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In vitro Evaluation of Efficacy of Dihydroartemisinin-Loaded Methoxy Poly(ethylene glycol)/Poly(L-lactic acid) Amphiphilic Block Copolymeric Micelles

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ABSTRACT: Dihydroartemisinin (DHA)-loaded methoxy poly(ethylene glycol)/poly(L-lactic acid) ($mPEG_{5000}$—$PLLA_{3200}$) amphiphilic block copolymeric micelles (DHA-CM) have been prepared using modified solvent evaporation method. Physicochemical properties of DHA-CM were investigated by using dynamic light scattering, transmission electron microscopy, high-performance liquid chromatography, and Fourier transform infrared. Polymers formed stable, spherical, and worm-like micelles with mean sizes smaller than 130 nm. *In vitro* release experiments revealed that DHA-CM provided a more solubilizing effect than DHA suspension; in addition, it was showed that drug release profiles highly depended on pH values of dissolution media. Various types of lyoprotectants were tested to improve the redispersion performance of the freeze-dried products. 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay was used to evaluate the cytotoxicity of micellar solutions of freeze-dried DHA-CM. The results showed that the IC_{50} values of DHA-CM and DHA suspension for KB cell lines were 18.70 and 24.55 μM, respectively. However, DHA-CM had little cytotoxicity for L02 cell lines. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: block copolymers; micelles; drug delivery systems

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INTRODUCTION

Artemisinin is a potent antimalarial drug isolated from the traditional Chinese medicinal herb, *Artemisia annua*. Dihydroartemisinin (DHA, Figure 1) is an artemisinin derivative with the C-10 lactone group replaced by hemiacetal and endoperoxide containing sesquiterpene lactone structure, which can be synthesized from artemisinin in fewer steps. It is the active metabolite of a number of artemisinin derivatives, which are widely used as antimalarial drugs in clinic with fewer adverse side effects.[1] Recently, certain artemisinin derivatives, especially DHA, are shown to have anticancer effects in a wide variety of human and animal cancer cells.[2] The mechanisms of action for their antitumor activities are not fully understood, but it might be due to transferrin conjugates.[3] Furthermore, it is reported that DHA has part therapeutic significance *in vivo*.[4,5] However, DHA is susceptible to light, heat, and oxygen for its special peroxy bond. Also, DHA has low bioavailability for oral administration,[6] due to its poor water solubility (0.168 mg mL^{-1} at 30°C).[7] Thus, the development of the new formulation of DHA to enable higher availability is in great need.

In recent years, amphiphilic block copolymeric micelles have received growing scientific attention because of its excellent biocompatibility and biodegradability.[8] It could be hydrolyzed by enzyme *in vivo* before absorbing or eliminating.[9] The amphiphilic block containing hydrophobic and hydrophilic parts could be self-assembled to form micelles at or above the critical micelles concentration (CMC).[10] In addition, amphiphilic diblock copolymers generally self-assemble in dilute aqueous solution into three basic morphologies: spherical micelles, worm-like micelles, and vesicles.[11] Extensive theoretical as well as general experimental studies of amphiphilic block copolymer have established that aggregate morphology, in dilution, is principally determined by the weight faction of its hydrophilic "head" group with poly(ethylene glycol) (PEG) chains (equivalent to polyethylene oxide, PEO, f_{EO}).[12–14] Vesicles are favored when f_{EO} = 20–40%, worm-like micelles are predominantly formed at f_{EO} = 40–50%, whereas when f_{EO} > 50% spherical micelles are the predominant morphology for a variety of diblock copolymers. The fabrication process also plays an important role in aggregate morphology of diblock copolymer. Drugs such as paclitaxel have now been loaded into those worm-like and

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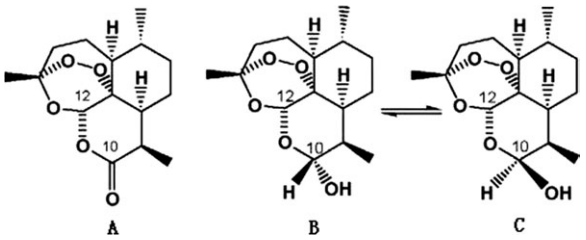

Figure 1. Structures of artemisinin (A), α-epimer (B) and β-epimer (C) of dihydroartemisinin (DHA).

spherical micelles prepared by different methods.[15] Spherical micelles have a core-shell type polymeric nanosphere system, consisting of a hydrophilic outer shell and hydrophobic inner core which provides a promising drug carrier system for poorly soluble anticancer drugs.[16] As a result, copolymeric micelles improve their solubility, stability, circulation, and half life in biological media on one hand and lower interaction with the reticuloendothelial cell system due to their small size on the other hand.[17] Besides, polymeric micelles have proved a great potential in parenteral delivery of the hydrophobic agents.[18,19]

To the best of our knowledge, little work had been done on the novel formulation of DHA using polymeric micelles to achieve its anticancer effects. Here, we used different methods to prepare DHA-loaded $mPEG_{5000}$-poly(L-lactic acid) $(PLLA)_{3200}$ amphiphilic block copolymeric micelles and to look in particular at its physicochemical characterization that is, size, morphology, CMC, cumulative release properties, and lyophilization. Furthermore, the development and validation of a high-performance liquid chromatography (HPLC)-UV method (210 nm) for DHA-CM were described to calculate drug encapsulation efficiency (EE) and drug loading efficiency (DL) of DHA-CM. Also, the cytotoxicity of freeze-dried DHA-CM was reported here. The objectives of this study were to improve water-solubility and bioavailability of DHA, explore anticancer effect of freeze-dried DHA-CM *in vitro*. Results of *in vivo* studies of DHA-CM will be described in a future publication.

EXPERIMENTAL

Materials

The methoxy poly(ethylene glycol)/poly(L-lactic acid) (M_n = 5000–3200, calculated by ^{1}H NMR, weight fraction of PEO f_{EO} = 0.61, denoted $mPEG_{5000}$-$PLLA_{3200}$) was purchased from Ji'nan Daigang Biomaterial, China. DHA (>98.0% purity), dialysis membranes made up of Cellulose ester (MWCO: 3000 and MWCO: 10000) were obtained from Guangzhou Qiyun Biomaterial, China. DHA reference standard (National Institutes for the Control of Pharmaceutical and Biological Products, batch number: 100184-200401, China) was used for HPLC assay. Acetonitrile (HPLC grade, o&pudtion, Sweden) was gained from Guangzhou Dongju Experimental Apparatus, China. Pyrene (AccuStandard, USA) was used for CMC determination. Phosphate buffer solution (PBS) with 0.3% sodium dodecyl sulfate (SDS, Tianjin Damao Chemical Reagent Factory, China) was used for the preparation of the drug-releasing media. The culture medium used was composed of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) and 10% fetal bovine serum (FBS, Gibico BRL, USA). The trypsin-EDTA (0.25% trypsin, 0.02% EDTA tetra-sodium) was purchased from Hangzhou Jinuo Biomedical Technology, China. In addition, 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and DMSO were obtained from Sigma-Aldrich, (St. Louis, MO). The other chemicals used were of analytical reagent grade. Water used in the study was doubly distilled.

Preparation of DHA-CM

Drug-loaded micelles were prepared by modified solvent evaporation method and dialysis method. In the first method, 20 mg of $mPEG_{5000}$-$PLLA_{3200}$ copolymers and 2.5 mg DHA (11% theoretical loading) were dissolved in 2 mL dichloromethane. The mixture was dripped into 15 mL of distilled water at a speed of one drop per 10 s, mildly stirred for 24 h, and dialyzed for 3 h. Finally, the micellar dispersion was isolated by low-speed centrifugation and filtered through a 0.45 μm filter (Molipore) to remove aggregates, with no significant effect on the micelles yield. Dialysis method involved dissolving same component in 2 mL dichloromethane. The mixture was introduced into a pretreated dialysis bag (MWCO: 3000), and then dialyzed against 1 L of distilled water for 24 h, which was regularly renewed by fresh water. The micellar suspension was purified as described above. The drug-free micelles were prepared according to the same procedure. The freeze-dried product were obtained by freeze drying and kept in desiccator.

Partical Size Analysis

The average size and size distribution of micelles were measured by dynamic light scattering (DLS), using a Malvern Zetasizer 3000HS (Malvern Instruments, UK). All experiments were performed at (25.0 ± 0.1)°C with a 90° scattering angle after the micellar solution were filtered through a 0.45 μm filter (Millipore) and diluted adequately with double distilled water. The measurement yielded the mean size and polydispersity index (PI).

Transmission Electron Microscopy Examination

The morphological observations of blank and drug-loaded micelles were performed by transmission electron microscopy (TEM, Hitachi H-7650, Japan) operating at an accelerating voltage of 80 kV. For sample preparation, one drop of the micellar solution was placed onto a 400 mesh carbon-coated copper grid. On drying under room temperature, it was stained with 1% alkaline phosphotungstic acid (PTA) for several minutes and dried at room temperature before analysis.

Determination of Critical Micelles Concentration (CMC)

The critical micelles concentration (CMC) of the copolymers was determined by fluorescence spectroscopy using pyrene (AccuStandard, USA) as fluorescence probe. Briefly, a known amount of pyrene in acetone was added to each of a series of 10 mL vials, and then 2 mL of various concentrations of drug-free micellar micelles (3.05×10^{-5}–0.5 mg mL^{-1}) were added to each vial (the final concentration of pyrene was $6.14 \times 10^{-7} M$).The solutions were shaken and heated at 37°C for 5 h and then left to cool overnight at room temperature in dark. Pyrene fluorescence intensity at excitation wavelength of 333

and 338 nm were measured at emission wavelength of 390 nm. Pyrene moved into the inside of the micelles from the aqueous phase which resulted in an alteration in the intensity ratio (I_{338}/I_{333}) of pyrene fluorescence intensity.

Determinations of Drug EE and DL Percentage

The DHA entrapped in $mPEG_{5000}$-$PLLA_{3200}$ micellar solutions was diluted in a known amount mobile phase to extract DHA, and then measured using reverse-phase HPLC as described below to determine the amount of DHA. The DL and EE are calculated as follows:

$$\text{DL}\ (\%) = \frac{\text{weight of DHA in dried DHA-CM}}{\text{weight of the dried DHA-CM}} \times 100 \quad (1)$$

$$\text{EE}\ (\%) = \frac{\text{DHA encapsulated in dried DHA-CM}}{\text{DHA added theoretically}} \times 100 \quad (2)$$

The HPLC was performed with a LC-20 A apparatus (Shimadzu) equipped with a UV detection (SPD-20 A, Shimadzu) and an ECOSIL column (4.6 × 150 mm, pore size 5 μm, C_{18}, Japan). The mobile phase consisted of acetonitrile (HPLC grade) and water in a volume ratio of 60:40. Before use, the water was filtered through a 0.45 μm hydrophilic membrane filter. The mobile phase was delivered at a flow rate of 1.0 $mLmin^{-1}$. The detection wavelength was 210 nm at 25°C and sample injection volume was 20 μL.[20] The calibration curve was linear in the range of 10–400 $\mu g\ mL^{-1}$ with a correlation coefficient of $R = 0.9999$ ($n = 6$).

In Vitro Release Experiments

For drug release study, three release media at different pH (pH 6.5, pH 7.0, and pH 7.5), containing SDS solution (0.3%, w/v) were used. DHA-CM (4 ml) were transferred into a dialysis bag (MWCO: 10000) and then placed in well-closed glass tubes, and then it was placed in a shaker(Spx-100 b-D, Shanghai Boxun Holdings, China) and shaken horizontally at 37°C and 100 strokes per min. At predetermined time intervals, the release media were taken out and replaced by 5 mL of fresh PBS. All the experiments were performed in dark. The concentrations of released DHA were determined by HPLC method described above. Furthermore, a pure drug suspension was prepared as control according to the previous research.[21] The release data of all formulations were analyzed using different established models-zero order, first order, second order, and Higuchi to find out the release mechanism.[22]

Lyophilization and Reconstitution of DHA-CM-NPs

DHA-CM (2.5 mL) were placed in 10 mL vials and then added 0.5 mL different cryoprotectants, such as trehalose, mannitol, and galactose at a final concentration of 5% (w/v). The mixtures were fast frozen under −80°C in a ULT 25865-A14 freezer (Revco scientific, Asheville NC) for 5 h. Afterwards, the frozen samples were lyophilized using a freeze-drier (FD-1-50, Beijing Boyi Experimental Instruments, China) for 48 h to obtain DHA-CM powder. Reconstitution of the lyophilized samples was performed by addition of 5 mL of distilled water with manual shaking. FTIR of blank micelles, DHA-CM, and the mixture of DHA and blank micelles were taken with KBr pellets on a Thermo NICOLET 6700 FTIR spectrometer, USA.

In Vitro Cytotoxicity of DHA-CM

Human oral carcinoma KB cells and human hepatocyte L02 cells were kindly gifted by Clinical Medical Experimental Research Center of Nanfang Hospital, Guangzhou, China. The cells were cultivated in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 in a humid dark environment. All the experiments were performed with cells in the logarithmic growth phase. The growth inhibitory effects of micellar solution of dried DHA-CM and DHA suspension on cells were assessed by the MTT method.[23] The cells were seeded in 96-well plates (Corning Costar, USA) at the density of 5×10^3 viable cells/well. The wells were divided into several groups for corresponding samples: (1) black zero groups (5 wells): only culture medium added; (2) control groups (5 wells): test cells and equivalent of dissolvent (DMSO) added; (3) DHA suspension groups (5 wells each concentration): DHA was dissolved in DMSO at a calculated concentration, diluted with PBS and added into the wells (DMSO less than 0.1%). The final DHA concentrations were 2.125, 4.25, 8.5, 17, and 34 $\mu g\ mL^{-1}$, respectively; (4) DHA-CM groups: DHA-CM groups went through the same procedure as DHA suspension groups. The cells cultured 24 h to allow cell attachment, then incubated with the DHA-CM or DHA suspension for 24, 48, and 72 h. At designated time intervals, 20 μL of MTT (5 mg ml^{-1} in PBS) were added to each well. After incubation at 37°C for 4 h, the supernatant in each well was removed carefully. Formazan crystals were dissolved in 150 μL DMSO and shook for 10 min. The absorbance of each well was then read at 490 nm using a microplate reader (SpectraMax M5, USA). Cell inhibition ratio was calculated by the following equation:

$$\text{Inhibition ratio}\ (\%) = \left(1 - \frac{\text{Int(s)}}{\text{Int(control)}}\right) \times 100 \quad (3)$$

Where Int (s) is the fluorescence intensity of the cells incubated with DHA-CM or DHA suspension and Int (control) is the fluorescence intensity of the cells treated with equivalent DMSO solution. All of the experiments were performed thrice, and the mean, S.D., and IC_{50} value were calculated.

Statistical Analysis

All analyses represented in at least triplicate experiments, and data were expressed as mean ± S.D. IC_{50} values were evaluated by independent samples T-test using SPSS 13.0 for window for determination of significant differences. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Preparation of DHA-CM

Two main classes of DL procedures could be applied: direct dissolution and dissolution with the help of an organic solvent common to both the copolymer and the drug. Common drug-loading procedures were illustrated in detail in previous work.[24] Two methods using organic solvents were used because the amphiphilic copolymer and drug used in this study were not readily soluble in water. High EE level and suitable size distribution were important requirements for micelles applied in drug delivery, so these two parameters were selected as indexes to

Table I. Mean Size, PI, and EE of DHA-CM Prepared via Modified Solvent Evaporation Method (A) and Dialysis Method (B)

Methods	Size (nm)	PI	EE (%)
A	96.9 ± 4.2[a]	0.390 ± 0.028	50.68 ± 0.2
B	195.5 ± 3.8	0.407 ± 0.033	32.89 ± 0.3

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

evaluate the formulation (Table I). The results showed modified solvent evaporation method had an advantage over dialysis method. So, we chose the first method to prepare micelles in the subsequent study.

In modified solvent evaporation method, organic solvent was gradually replaced by stirring and evaporation and micellar core was progressively formed, which were slow and sustaining processes. As DHA was hardly soluble in water, the most of solubilized DHA molecules would migrate into the hydrophobic core of micelles slowly and effectively, and few DHA molecules solubilized in water and little solvent removed by dialysis method. Subsequent processes, that is, low-speed centrifugation and filtration were used for getting rid of aggregate copolymers. All purified procedures had no significant effect on micelles yield. In preliminary trials, using other organic solvent, that is, tetrahydrofuran, acetonitrile, and dimethyl sulfoxide in the preparation obtained DHA-CM with either large size and size distribution or low drug EE (date not shown), so we used dichloromethane instead.

Furthermore, supplemental experiments revealed the formula described above was reliable and reproducible to prepare DHA-CM. The mean size, DL, and EE of optimal DHA-CM were (119.6 ± 5.8) nm, (13.57 ± 2.68)%, and (94.23 ± 2.13) %, respectively.

Characterization of DHA-CM

The size and size distribution of the micelles were obtained by DLS measurement. Size distribution and mean size of the optical DHA-free and DHA-loaded micelles were given (Table II). Results showed that micelles had mean sizes of around 82.3 nm which increased to around 119.6 nm after DHA encapsulation. The sizes of the micelles were enlarged after DHA incorporation into the hydrophobic core, which was in accord with the previous work.[25] The particle sizes of micelles played an important role in determining its fate in the body. It was reported that smaller particles tended to accumulate in the tumor sites due to the facilitated extravasation and a greater internalization was also observed.[26,27] It was interesting to note that, in literature, smaller sizes and PI values were reported for micelles derived from DLS for various PLA/PEG block copolymers.[28,29] In this work, micelles were made of $mPEG_{5000}$-$PLLA_{3200}$ with a $f_{EO} = 0.61$ and formed by stirring moderately. It was normally to find the coexistence of disparate morphologies for a single block copolymer.[30] So, it was reasonable to assume that spherical and worm-like micelles coexisted in the micellar soution.[31] As a consequence, the sizes and PI values were larger than those obtained by other researchers.

TEM was used to take photos of the optimal blank and DHA-loaded micelles to provide information on the morphology, as shown in Figure 2. These images were typical of those obtained for all the samples, confirming that the $mPEG_{5000}$-$PLLA_{3200}$ copolymers form spherical micelles coexisting with short cylindrical micelles in aqueous media. We could even detect a core-shell like structure for spherical micelles using PTA. The coronal domains where the hydrophilic PEG block were diffusively distributed provided a marginal contrast and appeared as gray haloes surrounding the bright hydrophobic PLLA core. The similar morphology of blank and DHA-loaded micelles was provided. The sizes of micelles estimated from the TEM images were approximately (60–80) nm, that is, lower than that from DLS measurements. 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.[32]

CMC of Copolymer

The CMC of $mPEG_{5000}$-$PLLA_{3200}$ copolymer was determined by fluorescence probe techniques for its functional, versatile, and easy application. The intensity ratio (338 nm/333 nm) from pyrene emission spectra versus logarithm of the copolymers concentration was plotted, as shown in Figure 3. Below the CMC, the I_{338}/I_{333} value was nearly a constant, but above this concentration, this value increased extremely. Thus, a graph with two linear segments having different slopes was formed. The intersection point of these two segments gave CMC value. And in Figure 3, the CMC value of copolymer was calculated to be $2.32 \times 10^{-7} M$.

CMC was an important stability indicator for copolymer micelles. In this work, fluorescent spectroscopy was chosen for its sensitive and precise. Pyrene molecules had a strong hydrophobic character with very low solubility in water and preferentially solubilized into the hydrophobic core of micelles, so its fluorescence changes depend on the surroundings.[33] Below the CMC, there was no micelles in the polymer solution, so the fluorescence intensity was very low. However, the excitation spectrum of pyrene exhibited a red shift with increasing the concentration of copolymer micelles. The maximum excitation wavelength in water changed from 333 to 338 nm, which gave rise to the change of intensity ratio (338 nm/333 nm).[34] The low CMC value of copolymeric micelles showed that they formed stable formations and kept their intact structure on dilutions with body liquids. And micelles formed from $mPEG_{5000}$-$PLLA_{3200}$ copolymer in this study as drug carrier was susceptible to preserve thermodynamic stability even after intravenous injection which induced severe dilution because of the remarkably low CMC.[35]

Table II. Mean Size and PI of the DHA-free and DHA-loaded Micelles

Samples	Size (nm)	PI
DHA-free micelles	82.3 ± 8.4[a]	0.377 ± 0.045
DHA-loaded micelles	119.6 ± 5.8	0.520 ± 0.063

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

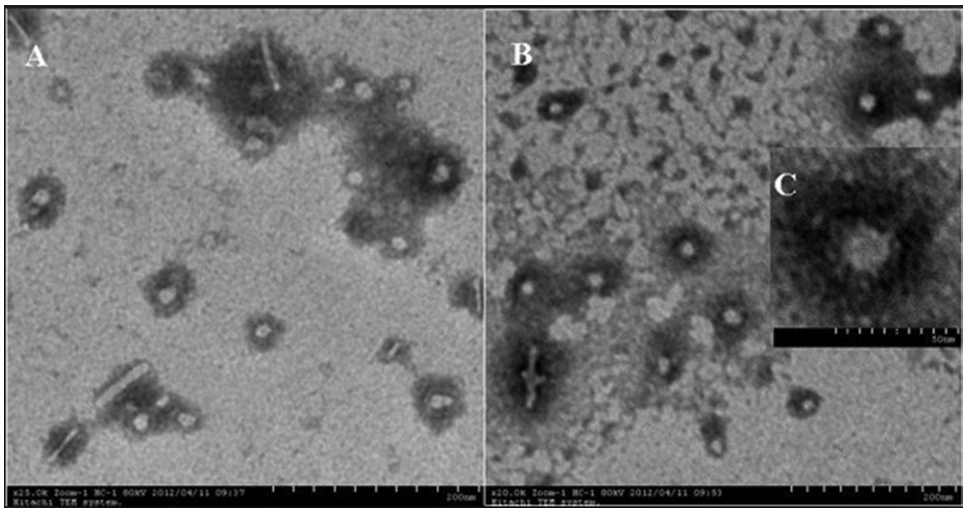

Figure 2. TEM images of drug-free micelles (A × 20,000), DHA-loaded micelles (B × 20,000), and enlarged view of DHA-loaded micelles (C × 60,000).

Release Experiments

Figure 4 presented comparatively the drug release profiles from micelles in various release media composed of 0.3% SDS and PBS (0.1 *M*) at different pH values (6.5, 7.0, and 7.5), together with the drug release profile from DHA suspension. In all cases, the initial burst release during the first 8 h was observed. And another slightly fast release was observed in media at pH 6.5 and 7.0. It was notable that the drug release rate in PBS at pH 6.5 exhibited a significantly faster release rate compared with that in control group, and the accumulate release amount decreased with the increased pH values. It could be seen that the drug releasing amounts from DHA-CM in PBS at pH 6.5, 7.0 and 7.5 were 79.36%, 65.50%, and 59.86% after 48 h incubation, respectively.

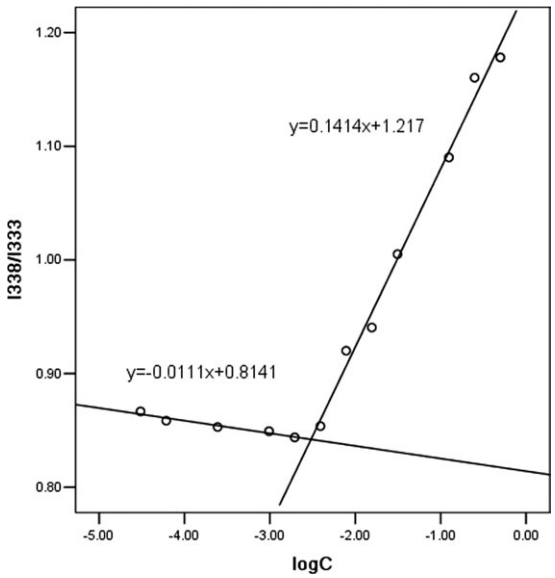

Figure 3. Plots of the intensity ratio (338 nm/333 nm) from pyrene emission spectra versus logarithm of the copolymers concentration. The intersection point of these two segments gave CMC value of mPEG5000-PLLA3200 micelles.

Further, results of optimum modeling of DHA release profiles were given in Table III. Release modes in release media with pH 6.5 and 7.0 exhibited an approximately first-order release, while in release medium at pH 7.5 was Higuchi release.

Release study was carried out by dialysis method in PBS adding 0.3% SDS, a anionic surfactant, to solubilize DHA and meet therapeutic leakage trough conditions. The micellar solution was incubated at 37°C in PBS at pH 7.5, 7.0, and 6.5, to examine the influence of pH values on the release of DHA from micelles. The release of drug from block copolymer micelles depended on the rate of diffusion of the drug from the micelles, micelle stability, and the rate of biodegradation of the copolymer. Although if the micelles were stable, the polymer–drug interactions, the physical state, and length of the hydrophobic block, the localization of the drug within the micelles were major factors to influence drug release kinetics.[36] It was hypothesized that the localization of DHA within the outer corona region or at the interface of micelles would account to some extent for the faster rate of release of DHA at the beginning of the release study. Because the outer corona region and

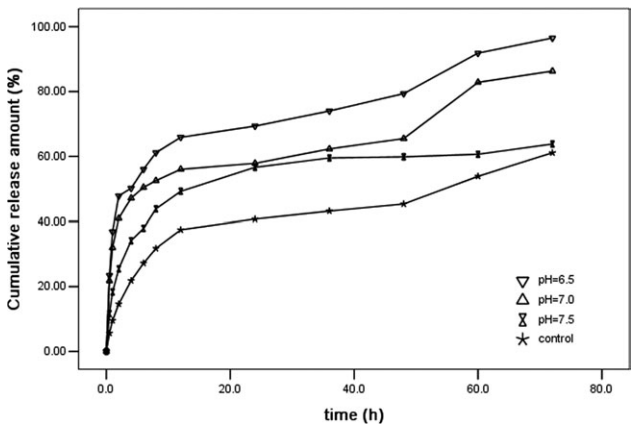

Figure 4. *In vitro* drug release profile from the optimized DHA-loaded micelles (mPEG5000-PLLA3200, loading: 13.57%) in PBS at different pH values and the suspension of DHA at pH 7.0 as control group.

Table III. Optical Modeling of Drug Release from DHA-CM in Different Release Media

Release media	Release equation	R^b	Model
pH6.5PBS(0.3%SDS)	$\ln(1-Q) = 4.1966-0.0350t^a$	0.9513	First-order release
pH7.0PBS(0.3%SDS)	$\ln(1-Q) = 4.2158-0.0207t$	0.9332	First-order release
pH7.5PBS(0.3%SDS)	$Q = 15.0278+6.7956t^{1/2}$	0.9213	Higuchi release

[a]Q is cumulative release of DHA from mPEG-PLLA micelles; t represents release time.
[b]R is the correlation coefficient for respective model.

the interface of micelles were quite mobile, DHA released from these areas would be rapid. After that, a sustained release indicated that a portion of DHA interacted strongly with the hydrophobic PLLA block. Because of the long diffusion or erosion process, the slightly fast release occurring between 48 and 60 h at pH 6.5 and 7.0 most likely originated from disintegration of the micelles structure.[37] Hence, DHA rapidly dissociated from the hydrophobic core of micelles. However, the structure of DHA-CM seemed to be stable after 72 h incubation in the buffer at pH 7.5.

As shown in Figure 4, the percentage of DHA released from the DHA-CM increased as the pH value decreased from 7.5 to 6.5. This pH sensitivity of the release rate of DHA from the DHA-CM could be probably attributed to the sensitivity of the cleavage of the ester bonds to pH value. And polyester hydrolysis was well known to be accelerated by low pH.[38] At the beginning of degradation, the ester bonds of PLLA block were hydrolyzed and detained in the hydrophobic core. Afterwards, progressive appearance of water-soluble oligomers facilitated this degradation process. The degradation of polymer made contribution to the drug release at pH 6.5 and 7.0. However, it was expected that PLLA block was more stable under physiological condition (PBS, pH 7.5, 37°C), and DHA release behavior at pH 7.5 mostly depended on the rate of its diffusion from the micelles. Because extracellular pH of tumor tissue is significantly lower than that of normal tissue.[39] This character could make drug selectively target tumor cells, which could reduce cytotoxicity to normal cells and tissues. In the other hand, the drug released from DHA-CM was more than that from pure drug suspension. It indicated that this novel carrier could improve the solubility of DHA. To further understand DHA release mechanism, we used some mathematical models, suggesting DHA released from DHA-CM not only based on a diffusion process, but also a degradation of DHA-CM following by erosion phenomena. These results were consistent with above discussion. The *in vivo* pharmacokinetics and efficacy study are under progress which will further reveal the actual fate of the formulation.

Lyophilization and Reconstitution

To produce the lyophilized products with better redispersion performance, high entrapment efficiency, trehalose, mannitol, and galactose were chosen as lyoprotectants and added to have a final concentration of 5% (w/v). The results demonstrated that trehalose was the most suitable lyoprotectants in stabilizing the micelles (Figure 5). The appearance of freeze-dried products revealed that DHA-CM protected by trehalose and mannitol were white, caky, and well-distributed evenly. In reconstitution study, galactose did not show sufficient effect on the improvement of redispersibility of the lyophilizates. Samples included the first two cryoprotectants could redisperse easily in water in 15 s by handing shaking without any visible precipitates, whereas the other two samples (without cryoprotectant and with galactose) redissolved with the help of vortexing process. Furthermore, in Figure 5, the products added mannitol and galactose as additives had a decreased EE and increased mean sizes. This might attribute to leakage of drug and aggregation of polymer in the lyophilized process.

The physicochemical stability of DHA-CM was a big problem in the case of copolymer hydrolysis or aggregation in aqueous suspension when conserved for a long time.[40] Freeze-dried procedure was important for intravenous administration of copolymeric micelles. Based on the clinical needs, the DHA-CM powder must have a good redispersibility. Saccharides were widely used reagents to protect polymeric micelles during freezing and drying.[41] In this study, the DHA-CM powder were freeze-dried with three different types of cryoprotectants: a monosaccharide (galactose), a disaccharide (trehalose), and a polyol (mannitol). In view of the clinic use of DHA-CM powder, the concentration of each cryoprotectant was 5% (w/v) to attain isotonic solution after dissolving according to the previous work.[42]

In Figure 6, absence of free drug in dried DHA-CM was confirmed by FTIR study. The peaks in IR spectrum of blank micelles [Figure 6(A)] were similar to that reported in previous papers.[43,44] The main four differences among those spectra were

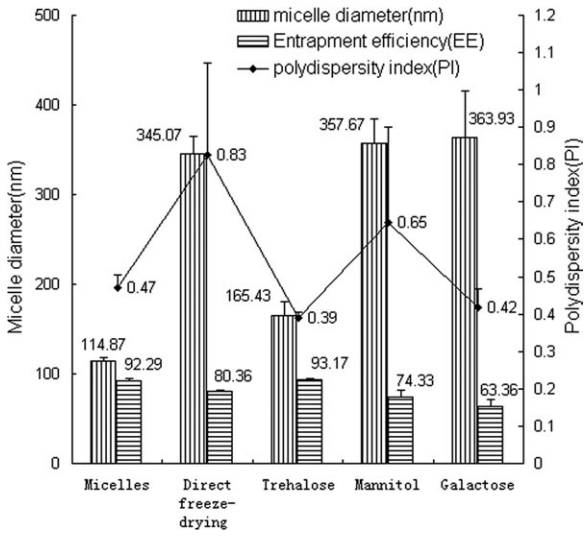

Figure 5. Mean size, PI, and EE of freeze-dried DHA-CM prepared from mPEG5000-PLLA3200 with different lyoprotectants.

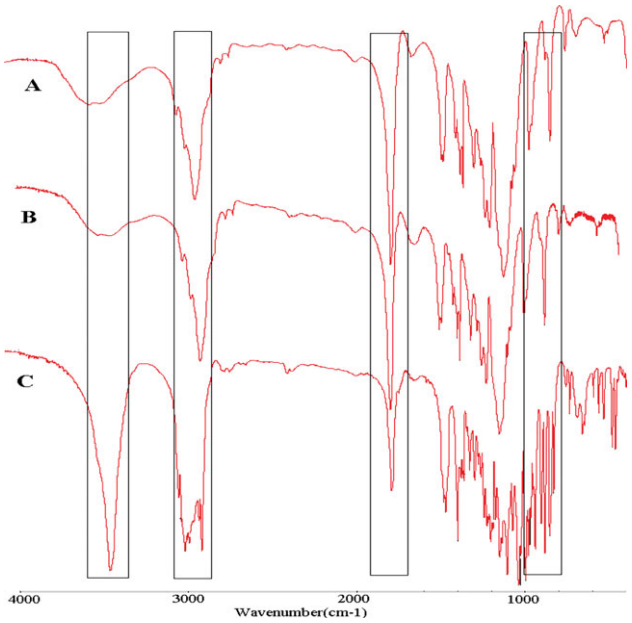

Figure 6. FT-IR spectra of blank micelles (A), DHA-loaded micelles (B), the mixture of blank micelles, and DHA (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

highlighted by rectangle. First of all, band at 3378.4 cm^{-1} was assigned to stretching vibration of O—H groups in DHA, which appeared only in IR spectrum of the mixture [Figure 6(C)]. Second, peaks in the region 3000–2800 cm^{-1} in IR spectra of blank and DHA-loaded micelles [Figure 6(A, B)] were obviously different from that in Figure 6(C). And third, peaks at about 1756.9 cm^{-1} together with 2820 cm^{-1}, 2720 cm^{-1} in Figure 6(A, B) were assigned to carbonyl groups in the copolymer, which were not found in spectrum of DHA reference standard.[45] Finally, the absorption at about 824 cm^{-1} was characteristic absorption peak of peroxy bonds in structure of DHA, whereas did not appear in Figure 6(A, B). In our study, Figure 6(A, B) were alike and different from Figure 6(C). And the characteristic absorption peaks of DHA in Figure 6(B) were almost negligible as compared to Figure 6(C), which indicated that drug was encapsulated in carriers. And the absence of free drug was also substantiated.

In Vitro Anticancer Effects

Figure 7 showed the *in vitro* cytotoxic effects of DHA-CM (DL % was 13.57%) and DHA suspension on KB cells and L02 cells after 72 h of incubation ($n = 3$), respectively. Under the same drug concentration, DHA-CM and DHA suspension exhibited a significantly higher cyctotoxic activity on KB cells than L02 cells, whereas the anticancer effects of DHA-CM and DHA suspension groups were similar for the same cell lines. The viability of KB cells had marked reductions from 77.69 to 52.54% when incubated with 4.25 $\mu g\ mL^{-1}$ DHA-CM. And the cell viability decreased from 52.54 to 30.72 % when incubated with 8.5 mg mL^{-1} DHA-CM. As for DHA suspension groups, the cell viability decreased from 77.64 to 53.21 % and then decreased from 53.21 to 42.12 %. However, the cell viability changed very little for L02 cells.

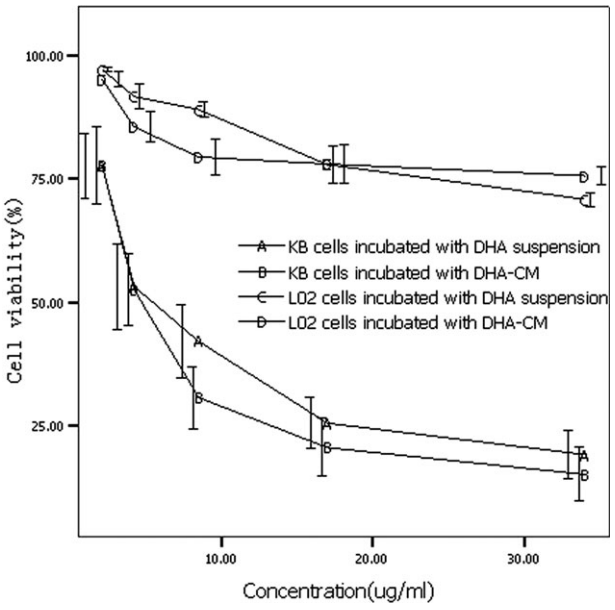

Figure 7. Cyctotoxic effect of DHA suspension and DHA-CM (DL% was 13.57%) incubated with KB (A, B) and L02 (C, D) cells ($n = 3$).

IC_{50} gave the drug concentration at which 50% cells were inhibited in growth. The IC_{50} values were calculated and listed in Table IV to make comparison between DHA suspension and DHA-CM. After 72 h incubation with KB cells, the IC_{50} values decreased from 21.55 for DHA to 18.70 μM for DHA-CM. And there was significant difference between the IC_{50} values.

DHA has drawn much attention in recent years because the strong anticancer activity for many cell lines.[46] The results of anticancer effect *in vitro* certified that DHA and its formulation exerted potent cytotoxicity on human oral carcinoma KB cells but minimal effects on normal human hepatocyte L02 cells. DHA-CM were deemed safe and effective enough for anticancer use *in vitro*. The data in Table IV showed that DHA-CM exhibited a inferior effect for 48 h incubation from IC_{50} values ($P < 0.05$). However, DHA-CM were significantly better than DHA in restraining the growth of KB cells after 72 h incubation ($P < 0.01$). The results indicated that the novel DHA formulation in this study had exhibited excellent inactivation effect on KB cells. Our work may provide evidence for further studies of DHA-CM as a possible anticancer drug in the clinical treatment

Table IV. IC_{50} of DHA Suspension and DHA-CM (DL% was 13. 57%) Incubated with KB Celles

	IC_{50} (μM)	
Incubation time(h)	DHA suspension	DHA-CM
24	100.34 ± 3.87[a]	ND[b]
48	46.97 ± 1.42[c]	50.37 ± 1.08
72	24.55 ± 1.13[d]	18.70 ± 0.98

[a]Date represent mean value ± S.D., $n = 3$.
[b]Not determined.
[c]$P < 0.05$ versus DHA-CM groups by independent samples *T*-test.
[d]$P < 0.01$ versus DHA-CM groups by independent samples *T*-test.

of oral carcinoma. Further research is needed to confirm such preliminary results.

CONCLUSIONS

The research work presented the development of copolymeric micelles formulation of DHA by two different methods. The emphasis was given to *in vitro* investigation on $mPEG_{5000}$-$PLLA_{3200}$ copolymeric micelles fabricated by modified solvent evaporation method. The experimental values of the DHA-CM gave rise to the EE of (94.23 ± 2.13)%, DL of (13.57 ± 2.68) %, mean size of (119.6 ± 5.8) nm, and PI of 0.520 ± 0.063. The sizes of micelles were enlarged after drug-loading as determined using DLS. TEM confirmed micelles formed spherical micelles coexisting with short cylindrical micelles in aqueous media. The formulation containing 5% (w/v) trehalose presented the best characteristics before and after reconstitution. The blank $mPEG_{5000}$-$PLLA_{3200}$ micelles had a low CMC value (2.32×10^{-7} M). The drug release behavior from DHA-CM exhibited a biphasic pattern with burst release at the initial stage and sustained release subsequently. The *in vitro* anticancer effects showed that DHA and its formulation had similar effect on human oral carcinoma KB cells and normal human hepatocyte L02 cells. The low IC_{50} value of KB cells implied that $mPEG_{5000}$-$PLLA_{3200}$ copolymeric micelles were a great potential drug delivery system for DHA in anticancer use. However, further investigation in animal test is needed to confirm the preliminary, yet encouraging results obtained in this research.

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