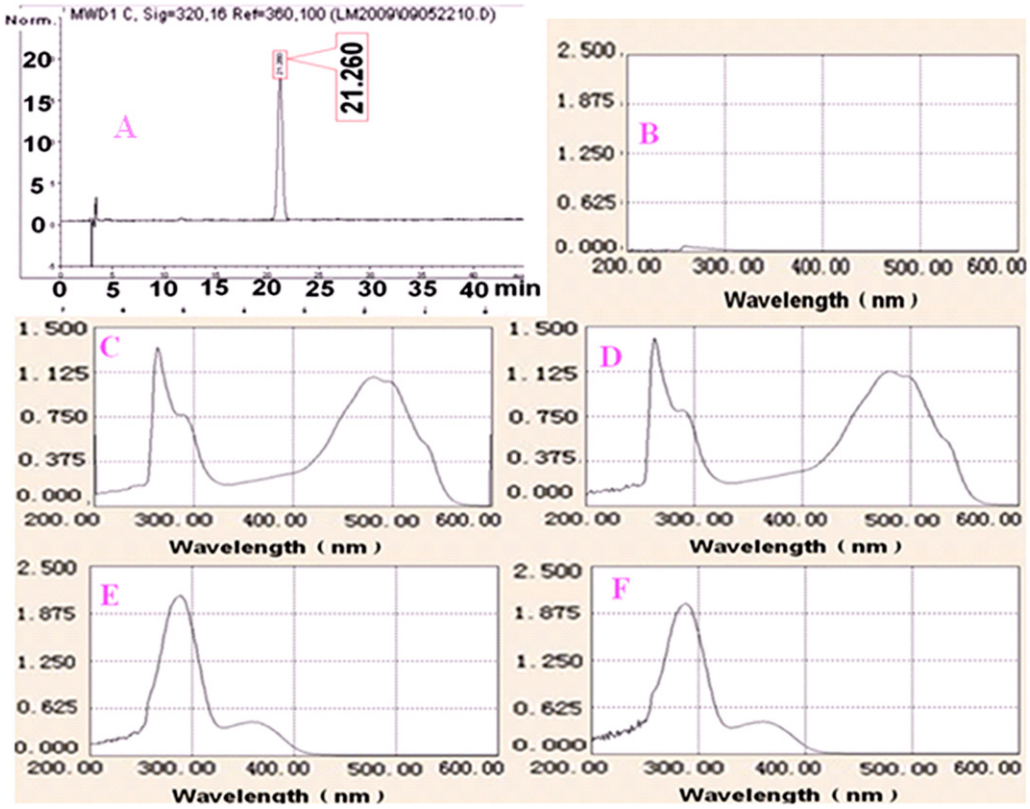


Fig. 2. Retention time of BBSKE in HPLC and UV–vis for folate and DOX.

be easier to expose to the environment and thus they can search the folate receptor and perform a targeting function more effectively.

3.2.1. Analysis of critical micelle concentration

The CMC values of tri-block copolymers were determined by a fluorescence spectroscopy measurement. Pyrene was chosen as a fluorescent probe because its photochemical properties are suitable for an effective probe (Kwon et al., 1993; Hagan et al., 1996; Yoo and Park, 2001; Jaeyoung et al., 2004; Xinru et al., 2009). The CMC values of tri-block copolymers PLA3000-PEG6000-PLA3000 was 1.74×10^{-7} mol/L and the CMC values of DOX-PLA3000-PEG6000-PLA3000-DOX was 3.89×10^{-7} mol/L. The CMC values

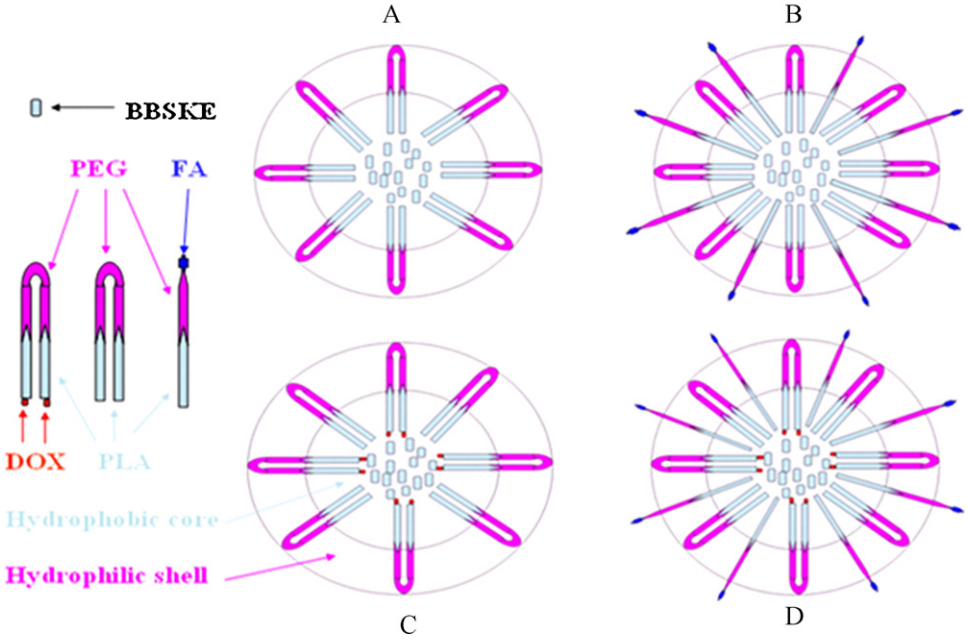


Fig. 3. The structure of copolymer micelles. (A) Structure of PEG-PLA/BBSKE copolymer micelles; (B) structure of folate-PEG-PLA/BBSKE copolymer micelles; (C) structure of PEG-PLA-DOX/BBSKE copolymer micelles; and (D) structure of folate-PEG-PLA-DOX/BBSKE copolymer micelles. PEG: polyethylene glycol; PLA: polylactic acid; DOX: doxorubicin; and BBSKE: ethaselen.

Table 1
Results of particle size determination. The value was mean ± SD ($n \geq 3$).

Particles	Size (nm)
Blank polymer micelle	31.03 ± 1.90
BBSKE polymer micelle	71.25 ± 2.75
FA-BBSKE polymer micelle	75.68 ± 2.34
DOX-BBSKE polymer micelle	83.28 ± 5.44
FA-DOX-BBSKE polymer micelle	85.43 ± 3.86

PEG: polyethylene glycol; PLA: polylactic acid; DOX: doxorubicin; BBSKE: ethaselen; and FA: folate.

of the tri-block copolymers that we synthesized was pretty low, which is an advantage of polymeric micelles as drug carriers. The CMC values of these tri-block copolymers were slightly increased when DOX was connected to the hydrophobic block. This may be attributed to the increase in the hydrophilicity of hydrophobic block with the connection of DOX. Because the absolute value of CMC is very low, however, the stability of nanoparticles was not affected by the connection of DOX.

3.2.2. Drug content and solubility analysis

The data in our experiments suggested that when BBSKE was packaged in the polymer micelle, the water solubility of BBSKE reached to 0.21 mg/ml ($n \geq 3$). Compared with the original situation where BBSKE was insoluble in water (2.57 μg/ml) (Xinru et al., 2009), polymer micelles significantly improved the water solubility of BBSKE, which enabled us to choose the method of injection as the route of administration. This will help BBSKE to perform better in its anti-tumor effect.

We prepared drug-loaded polymer micelle by the dialysis method and determined the drug content with liquid chromatography. In our condition the retention time of BBSKE was 21 min as shown in Fig. 2A. The drug-loaded content of tri-block copolymer micelles in the absence of folic acid was 4.91% ($n \geq 3$) and the drug-loaded content of copolymer micelles coated with folic acid was 4.73% ($n \geq 3$) (Weiyang et al., 2007; Xinru et al., 2009).

3.2.3. Size of nanoparticles and morphology analysis

Dynamic light scattering analysis was applied to measure the size of various polymeric micelles. Table 1 was the result of analysis of particle size for five different nanoparticles. From Table 1 we could observe that size of blank micelles was approximately 30 nm. However the size of particles was increased significantly to approximately 70 nm when it came to the polymeric micelles loading BBSKE. The size of drug-loaded micelles was bigger than that of drug-free micelles, which suggested that BBSKE molecules were trapped in the hydrophobic inner cores and that these entrapped BBSKE molecules increased the average size of BBSKE-loaded tri-block copolymer micelles. Meanwhile, the size of polymer micelles with folic acid as a target was slightly larger than that of non-folate micelles. This maybe due to folate-PEG4000-PLA4000 being longer than folded PEG6000-PLA6000. Another increase in size was observed when DOX was connected to the materials as fluorescence signal for detection.

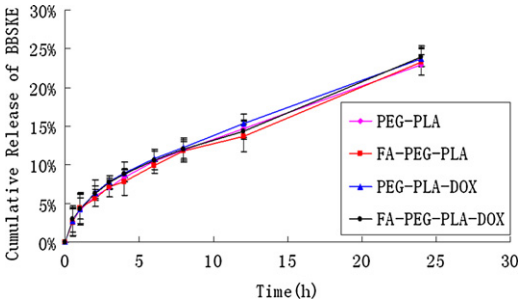


Fig. 5. In vitro release profiles of BBSKE from polymeric micelles (mean ± SD, $n = 3$).

Scanning electron microscope (SEM) was used to study morphology character of polymeric micelles. The SEM experimental results are vividly shown in Fig. 4. Fig. 4I gives the view of blank polymeric micelles investigated through SEM. Fig. 4II shows the shape of polymer micelles loaded BBSKE. Fig. 4III shows the result we obtained by observing polymer micelles loaded BBSKE and coated with folic acid using SEM. From the SEM experimental results, we can readily elicit a conclusion that the blank micelles, micelles loading drugs and micelles loading drugs and coated with folic acid are all spherical in shape and their particle sizes are less than 100 nm. Meanwhile, the results of their particle sizes shown in Fig. 4 are in accord with the data shown in Table 1.

3.2.4. In vitro release profiles of BBSKE from polymeric micelles

The results of in vitro BBSKE release experiments are presented in Fig. 5. During 24 h, the contents that BBSKE released from four kinds of polymeric micelles were all about 23%, in the media PBS (pH 7.4) with 50% fetal bovine serum. As shown in Fig. 5, the modification of targeting molecular folate has no affect on the release activities of BBSKE from polymeric micelles. Besides, the release of BBSKE from polymeric micelles modified with DOX was also very similar to the release of BBSKE from polymeric micelles without the modification, which means release activities of BBSKE from polymeric micelles were not affected by the connection of DOX.

3.3. MCF-7 cell growth inhibition study

In our study, we applied the novel anti-tumor medicine BBSKE and BBSKE packaged in tri-block copolymer micelles to inhibit the growth of MCF-7 breast cancer cells and MTT assay to analyze the treated cancer cells. Why did we choose MCF-7 cells as the candidate for our study? This was because the folate receptor was highly

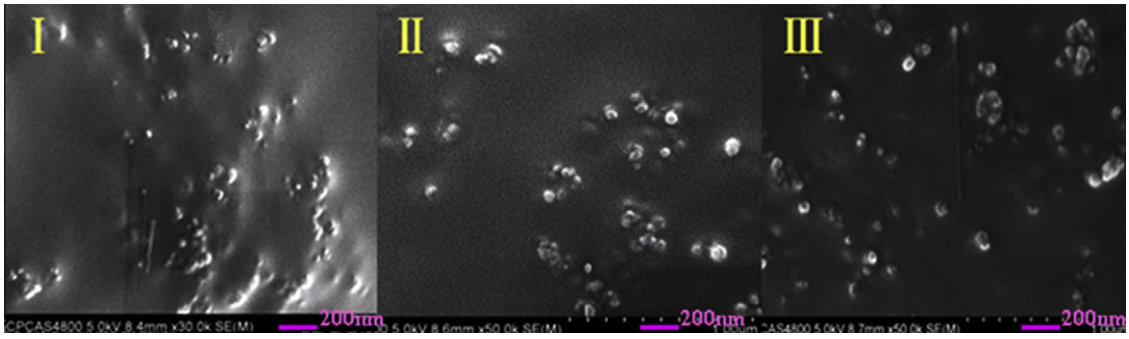


Fig. 4. SEM photography of polymeric micelles. (I) Blank polymeric micelles; (II) polymeric micelles loaded BBSKE; and (III) polymeric micelles loaded BBSKE and coated with folic acid.

Table 2
Investigation of cell growth inhibition for 24 h, 48 h, 72 h, $IC_{50} \pm SD$ (μM).

Group	24 h	48 h	72 h
BBSKE	7.14 ± 1.04	5.05 ± 0.16	4.23 ± 0.40
PM	10.54 ± 1.81	10.13 ± 1.20*	7.29 ± 0.64*
FA + PM	4.14 ± 0.06*,#	3.99 ± 0.10*,#	3.43 ± 0.31#
FA + PM + free FA (1:10)	7.84 ± 0.11†	6.88 ± 0.18*,#,†	6.30 ± 0.73†

Note: FA: folic acid; PM: BBSKE polymer micelles; FA + PM: BBSKE polymer micelles coated with folic acid; FA + PM + FA (1:10): BBSKE polymer micelles coated with folic acid as target and with free folic acid (1:10) as antagonist. Data are presented as means ± SD ($n = 3$).

* $P < 0.05$ (Student's t-test) significantly different from BBSKE group.

\# $P < 0.05$ (Student's t-test) significantly different from PM group.

† $P < 0.05$ (Student's t-test) significantly different from FA-PM group.

expressed on the surface of these breast tumor cells and one group of our polymeric micelles containing anticancer drug was coated with folate. Thus the polymeric micelles coated with folate can be absorbed into the tumor cells through receptor-mediated way, allowing it to function more effectively in killing the tumor cells. Our idea was confirmed by the data we acquired from the MTT experiment.

We have tested the cell growth inhibition of BBSKE for three different time intervals: 24 h, 48 h, and 72 h. From Table 2 it can be viewed that with the extension of time, the inhibition effect of BBSKE to tumor cells by anti-tumor drugs naked or loaded in copolymer micelles were increasingly elevated, which illustrated a certain degree of time-dependent manner. In the four groups, the inhibition effect to tumor cells growth was FA + PM group > BBSKE group > FA + PM + free FA (1:10) group > group of PM. IC_{50} of naked BBSKE was 7.14 μM, 5.05 μM and 4.23 μM when MCF-7 cells were treated with BBSKE for 24 h, 48 h and 72 h, respectively. Compared with naked BBSKE, the IC_{50} value of FA + PM group significantly declined when treatment time was 24 h (4.14 μM) and 48 h (3.99 μM). However, when the time was extended 72 h, a significant difference was not witnessed between naked BBSKE group and FA + PM group, though FA + PM group (3.43 μM) has a slightly lower IC_{50} than naked BBSKE group (4.23 μM). The difference between naked BBSKE group and FA + PM group became smaller as the time was extended from 24 h to 72 h. This result implied that copolymer micelles loading BBSKE and coated with folate can be absorbed into MCF-7 cells faster than naked BBSKE due to the interaction between folate coated on the surface of micelles and folate receptors situated at the surface of MCF-7 cells. It is the interaction between folate and folate receptors that ensured that more drugs were pumped into the tumor cells and showed a better anticancer effect (Hyuk and Tae, 2004).

When the inhibition effect of FA + PM group was compared with that of FA + PM + free FA (1:10) group, we found that when tumor cells were treated with drugs for 24 h, the inhibition effect of FA + PM was 2.55 times larger than that of PM. As time extended to 48 h and 72 h, the inhibition effect of FA + PM was 2.54 times and 2.13 times larger than that of PM, respectively. This means that free folate weakened the inhibitory effect significantly. This result can be attributed to the fact that excessive amount of free folic acid pre-occupied the folate receptors on the cell surface, which led the decreased interaction between folate coated copolymer micelles and folate receptors. Thus the data above offered us an evidence to elicit the conclusion that the folate employed as a targeting moiety significantly improved the inhibition effect of copolymer micelles loading BBSKE through the approach of receptor-mediated endocytosis.

As shown in Table 2 the inhibition effect to MCF-7 cells of group BBSKE polymeric micelles without folate (PM) was not as well as naked BBSKE. When BBSKE polymer micelles enter into cancer cells, they have to release BBKE first and then the drug can perform the function of killing the cancer. However, the naked BBSKE entered into cancer cells can kill them directly. Thus, at the cell level, BBSKE polymer micelles have a weaker suppression of tumor cell growth than naked BBSKE.

3.4. Confocal and flow cytometry study

In the experiment of Confocal and flow cytometry we also selected MCF-7 cells as the candidate cancer cells for our study. As shown in Fig. 6A, hoechst33342 is used to label the nucleus and its

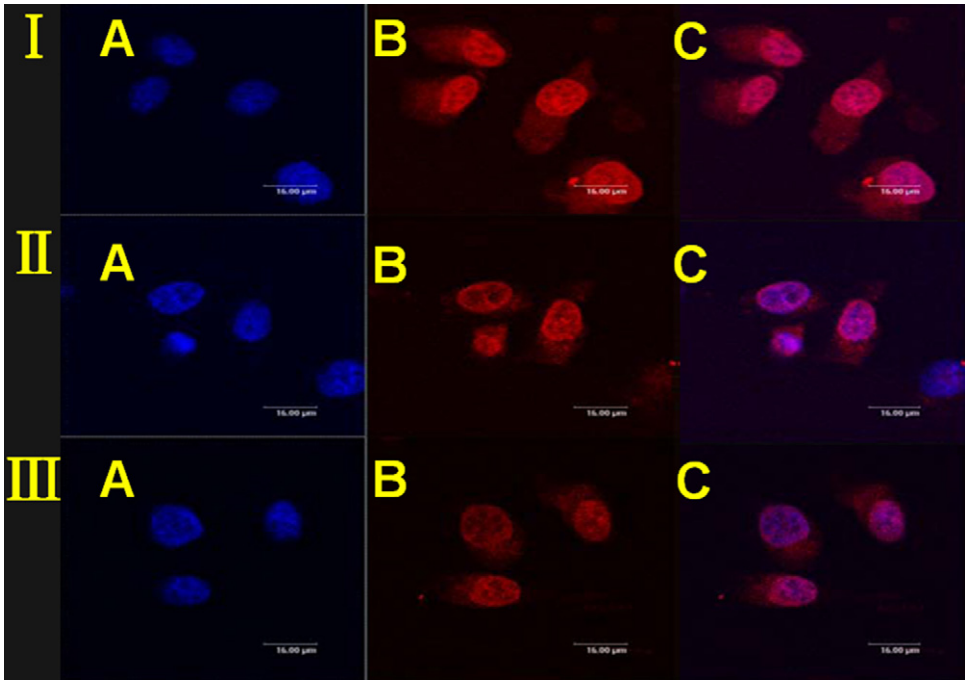


Fig. 6. Confocal microscopic images of MCF-7 cells incubated with polymeric micelles in the medium. (I) Folate-PEG-PLA-DOX/BBSKE; (II) PEG-PLA-DOX/BBSKE; and (III) free folate and folate-PEG-PLA-DOX/BBSKE. (A) Signal of hoechst33342; (B) signal of DOX; and (C) combination signals of hoechst33342 and DOX.

signal is blue, this signal can help us to locate a cell. Fig. 6B depicts the situation of polymer micelles entered into the cells with DOX as fluorescent indicator (red signal). Through the strength of DOX fluorescent signal we can acquire the information about how much copolymer micelles entered into cancer cells. The stronger the signal of DOX is, the more copolymer micelles are absorbed into cancer cells. Meanwhile, we can observe combination signals of hoechst33342 and signal of DOX in Fig. 6C. Group I in Fig. 6 are MCF-7 cells treated with FA-PEG-PLA-DOX/BBSKE. While group II in Fig. 6 and group III in Fig. 6 are MCF-7 cells treated with PEG-PLA-DOX/BBSKE and folate-PEG-PLA-DOX/BBSKE + free folate, respectively. Through the comparison of the three groups, we can clearly see that the cells treated with the polymer micelles coated by folate have stronger fluorescent signal than the cells treated by the polymer micelles without folate. This result indicated that the number of polymer micelles with folate entered into the cells were greater than that of polymer micelles without folate. However, when it comes to the cells treated with drug-loaded polymer micelles coated by folate together with free folate, the signal diminished conspicuously, which indicated the free folate and folate coated on the surface the micelles compete to anchor the folate receptor located at the surface of cancer cells. Thus the conclusion was self-evident, that micelles coated with folate can entered into the cancer cells through the access of receptor-mediated endocytosis. In addition, the results of Confocal are consistent with the results of cancer cell growth inhibition study, and they provide us evidence to explain the improved inhibition effect of BBSKE copolymer micelles with folate to MCF-7 cell growth.

Flow cytometry study was employed to verify the Confocal results. As shown in Fig. 7, Fig. 7I shows MCF-7 cells incubated with FA-PEG-PLA-DOX/BBSKE, Fig. 7II shows MCF-7 cells incubated with PEG-PLA-DOX/BBSKE and Fig. 7III shows MCF-7 cells incubated with free folate and folate-PEG-PLA-DOX/BBSKE. The study of the flow cytometry revealed the same result as to Confocal that is the folate-mediated active targeting of drugs increased the amount of drugs pumped into cancer cells. As a result polymer micelles with folate demonstrated better anti-tumor effect in comparison to naked drugs.

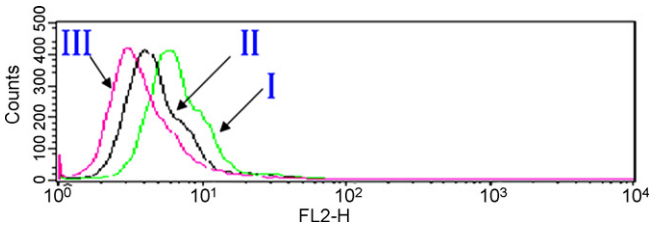


Fig. 7. Flow cytometry results of polymeric micelles. (I) FA-PEG-PLA-DOX/BBSKE; (II) PEG-PLA-DOX/BBSKE; and (III) free folate and folate-PEG-PLA-DOX/BBSKE.

3.5. Study of tissue distribution by imaging in vivo method

Imaging in vivo method was employed to study the tissue distribution of BBSKE polymeric micelles in nude mice with and without tumor. Through the comparison of these two situations, we can obtain a conclusion that polymeric micelles can aggregate in tumor site.

3.5.1. Tissue distribution of BBSKE polymeric micelles in mice without tumor

Fig. 8 depicted the distribution of polymer micelles after the same amount of micelles were injected into nude mice without tumor through the access of intravenous for 2.5 h and 24 h. Fig. 8A and B shows the situation of nude mice when they were viewed

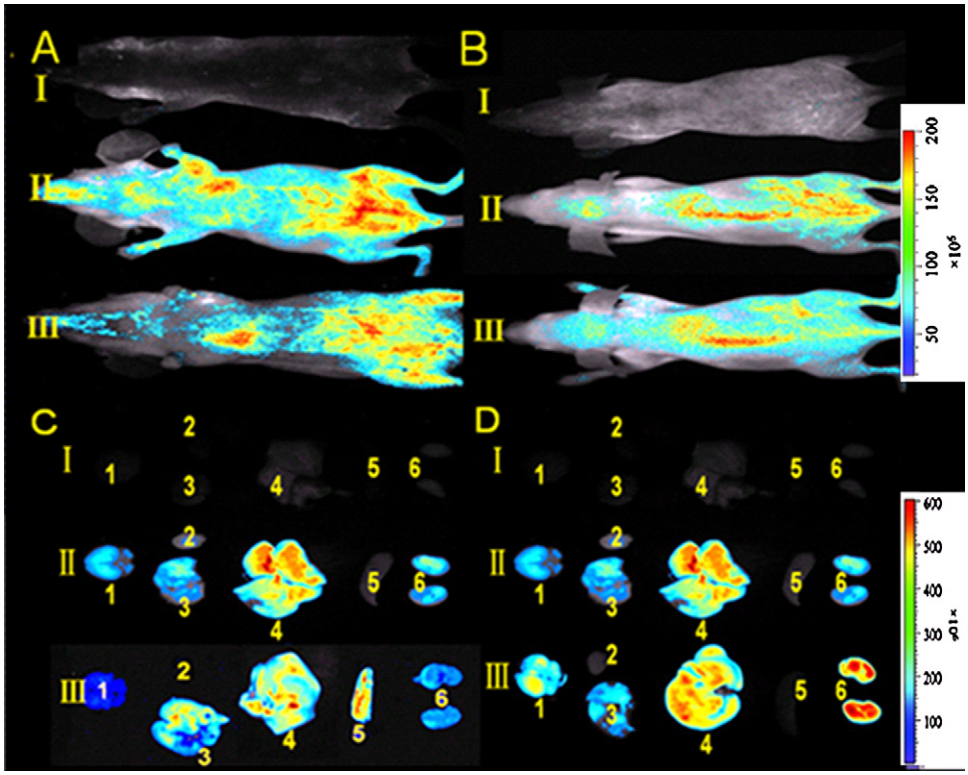


Fig. 8. Tissue distribution of BBSKE polymeric micelles in nude mice without tumor. (A) Viewed from abdomen; (B) viewed from back; (C, D) viewed from organs. (I) Group control; (II) group intravenous injected with PEG-PLA-DOX/BBSKE, 2.5 h; (III) group intravenous injected with PEG-PLA-DOX/BBSKE, 24 h, for A, B and C; (III) group administrated with PEG-PLA-DOX/BBSKE orally, 4 h, for D. 1: Brain; 2: heart; 3: lung; 4: liver; 5: spleen; and 6: kidney.

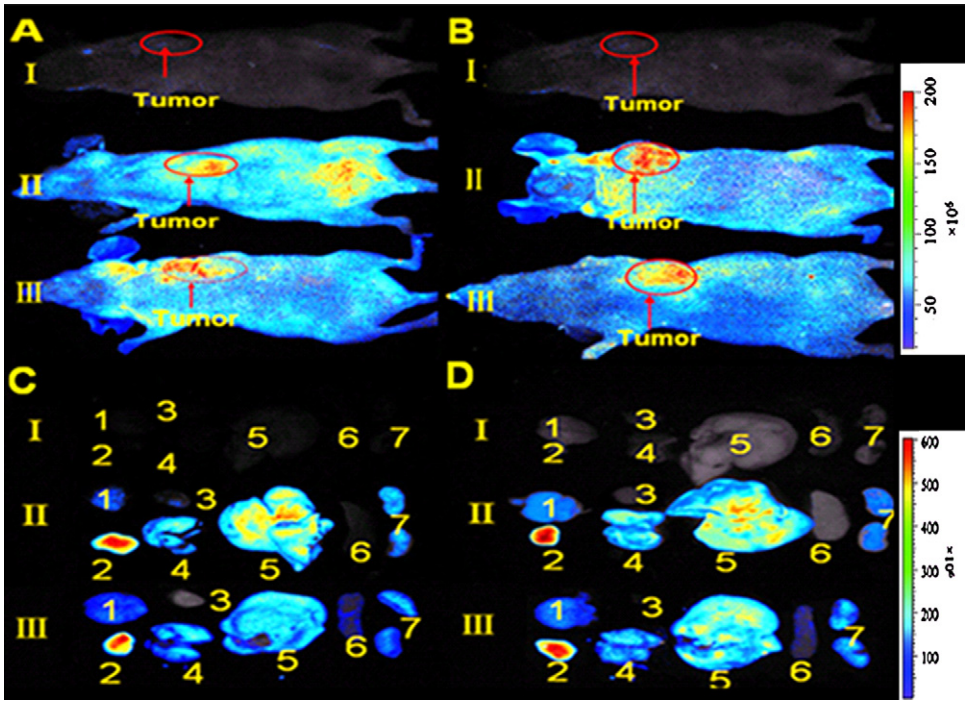


Fig. 9. Tissue distribution of BBSKE polymeric micelles in nude mice with tumor. (A, C) Group intravenous injected with PEG-PLA-DOX/BBSKE; (B, D) group intravenous injected with folate-PEG-PLA-DOX/BBSKE. (I) Group control; (II) group treatment (intravenous injection, 2.5 h); (III) group treatment (intravenous injection, 24 h). 1: Brain; 2: tumor; 3: heart; 4: lung; 5: liver; 6: spleen; and 7: kidney.

from abdomen and back, respectively. While, the tissue distribution of BBSKE polymer micelles in nude mice viewed from organs are shown in Fig. 8C. In Fig. 8A–C, I is group control; II is group intravenous injected with PEG-PLA-DOX/BBSKE and had taken a photograph after 2.5 h; III is group intravenous injected with PEG-PLA-DOX/BBSKE and take a photograph after 24 h. There are no micelles distributed into heart neither for 2.5 h nor for 24 h. However, an obvious difference in distribution of micelles between 2.5 h and 24 h can be observed from Fig. 8. As shown in Fig. 8C.II, there were no polymeric micelles distributed into spleen after polymeric micelles were intravenously injected into nude mice after 2.5 h. However, when time was extended to 24 h, the polymeric micelles distributed in the spleen were significantly increased. Meanwhile, the amount of micelles in the lung administrated after 24 h only enhanced slightly as compared with the situation after treated 2.5 h. However, the distributions of micelles in other organs were weakened to varying degrees. An organ that should draw our especial attention was the brain, whose blood–brain barrier causes the drugs in general to have difficulty entering the brain. But as shown above tri-block copolymer micelles can enter into the brain in a certain degree. This was a matter of great significance, which indicated that copolymer micelles provided an alternative method for future treatment of brain tumors.

Fig. 8D vividly shows us the tissue distribution comparison between two types of administration of polymer micelle – intravenous and oral. I is group control; II is group intravenous injected with PEG-PLA-DOX/BBSKE and a photograph taken after 2.5 h; III is group treated with PEG-PLA-DOX/BBSKE through oral approach and a photograph taken after 4 h. Oral administration had a process of absorption, so we observed the distribution of oral administration after 4 h, and the dose of oral administration was 2.5 times larger than that of the injection. From the results we observed that the differences between oral administration of micelles and intravenous administration of micelles mainly exist in two organs – brain and kidney. As shown in Fig. 8D, when polymeric micelles were administrated orally the quantity of polymeric micelles gathered in kidney increased tremendously, compared with the situation when polymeric micelles were administrated through injection. In addition, the quantity of polymeric micelles gathered in brain was slightly increased than when they were administrated orally. The result that micelles are distributed in the brain prompts us to suppose that micelles are absorbed into the blood circulation as an integrated form. They could not penetrate the BBB and accumulate in brain if they were not absorbed into the blood circulation as a nanoparticle.

3.5.2. Tissue distribution of BBSKE polymeric micelles in mice with tumor

As shown in Fig. 9, tissue distribution study of BBSKE polymer micelles in nude mice with tumor revealed the fact that tri-block copolymer micelles have the trend of aggregating in the tumor site. Fig. 9A and C shows the results when nude mice with tumor were intravenously injected with PEG-PLA-DOX/BBSKE. Fig. 9B and D shows the results when nude mice with tumor were intravenously injected with folate-PEG-PLA-DOX/BBSKE. In Fig. 9, I is group control; II is group that was intravenously injected with polymeric micelles and a photograph taken after 2.5 h; III is group that was intravenously injected with polymeric micelles and a photograph taken after 24 h.

Fluorescence was employed as a detection signal. So the stronger the florescence signal of an organ was, the larger the quantity of polymeric micelles accumulated in the organ was. From Fig. 9 we can find that polymeric micelles have a high concentration in tumor site. For both folate modified polymeric micelles and polymeric micelles without folate, the accumulation amount in the tumor was comparatively higher than in any other organ. At the same time, polymeric micelles scattered into the brain, kidney, liver and spleen were reduced compared with the situation in nude mice without tumor. Among these organs, polymeric micelles distributed in spleen reduced the most significantly. Meanwhile, the quantity of polymeric micelles aggregated in liver also declined conspicuously. When we compared Fig. 9A and C with B and

D, respectively, we found that nude mice injected with folate-PEG-PLA-DOX/BBSKE have stronger fluorescence signal in tumor tissue than nude mice injected with PEG-PLA-DOX/BBSKE, both at 2.5 h and 24 h. This result indicates that folate modified polymeric micelles performed a stronger aggregation behavior in tumor site than polymeric micelles which were not coated with folate, when the same amount of polymeric micelles were injected into nude mice.

The results above offer us evidence to elicit a conclusion that polymeric micelles tend to accumulate at solid tumors. We can attribute enhanced permeation and retention (EPR) effect (Kataoka et al., 1992; Colin et al., 1994; Kwon and Okano, 1996; Minko et al., 1998; Yoo et al., 1999) as a major mechanism for their unique bio-distribution profile in the tumor tissue. 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 neo-vascularization. As a result, many new vessels are formed, but their cell junctions are not as tight as those of normal tissues. Thus, polymeric micelles having a size of about 100 nm were likely to freely pass through the endothelial junctions of the capillaries in tumor tissue, but not in normal tissue. In our study, the particle size of polymeric micelles we prepared was less than 100 nm, so they can pass the vascular endothelial in tumor tissue and accumulate in tumor tissues. Moreover, because polymeric micelles were decorated with PEG chains on the surface, their circulation time in the blood stream was greatly prolonged which is also beneficial by accumulating in tumor tissue (Klibanov et al., 1990). In addition, folate modified polymeric micelles that were accumulated in the solid tumor region might be more readily taken up by tumor cells by a receptor-mediated endocytosis process. This indicates that PEG-PLA tri-block polymeric micelles without targeting motif can gather in tumor site through passive targeting-EPR effect and long circulation effect, while a ligand-mediated polymeric nanoparticle system for anticancer drug delivery with both a passive targeting property through a size effect, and an active targeting activity through specific recognition of ligand. The passive targeting allowed folate modified polymeric micelles aggregate in the tumor site, while the active targeting permitted them to be readily taken up by tumor cells at the site. Thus the combined passive and active targeting effects were likely to act synergistically, and they were mainly responsible for accumulation trends to tumor tissue. Therefore, polymeric micelles coated with folate have a slightly stronger accumulation trends than polymeric micelles that were not modified by folate.

4. Conclusion

In our study, we prepared a kind of tri-block copolymer micelles as carriers for novel anticancer drug BBSKE, utilizing the material PEG-PLA that we synthesized. The polymeric micelles improved the water solubility of BBSKE. Therefore, it was made to be a more promising formulation for this new anticancer drug. Folate was coated on the surface of this nanoparticle, which made the inhibition effect of copolymer micelles to MCF-7 tumor cells significantly better. By adopting a new method of imaging in vivo, we studied the distribution of micelles in nude mice with MCF-7 tumor and without MCF-7 tumor, which showed that this polymer micelles loading BBSKE could accumulate into tumor efficiently.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.03.001.

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