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Research Paper

Novel Biodegradable Polylactide/poly(ethylene glycol) Micelles Prepared by Direct Dissolution Method for Controlled Delivery of Anticancer Drugs

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Purpose. The aim of this study is to develop novel polylactide/poly(ethylene glycol) (PLA/PEG) micelles as carrier of hydrophobic drug (paclitaxel) by direct dissolution method without using any organic solvents. The *in vitro* and *in vivo* release properties were studied in comparison with micelles prepared by dialysis.

Methods. Drug encapsulation efficiency (EE) and loading content (LC) of the micelles were evaluated by high-performance liquid chromatography. Micelle diameters and structures were determined by dynamic light scattering and transmission electron microscopy. *In vitro* release was performed in phosphate-buffered saline (pH 7.4) at 37°C, and *in vivo* experiments were realized in lung cancer-bearing mice.

Results. Similar EE and LC values were obtained for micelles by direct dissolution method and those by dialysis. L- and D-PLA/PEG mixed micelles present higher drug encapsulation ability than separate micelles due to stereocomplexation. Micelle diameters are enlarged by drug-loading. Faster drug release was obtained for micelles by direct dissolution than those by dialysis. Compared with current clinical formulation and micelles by dialysis, paclitaxel-loaded micelles by direct dissolution showed the highest antitumor ability.

Conclusion. The L- and D-PLA/PEG mixed micelles by direct dissolution method present many advantages such as easy formulation and absence of toxic organic solvents, which shows great potential as carrier of hydrophobic drugs.

KEY WORDS: drug delivery; micelle; paclitaxel; polylactide; poly(ethylene glycol).

INTRODUCTION

Paclitaxel, derived from the bark of the pacific yew tree *Taxus brevifolia*, is regarded as one of the most efficient anticancer drugs. It has been widely used for the treatment of various tumors, such as ovarian cancer, metastatic breast cancer, non-small cell lung cancer, head and neck malignancies and other cancers (1–5). However, the clinical application of paclitaxel is limited by its poor aqueous solubility, which is below 0.5 μg/ml (6–8). A co-solvent formulation consisting of 50:50 Cremophor EL and dehydrated alcohol has been used for its clinical administration, called Taxol®. But serious side effects are associated with the use of Cremophor EL, including hypersensitivity reaction, nephrotoxicity, neurotoxicity and cardiotoxicity (9,10). Many novel delivery systems of paclitaxel have been investigated to reduce the side effects, such as parenteral emulsion (11), liposomes (12,13), nanoparticles (14–19), micro- or nanospheres (20–25), mixed-micelles (26), polymeric micelles (27–30) and conjugates (31–33). The drug encapsulation abilities of these systems are affected by many parameters, such as polymer composition, drug feeding amount, fabrication method, etc. Thus the encapsulation efficiency varies in a wide range and so does the cytotoxicity level. Although many systems have shown the potential as hydrophobic drug carriers, many problems should be resolved before clinical use, including phagocytic clearance during blood circulation, toxic side effects caused by its systemic spread, and exclusion from the cell by membrane transporters, etc. (33).

Colloidal systems, and especially polymeric micelles prepared from amphiphilic block copolymers, seem to be more promising and have been widely investigated as drug delivery systems (DDS) (34–36). A core-shell structure is usually formed through self-assembly in aqueous media: the hydrophobic blocks aggregate to form an inner core which is able to encapsulate hydrophobic drugs with improved solubility; the hydrophilic shell consists of a brush-like protective corona that stabilizes the micelles in aqueous solution. 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 (37,38). Furthermore, the nano-scale size range with a narrow distribution enables these micelles to achieve higher accumulation at the target site through an enhanced permeation retention (EPR) effect (38,39).

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Polylactide/poly(ethylene glycol) (PLA/PEG) copolymer micelles have been largely investigated as drug carriers (40–44). Bioresorbable PLA blocks constitute the hydrophobic core of the micelles, and hydrophilic PEG blocks form the hydrated outer shell. It is well known that PEG blocks can limit the opsonification and subsequent non-specific uptake by the reticuloendothelial system (RES), thus the drug circulation time in plasma could be consequently increased. Recently, a formulation of paclitaxel-loaded PLA/PEG micelles named Genexol-PM has been used in Phase II clinical trial for treatment of breast and lung cancer (45–47). However, it should be noted that in literature, the common methods to prepare micelles are solvent evaporation, dialysis and solution casting (48–51), all involving the utilization of organic solvents such as dichloromethane, acetone, DMF, and acetonitrile, which might cause side effects to human body. During micelle formation in such cases, the PEG blocks migrate from the organic phase to the water interface, and the PLA blocks remain in the organic phase. A packed and solidified PLA core is thus formed after solvent elimination. In contrast, micelles could be formed by directly dissolving the amphiphilic compound in an aqueous medium at a concentration above its critical micelle concentration (CMC). Such micelles exhibit a fluid structure (28).

In our previous work, we synthesized and characterized a series of PLA/PEG diblock and triblock copolymers. The properties of micelles directly prepared from aqueous solutions containing L-PLA/PEG or D-PLA/PEG or both were investigated to elucidate the effect of stereocomplexation (52). To the best of our knowledge, paclitaxel encapsulation and release properties from PLA/PEG micelles prepared by direct dissolution method have not been reported in literature, especially for the L-PLA/PEG and D-PLA/PEG mixed micelles. Although some reports did synoptically mention the direct dissolution method as one of the drug-loading procedures in micelle preparation (53,54), detailed encapsulation results were not given. In this work, we investigated the properties of PLA/PEG aqueous micelles formed by direct dissolution method as paclitaxel carriers, especially in the case of L-PLA/PEG and D-PLA/PEG mixed micelles. The drug encapsulation and release properties were compared with traditional dialysis micelle-formation method. The *in vivo* biodistribution and antitumor efficacy of the paclitaxel-loaded micelles were also investigated. The results are reported herein in comparison with literature data.

MATERIALS AND METHODS

Materials

L-lactide and D-lactide were obtained from Purac and recrystallized from ethyl acetate. Monomethoxy poly(ethylene glycol) (mPEG) with molar mass of 5,000 and dihydroxyl PEG with molar masses of 4,600 and 20,000 were supplied by Fluka. Zinc lactate was purchased from Sigma. Paclitaxel was obtained from Techwell Biopharmaceutical Ltd. (Shanghai, China). Acetonitrile of HPLC grade and all other organic solvents of analytical grade were supplied by Sinopharm Chemical Reagent Co. Ltd. and used as received. Female SPF C57BL/6J mice were supplied by Shanghai Cancer Institute, China. The animal experiments were carried out according to the guidelines of the Ethical Committee for the care of laboratory animals of the local government.

Synthesis of PLA/PEG Copolymers

PLA/PEG block copolymers were synthesized by ring-opening polymerization of L- or D-lactide in the presence of PEG homopolymer with zinc lactate catalyst, as described in detail in previous papers (52,55). Triblock and diblock copolymers were obtained with PEG or mPEG, respectively (Table I). The yield of the reactions ranged from 80 to 90%.

The composition of the copolymers was determined by proton nuclear magnetic resonance (^{1}H NMR), which was recorded at room temperature with a Bruker spectrometer operating at 250 MHz by using DMSO-d_6 as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference.

Preparation of Paclitaxel-Loaded Micelles

Two preparation methods (direct dissolution and dialysis) were applied to encapsulate paclitaxel within PLA/PEG micelles. A 10% theoretical loading (e.g. paclitaxel, 2 mg; L-PLA/PEG and D-PLA/PEG, 10 mg each) was used in all cases. The direct dissolution method involves dissolving paclitaxel and

Table I. Molecular Characteristics of PLA/PEG Block Copolymers

Acronym	Copolymer	PEG	EO/LA[a]	DP_{PEG}[b]	DP_{PLA}[c]	M_n[d]
1L	$L_{12}EO_{104}L_{12}$	PEG4600	4.2	104	24	6,400
1D	$D_{13}EO_{104}D_{13}$	PEG4600	4.1	104	26	6,400
2L	$L_{25}EO_{113}$	mPEG5000	4.5	113	25	6,800
2D	$D_{26}EO_{113}$	mPEG5000	4.3	113	26	6,870
3L	$L_{21}EO_{454}L_{21}$	PEG20000	11.0	454	42	23,000
3D	$D_{22}EO_{454}D_{22}$	PEG20000	10.5	454	44	23,100

[a] EO/LA, the molar ratio of ethylene oxide to lactate repeat units which was calculated from the integration of NMR signals belonging to PEG blocks at 3.6 ppm and to PLA blocks at 5.2 ppm
[b] $DP_{PEG} = M_{nPEG}/44$
[c] $DP_{PLA} = DP_{PEG}/(EO/LA)$
[d] $M_n = DP_{PEG}\cdot 44 + DP_{PLA}\cdot 72$

Table II. Drug Encapsulation Data of Paclitaxel-Loaded Micelles Prepared by Direct Dissolution and Dialysis Methods

Sample	Copolymer[a]	Micelle preparation method	EE (%)	LC (%)
S1	1L+1D	direct dissolution	52.2±0.2[b]	5.1±0.1
S2	1L+1D	dialysis	53.2±0.3	5.3±0.1
S3	1L	direct dissolution	32.1±0.1	3.2±0.1
S4	1L	dialysis	35.6±0.3	3.5±0.2
S5	2L+2D	direct dissolution	46.5±0.2	4.5±0.1
S6	2L+2D	dialysis	42.6±0.3	4.1±0.2
S7	3L+3D	direct dissolution	30.6±0.1	3.0±0.1
S8	3L+3D	dialysis	36.7±0.2	3.6±0.1

[a] PLA/PEG copolymers used to prepare paclitaxel-loaded micelles: L+D, using 50/50 w/w L-PLA/PEG and D-PLA/PEG; L, using L-PLA/PEG only. The acronyms of the copolymers are the same as in Table I.
[b] Data represent mean value±S.D., n=3

PLA/PEG copolymers in 2 ml of distilled water under stirring for various periods of time, as in the case of micelle preparation without using any organic solvents (52). In the dialysis method, both paclitaxel and PLA/PEG copolymers were first dissolved in 1-methyl-2-pyrrolidone (1 ml), a low toxic and water miscible solvent. The solution was then transferred into a pre-swollen dialysis membrane (MWCO=3,500) and dialyzed during 24 h against distilled water (1 l) which was regularly renewed by fresh water.

Encapsulation Efficiency of PLA/PEG Micelles

The drug-containing micellar solutions obtained above were then transferred into centrifugal tubes. After centrifugation (4,000 rpm, 10 min), the supernates were recovered, and the resulting sediments were measured using reverse-phase high-performance liquid chromatography (HPLC) as described below to determine the amount of free paclitaxel which was not encapsulated. The drug loading content (LC) and the drug encapsulation efficiency (EE) are calculated as follows:

$$\mathrm{LC} = \frac{\text{total paclitaxel amount} - \text{free paclitaxel amount}}{\text{amount of paclitaxel loaded micelle}} \times 100 \quad (1)$$

$$\mathrm{EE} = \frac{\text{total paclitaxel amount} - \text{free paclitaxel amount}}{\text{total paclitaxel amount}} \times 100 \quad (2)$$

where the amount of paclitaxel-loaded micelle was determined from the total amount of copolymer and loaded paclitaxel.

The HPLC was performed with a LC-10A apparatus (Shimadzu) equipped with a UV detection (SPD-10A, Shimadzu) and a 218MR54 column (4.6×250 mm, pore size 5 μm, C18, Vydac, USA). The detection wavelength was 227 nm. The sample solution of mobile phase (acetonitrile/water 55:45 *v*/*v*) was filtered through 0.22 μm filter before injection. The flow rate was 1.0 ml/min. Retention time was controlled at 5.3 min, and the calibration curve was linear in the range of 0.1–20 mg/l with a correlation coefficient of R^2= 0.9999. The area of each eluted peak was integrated and used for paclitaxel quantification. The analysis was performed in triplicate for each sample.

Size and Morphology

The size and size distribution of micelles were measured by using dynamic light scattering (DLS), which was carried out using a commercial laser light scattering spectrometer (Malvern Autosizer 4700, Malvern Instrument, Worcs, UK) equipped with a digital time correlator (Malvern PCS7132) and Compass 315 M-100 Diode-Pumped Laser as the light source (output power ≥ 100 mW, CW at λ_0=633 nm, Coherent Laser division, Santa Clara, CA). All the DLS measurements were made at 25.0±0.1°C with a 90° scattering angle, and the samples were filtered through a 0.45-μm filter (Millipore) before measurements. The autocorrelation functions from DLS were analyzed by using the constrained regularized CONTIN method to obtain the diameter distributions.

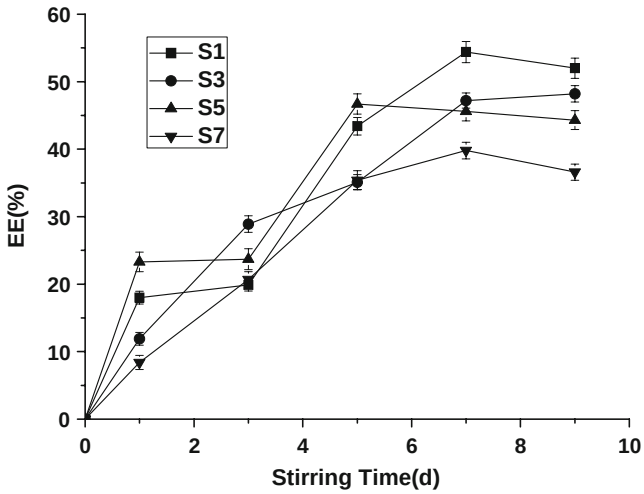

Fig. 1. Effect of stirring time on the drug encapsulation efficiency of micelle systems obtained by direct dissolution method (S.D. shown as error bars, n=3).

The morphology of micelles was observed by using transmission electron microscopy (TEM), which was performed on a Hitachi H-600 electron microscope, operating at an accelerating voltage of 75 kV. Specimens were prepared by dipping a copper grid into aqueous micelle solutions. The grid was then left to stand on a piece of filter paper and air dried before measurements.

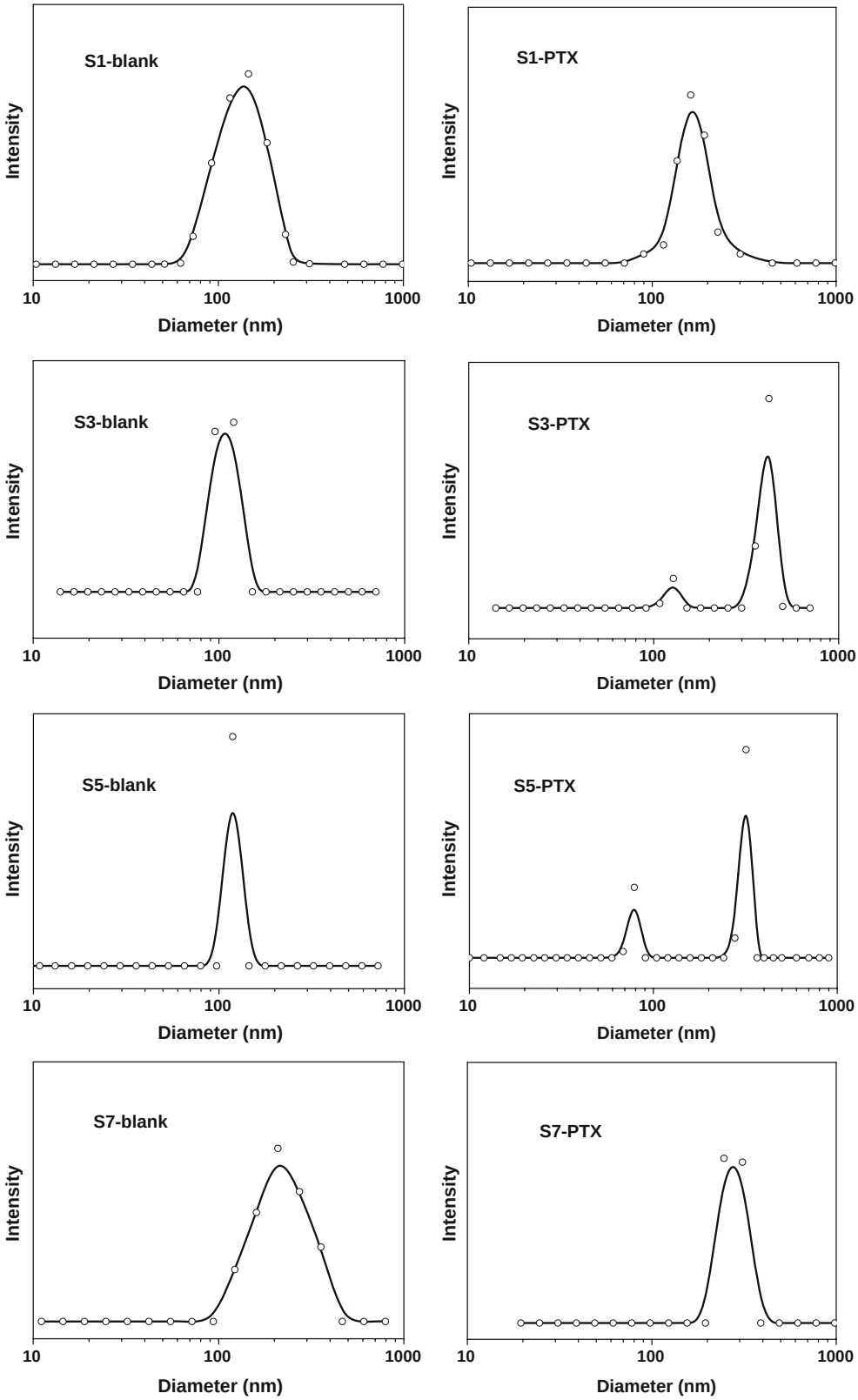

Fig. 2. Size distribution of blank and drug-loaded micelles, determined by DLS (-blank, blank micelles; -PTX, paclitaxel-loaded micelles).

Table III. Hydrodynamic Diameter and Polydispersity of Blank and Drug-Loaded Micelles

Sample	Diameter (nm)	Polydispersity
S1-blank	129.8±4.5[a]	0.17
S1-PTX	178.0±5.3	0.19
S3-blank	107.0±2.7	0.07
S3-PTX	204.9±6.9	0.19
S5-blak	101.4±1.5	0.06
S5-PTX	207.8±5.0	0.15
S7-blank	226.8±5.9	0.10
S7-PTX	276.4±7.5	0.24

[a] Data represent mean value ± S.D., $n=3$

In Vitro Release of Paclitaxel from Micelles

Paclitaxel-loaded micellar solution was transferred into a dialysis membrane (MWCO=3,500), which was then placed in 20 ml of phosphate-buffered saline (PBS, pH 7.4). *In vitro* drug release was allowed to proceed at 37°C under agitation (Heidolph unimax shaker 1010, 160/min). At various time intervals, the dialysis solution was taken out and replaced by 20 ml of fresh PBS, followed by analysis using HPLC.

In Vivo Pharmaceutical Properties

Female SPF C57BL/6 J mice (6–8 weeks, 20±5 g) were inoculated subcutaneously into the left back with 2×10^5 human Lewis lung cancer cells. When tumor size reached 400–1,000 mm^3, the animals were injected intraperitoneally with the following regimens, using a dose of 250 mg/m^2 for paclitaxel: (1) paclitaxel-loaded L- and D-PLA/PEG4600 mixed micelles derived from direct dissolution method; (2) paclitaxel-loaded L- and D-PLA/PEG4600 mixed micelles derived from dialysis method; (3) current clinical formulation of paclitaxel (1:1, v/v Cremophor EL:dehydrated alcohol). Control was realized with mice which were just injected with saline. Four mice were sacrificed at 2 h, 6 h, 1, 3, 5, 7d for (1) and (2) and 2, 6, 8 h for (3) after drug administration. Samples of plasma, heart, liver, spleen, lungs, kidneys and tumor were harvested and stored at −50°C before HPLC analysis to determine paclitaxel concentration.

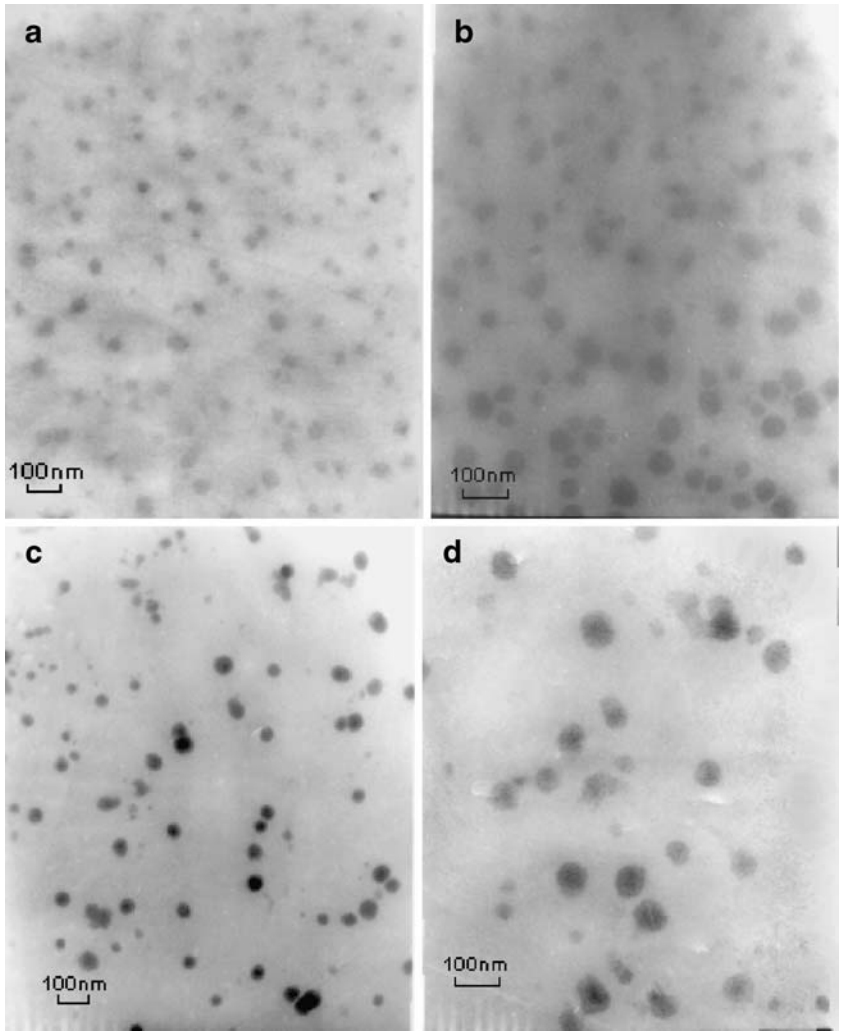

Fig. 3. Transmission electron micrographs of S1 blank (**a**, **b**) and drug-loaded (**c**, **d**) aqueous micelle solutions (**a**, **c**, magnification 50 k; **b**, **d**, magnification 100 k).

The antitumor activity was evaluated by measuring tumor size with a caliper at various time points after injection. Tumor volume was calculated by the formula $(a^2 \times b)/2$, where a is the width and b is the length in mm. The final inhibition ratio of tumor growth (IR) was calculated by the following equation:

$$\mathrm{IR} = \frac{\text{Tumor Volume of Control Group} - \text{Tumor Volume of Treatment Group}}{\text{Tumor Volume of Control Group}} \times 100 \quad (3)$$

RESULTS

Drug Encapsulation Properties

The drug loading results of micelles prepared via the two different routes, i.e. direct dissolution and dialysis are presented in Table II. The L/D mixed micelles of S1 exhibit a much higher drug loading efficiency than the separate micelles of S3, both prepared via direct dissolution. Similarly, S2 exhibits a higher drug loading efficiency than S4, both prepared via dialysis.

It is worth noting that the drug EE and LC values are comparable for micelles derived from the same copolymers but by different preparation methods. The direct dissolution method is able to yield micelles with comparable drug loading efficiency as the traditional dialysis method. In fact, the drug loading ability of the micelles prepared by direct dissolution highly depends on the stirring time. Fig. 1 shows the EE variation of different micelle systems prepared by using direct dissolution method as a function of stirring time. The EE of the micelles increased with stirring time in all cases and reached the maximum after 5 days for S5 but 7 days for the other three triblock copolymer systems.

It is also of interest to compare the drug loading abilities of triblock and diblock copolymers. As shown in Table II, S1 derived from PLA/PEG4600 triblocks exhibits higher EE and LC values than S5 which consists of PLA/mPEG5000 diblocks, although both copolymers present similar PEG length and EO/LA ratios. On the other hand, S1 presents much higher EE and LC than S7 derived PLA/PEG20000.

Size and Morphology

The size and size distribution of the micelles were obtained by DLS measurements. Fig. 2 shows the size distribution of blank and drug-loaded micelles prepared by direct dissolution method. Two populations of micelles were observed for drug-loaded S3 and S5 micelles, a smaller one around 100 nm and a larger one ranging from 200 to 300 nm, while only one population was detected for other micelle systems. From the hydrodynamic diameter results presented in Table III, it can be concluded that the drug-loaded micelles were enlarged as compared with the blank micelles.

The structures of the micelles was examined using TEM. Fig. 3 shows the micrographs obtained for S1 blank and drug-loaded micelles. Micelles are distinctly observed as dark and discrete spots. The inner core of air-dried micelles is clearly distinguished, especially for drug-loaded micelles. The diameter of micelles estimated from the TEM micrographs was approximately 50–60 nm for blank micelles and 70–80 nm for drug-loaded micelles, i.e. lower than that from DLS measurements.

In Vitro Drug Release Properties

Fig. 4 and Fig. 5 present comparatively the drug release profiles from micelles prepared by direct dissolution and dialysis methods. Similar parabolic release profiles are observed. However, micelles by direct dissolution method (Fig. 4) exhibit a significantly faster release rate as compared with those by dialysis (Fig. 5). Nearly 45% of drug release are detected after 30 days for S1 by direct dissolution, while only 16% is achieved for S2 by dialysis, both being derived from PLA/PEG4600 triblocks. On the other hand, it should be noticed that S3 from single L-PLA/PEG4600 copolymer exhibits faster release than S1 from mixed L-PLA/PEG4600 and D-PLA/PEG4600 copolymers (Fig. 4). Similar observations are made in the case of S2 and S4 (Fig. 5).

Differences are also detected between micelles derived from diblock and triblock copolymers. S1 derived from PLA/PEG4600 triblocks presents slower release rate than S5 which consists of PLA/mPEG5000. S7 initially presents faster release rate than S1, but drug release slows down beyond 10 days.

In Vivo Biodistribution Studies

The *in vitro* data showed that drug-loaded micelles could keep a sustaining release rate for over 30 days, thus it guaranteed the *in vivo* micelle formulations with a persistent release during the treatment period (7 days). Paclitaxel concentrations in plasma and various tissues of mice after administration of drug-loaded S1 and S2 micelles are presented in Tables IV and V, respectively. The *in vivo*

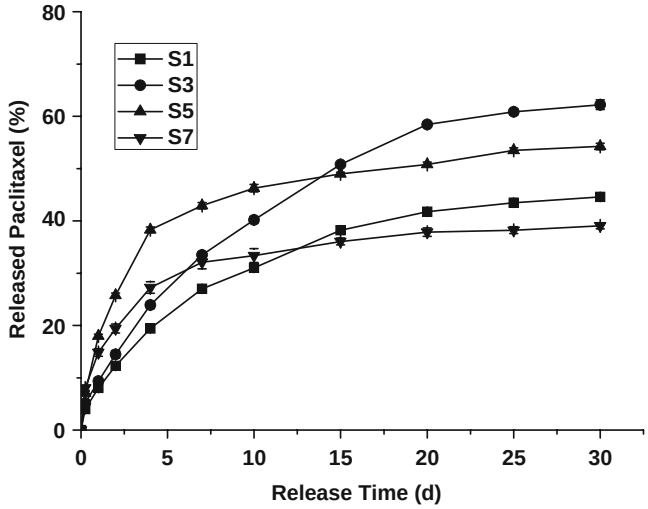

Fig. 4. *In vitro* release of paclitaxel from S1, S3, S5 and S7 micelles prepared by direct dissolution method (S.D. shown as error bars, $n=3$).

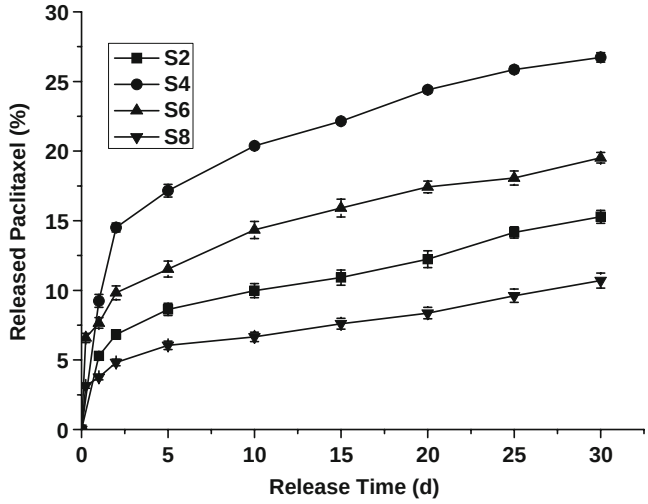

Fig. 5. *In vitro* release of paclitaxel from S2, S4, S6 and S8 micelles prepared by dialysis method (S.D. shown as error bars, $n=3$).

biodistribution of paclitaxel after administration of S1 appears very different from that of S2 at all time points. Paclitaxel concentrations reach a maximal value 2 h after administration of S1 in plasma and after 1d in tissues, and decrease progressively thereafter. The maximal values are obtained in plasma and in tissues after 1d and 3d, respectively, after administration of S2. On the other hand, the concentration of paclitaxel detected in plasma and tissues is always higher with S1 administration than with S2. In the whole period of 7 days, the paclitaxel concentration in tumor can be kept at a high level for mice with S1.

In Vivo Antitumor Efficacy

The antitumor efficacy of drug-loaded micelles was evaluated by measuring tumor volume after administration. Fig. 6 shows the tumor growth profile of the lung cancer-bearing mice treated by saline (control), S1 micelles, S2 micelles and clinical paclitaxel formulation (1:1, *v*/*v* Cremophor EL:dehydrated alcohol). Almost linear tumor growth is detected in the control group, while the S1 group presents the lowest growth rate during the whole treatment period. The clinical formulation of paclitaxel initially delays tumor growth as well as S1 micelles. However, rapid tumor growth is detected beyond 3 days in mice with the clinical formulation as in control mice. S2 micelles seem to show the inhibition ability against tumor 3 days after injection. The inhibition ratios of tumor growth are summarized in Table VI. The highest value is obtained in the case of S1 micelles administration.

DISCUSSION

The effect of stereocomplexation on the micelle stability has been previously investigated (52). The mixed micellar solutions containing both L-PLA/PEG and D-PLA/PEG copolymers exhibit lower CMC than separate solutions, in agreement with higher stability due to stronger interactions between L-PLA and D-PLA blocks. Therefore, the drug incorporation ability of the micelles can be greatly improved by stereocomplexation of enantiomeric PLA blocks. That is why the L/D mixed micelles exhibit higher drug-loading efficiency than the separate micelles. Kang *et al*. have reported on paclitaxel-loaded stereocomplex-type micelles formed from L-PLA/PEG and D-PLA/PEG, using paclitaxel as a prototype drug (56). Unfortunately, the fabrication method involved utilization of acetone. The LC value was 0.5% (*w*/*w*), which is almost 10 times lower than our LC values. However, the EE value was found to range from 87 to 92%, i.e. higher than values obtained in this work (Table II). This can be ascribed to two factors. First, as the denominator of Equation (2), the lower drug-feeding (0.05 mg/ml acetone solution of the drug) could improve the EE value but also would lead to a much lower LC value. Second, longer hydrophobic PLA blocks ($DP_{PLA}=75$) are believed to be able to encapsulate more drug in the core, leading to a higher EE.

The direct dissolution method is able to encapsulate a comparable amount of drug as traditional dialysis method. It is well-known that in the dialysis method, organic solvent in the dialysis membrane is gradually replaced by water due to osmosis, which is a slow and sustaining process. In this way, a compact solid micellar core is progressively formed, and the drug could be slowly and effectively incorporated during micelle formation. Similarly, it also takes time to encapsulate drug into micelles by direct dissolution method. As paclitaxel is hardly soluble in water, the few solubilized paclitaxel molecules will migrate into the hydrophobic core of micelles during stirring, and more molecules will be solubilized in the medium. Thus, a dynamic transport process of paclitaxel is established until saturation of the micelles is reached. However, differently from dialysis micelles, the inner core of these micelles is believed to be in a fluid state with continuous exchange between micelles and free molecules.

Table IV. Paclitaxel Distribution in Plasma and Tissues of Mice after Administration of Paclitaxel-Loaded S1 Micelles Prepared by Direct Dissolution Method (μg/ml or μg/g)

Time (d)	Plasma	Heart	Liver	Spleen	Lungs	Kidneys	Tumor
0.08	6.9±1.1[a]	2.0±0.6	7.3±1.7	1.1±0.2	3.8±1.1	4.1±0.6	2.4±1.0
0.25	3.1±0.6	3.7±0.8	14.1±3.2	3.7±0.8	6.9±1.5	13.9±2.1	8.1±2.2
1	2.5±0.5	9.5±1.6	127.0±11.8	16.7±2.9	17.5±3.3	18.1±3.5	12.4±3.5
3	2.1±0.5	7.3±1.0	84.9±6.6	11.3±2.5	13.8±2.7	17.5±3.6	12.3±3.3
5	1.9±0.4	4.7±0.6	21.3±3.6	2.2±0.7	9.7±2.4	15.5±2.5	7.2±1.5
7	0.5±0.2	1.3±0.5	7.7±1.0	0.5±0.2	3.9±0.7	6.4±1.6	6.2±1.6

[a] Data represent mean value ± S.E. for four mice

Table V. Paclitaxel Distribution in Plasma and Tissues of Mice after Administration of Paclitaxel-Loaded S2 Micelles Prepared by Dialysis Method (μg/ml or μg/g)

Time(d)	Plasma	Heart	Liver	Spleen	Lungs	Kidneys	Tumor
0.08	0.3±0.1[a]	0.5±0.2	2.4±0.5	1.6±0.4	4.7±1.1	1.2±0.4	0.5±0.2
0.25	0.3±0.1	1.6±0.3	3.4±0.9	3.2±0.9	6.8±1.2	1.4±0.5	1.1±0.3
1	1.5±0.3	4.3±1.3	13.4±2.7	4.6±0.8	7.7±2.3	5.3±1.3	1.6±0.4
3	1.5±0.3	9.7±2.5	31.0±6.0	16.1±3.9	11.6±3.3	6.0±2.3	11.2±1.6
5	0.5±0.2	3.7±0.5	26.3±4.8	4.3±1.1	9.2±2.1	5.7±1.6	8.2±1.7
7	0.4±0.1	2.8±0.7	7.6±2.2	3.6±1.1	5.1±1.4	2.4±0.6	5.3±1.2

[a] Data represent mean value ± S.E. for four mice

The structure of the copolymer micelles also has great influence on the drug encapsulation ability. It is generally assumed that diblock copolymers tend to form micelles with a stick-like structure, while triblock copolymers have to fold so as to insert the two lateral hydrophobic PLA blocks inside the micelles leading to a flower-like structure (57,58). The PEG blocks of diblock copolymers exhibit higher chain mobility and are more susceptible to interacting with surrounding water molecules by hydrogen bonding than those of the triblocks, leading to looser micelle structure. Accordingly, lower drug loading efficiency is obtained for diblock copolymer micelles. In addition, as shown in Fig. 1, it takes less time (5 days) to reach saturation of drug encapsulation for diblock copolymer micelles than for triblocks (7 days) due to their chain freedom differences. On the other hand, compared with PLA/PEG4600 micelles, the drug loading efficiency of PLA/PEG20000 micelles is quite lower. This might be ascribed to the high molar mass of PLA/PEG20000, which makes it difficult for the macromolecular chains to fold to form a perfectly dense micelle.

The size of the micelles is enlarged after paclitaxel incorporation into the hydrophobic core. For the drug-loaded S3 and S5 micelles, there are two populations detected on the DLS diagrams. The larger one could be attributed to the micelles that have encapsulated drugs, and the smaller one represents the blank micelles. As discussed above, the single L-PLA/PEG4600 triblock micelles (S3) and stick-like PLA/mPEG5000 diblock micelles (S5) both lead to less stable micellar systems with the coexistence of drug-loaded and blank micelles. In contrast, S1 and S7 drug-loaded micelles both exhibit a single population with narrow distribution. It has been reported that micelles with diameter below 200 nm can prevent spleen filtering and tend to accumulate in the tumor sites (15), thus the large hydrodynamic diameter of S7 (276.4±7.5 nm) might prevent its applications. From the TEM pictures, it is observed that the drug-loaded micelles appear larger than blank micelles, in agreement with DLS results. The size difference between TEM and DLS results could be assigned to the dehydration and shrinkage of the micelles during air-drying for TEM measurements.

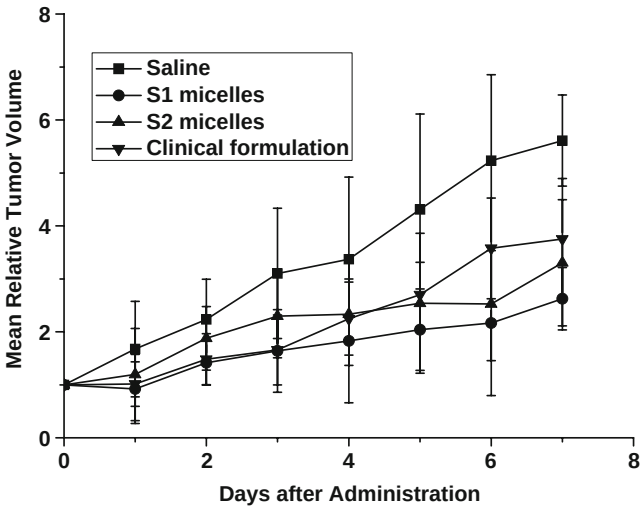

Fig. 6. Antitumor efficacy of paclitaxel-loaded S1 micelles (direct dissolution), S2 micelles (dialysis) and clinical paclitaxel formulation on female SPF C57BL/6 J mice bearing human Lewis lung cancer cells. Each point represents mean ± S.E.

The *in vitro* drug release strongly depends on the micelle fabrication method. It is shown that micelles formed by direct dissolution method exhibit a higher drug release rate than the dialysis micelles. This is because the osmosis process in dialysis method leads to a compact solid core which disfavors the diffusion of encapsulated drug. On the other side, drug loading in micelles prepared by direct dissolution is achieved via continuous migration of drug molecules into the self-assembled fluid core during stirring, as described before. Thus, loaded drug molecules can easily escape from the micelles with a looser structure.

The stereocomplexation of L-PLA/PEG and D-PLA/PEG copolymers has much effect on the drug release rate. Due to the strong interaction between L-PLA and D-PLA blocks leading to a more compact core, micelles consisting of L-PLA/PEG and D-PLA/PEG copolymers show a slower release rate than those of single L-PLA/PEG copolymers. Indeed, the much lower drug loading content of the latter could also account for its faster release rate.

Although composed of copolymers with similar PEG block length and EO/LA molar ratios, the diblock copolymer micelles of S5 present a higher release rate than triblock micelles of S1. This should be attributed to the different

Table VI. Inhibition Ratio of Tumor Growth (IR) for the Treatment Groups after 7 Days Period of Treatment

Treatment group	Tumor volume (mm^3)	IR (%)
Saline	7,291±390[a]	–
S1 micelles	4,176±1,132	42.7
S2 micelles	5,049±1,372	30.8
Clinical formulation	5,520±1,804	24.3

[a] Data represent mean value ± S.E. for four mice

structures of diblock and triblock copolymer micelles, as mentioned above. With the folded lateral hydrophobic blocks and flower-like structure, triblock copolymer micelles might provide a more compact inner core for drug molecules to diffuse out than diblock micelles. In addition, due to the looser configuration, the free drug molecules adsorbed at the core-shell interface of diblock S5 micelles can be easily leaked out and lead to an initial fast release (Fig. 4). Similar initial fast release is found for S7 of triblock PLA/PEG20000 micelles. As discussed above, it is very hard to form a perfectly dense micellar core with such long molecular chains. Thus, a certain amount of drug might be retained in the peripheral "corona" or core-shell interface of the micelles and can be easily released out. Nevertheless, it is not easy for drug molecules encapsulated in the core of the micelles to diffuse out, because the entanglement of long copolymer chains constitutes an obstacle for diffusion. Therefore, there is an almost constant release trend after the initial burst for S7. S1 of triblock PLA/PEG4600 copolymers with intermediate block length can easily fold the hydrophobic blocks into a compact core, thus allowing a good control on the drug diffusion process. S1 is believed to be a better controlled drug-release system as it displays approximately first-order release pattern.

The *in vivo* results found that after injection, paclitaxel is widely distributed into most tissues. However, paclitaxel in plasma and organs could be barely detected after 8 h for the group with clinical formulation (data not shown). This might be because the vehicle (1:1, *v*/*v* Cremophor EL:dehydrated alcohol) has a very short blood circulation time and the drug is eliminated by metabolism soon after administration. Thus, frequent drug-feeding is needed in clinical applications. In contrast, paclitaxel concentrations in various tissues are kept at high levels for micelles administration groups during the treatment period, especially for mice injected with S1. This is assigned to the hydrophilic shell of the micelles containing PEG blocks, which can prolong the circulation time in plasma and help to realize controlled drug release. The *in vivo* release of paclitaxel from S1 micelles formed by direct dissolution method is faster than from S2 micelles of dialysis method, in agreement with *in vitro* results. As a consequence, it takes a shorter time to reach maximal paclitaxel concentrations in tissues for S1 than for S2, and the concentrations are higher with S1 than with S2.

The tumor growth profile and inhibition ratio results also indicate that S1 administration has the highest antitumor ability. Although the clinical paclitaxel formulation shows comparable antitumor effect with S1 in the first 3 days, it could not control tumor growth at all thereafter due to fast elimination. However, for the S2 group, it takes 3 days to exhibit antitumor effect because of the more compact micelle structure and lower drug release rate as discussed above. Therefore, with relatively fast drug release rate and prolonged blood circulation time, S1 micelles derived from direct dissolution method are believed to be a promising candidate as hydrophobic drug carriers.

CONCLUSION

Biodegradable polymeric micelles were prepared in the present study from PLA/PEG diblock and triblock copolymers by two different methods (direct dissolution and dialysis) for controlled release of paclitaxel. The EE and LC values of paclitaxel are higher for L-PLA/PEG and D-PLA/PEG mixed copolymer micelles than for separate micelles due to the stereocomplexation effect. Micelles prepared by direct dissolution method present comparable EE and LC levels as those by traditional dialysis method. The concentrations of solubilized paclitaxel are 1,000-fold higher than the saturation solubility of paclitaxel in water. Drug encapsulation efficiency is greatly affected by the structure and composition of the micelles. The micelles' diameters are enlarged after drug-loading as determined using DLS. TEM confirms the structure of micelles, but the size estimated from TEM is smaller than from DLS due to the dehydration and shrinkage during drying. The drug release properties of the micelles strongly depend on the micelle fabrication method and copolymer composition. Faster release was obtained for micelles derived from direct dissolution than those from dialysis. The *in vivo* experiment shows that paclitaxel could be distributed widely and kept at high concentration levels in various tissues after administration with drug-loaded micelles. Compared with the current clinical formulation, which is eliminated fast by metabolism, and micelles by dialysis, which have a slower release rate, direct dissolution micelles with paclitaxel exhibit the highest antitumor ability. Therefore, the L- and D-PLA/PEG mixed micelles by direct dissolution method present great interest as injectable drug carriers because of the advantages as compared with other drug-delivery systems, especially easier formulation and absence of toxic organic solvents.

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