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Amphotericin B-incorporated polymeric micelles composed of poly(D,L-lactide-*co*-glycolide)/dextran graft copolymer

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Abstract

In this study, we prepared amphotericin B (AmpB)-encapsulated polymeric micelle of poly(D,L-lactide-*co*-glycolide) (PLGA) grafted-dextran (DexLG) copolymer and characterized its physicochemical properties *in vitro*. The average particle size of AmpB-encpasulated DexLG polymeric micelles was around 30–150 nm while particle size of empty polymeric micelles was below 100 nm according to the copolymer composition. The morphology of AmpB-encapsulated polymeric micelle of DexLG copolymer was spherical shapes at transmission electron microscopy (TEM) observation. At ^{1}H NMR study, specific peaks of AmpB and DexLG copolymer was obtained at DMSO but specific peaks characterized to AmpB and PLGA was disappeared at D_2O environment. These results indicated that AmpB was encapsulated into the micellar core of polymeric micelle. XRD results also support these results, indicating that specific crystal peaks of AmpB and broad peaks of DexLG copolymer were obtained but specific peaks of AmpB was disappeared at polymeric micelles while physical mixture of AmpB/empty polymeric micelles showed both specific peaks. Drug release rate was decreased according to the increase of drug contents and increase of PLGA component of DexLG copolymer. At the minimal inhibition concentration (MIC) study using *Candida albicans*, AmpB-encapsulated polymeric micelle showed almost similar effectives on the growth inhibition of microorganisms. These showed that AmpB-encapsulated polymeric micelle of DexLG copolymer can be considered to potential antifungal agent carriers.

Keywords: Amphotericin B; Dextran; Polymeric micelle; Poly(D,L-lactide-*co*-glycolide); *Candida albicans*

1. Introduction

Dextrans are characterized to a colloidal and water-soluble polysaccharide consisting of glucose molecules through the 1,6-glucosidic linkages. Since dextrans are inert in biological systems and do not affect cell viability, it has been extensively used as a drug carrier system, including for antidiabetics, antibiotics, anticancer drugs, peptides and enzymes (Akiyoshi et al., 1998; Ichinose et al., 2000; Mehvar, 2003; Molteni, 1985; Nishikawa et al., 1994). Especially, dexran or dextran derivatives are widely used for passive-drug targeting or nanoparticle formation (Ichinose et al., 2000; Jung et al., 2005).

Polymeric micelle-based drug delivery system has been extensively investigated due to their potential in targeting potential to wanted site of action (Allemann et al., 1993). Since nanoparticulate carriers are characterized as small particle size below 1000 nm, they can be primarily considered as devices for parenteral injection (Kreuter, 1988). Nanoparticulate carriers have various advantages such as drug targeting via active or passive targeting mechanism, reduction in the amount of drug administered, minimizing of irritation at injection site, and minimizing side effects. For parenteral injection of drug, various carriers have been developed such as liposomes (Estey et al., 1996), polymeric micelles (Kataoka et al., 1993; La et al., 1996), and core-shell type nanoparticles, (Gref et al., 1994; Jeong et al., 1998) have been reported. Especially, polymeric micelles (Kataoka et al., 1993; Lehn, 1993), core-shell type nanoparticles (Gref et al., 1994; Jeong et al., 1998), and hydrophobized

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polysaccharides (Akiyoshi et al., 1998) have received great attention due to their self-assembling characteristics in aqueous environment. Self-assembled nano-carriers are generally characterized of a hydrophobic core and a hydrophilic shell, i.e. hydrophobic core acts as a drug incorporation part and outer-shell acts as a safe-guard from the attack of reticuloendothelial system. Therefore, they are considered as superior drug carriers and developed by several researchers (Kataoka et al., 1993; La et al., 1996; Gref et al., 1994; Jeong et al., 1998; Yokoyama et al., 1991).

Previously, we reported that DexLG copolymer can form self-assembling nanoparticles as drug-carrying vehicles (Jeong et al., 2006). Dextran part in DexLG copolymer would form the hydrophilic outer-shell, due to its aqueous solubility, while PLGA has formed the inner core of the self-assembly, due to its hydrophobic properties.

In this study, we prepared polymeric micelle of DexLG graft copolymer and antimicrobial agent, amphotericin B (AmpB), was incorporated into polymeric micelle. AmpB-incorporated polymeric micelle of DexLG copolymer was characterized and antimicrobial activity was evaluated *in vitro*.

2. Materials and methods

2.1. Materials

Dextran from *Leuconostoc mesenteroides* (average molecular weights (M_w): 77,000) and amphotericin B (AmpB) were purchased from Sigma Chem. Co. (St. Louis, USA). *N*,*N*′-dicyclohexyl carbodiimide (DCC) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) were purchased from Aldrich Chemical Co., USA. Poly(D,L-lactide-*co*-glycolide) (PLGA 5005, M_w: 5100) was purchased from Wako Pure Chem Co., Japan. The dialysis membranes with a molecular weight cutoff (MWCO) of 8000 g/mol were purchased from Spectra/Pro™ Membranes. Dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were of HPLC grade and used without further purification.

DexLG copolymers were synthesized as reported previously (Jeong et al., 2006). Briefly, Dextran and PLGA were dissolved separately in dried DMSO. About 1.5 equiv. amount of DCC were added to the PLGA/DMSO solution, which was then stirred for 30 min to activate the carboxyl group of the PLGA. The resulting solution was added to the Dextran/DMSO solution containing DMAP, and the reaction was allowed to continue at room temperature for 12 h. The reaction mixture was filtered off to remove the byproducts and then exhaustively dialyzed against deionized water for 3 days. Following this, dialyzed solution was lyophilized for 3 days and then solid was precipitated three times in DCM to remove unreacted PLGA and dried in a vacuum oven for 2 days. After that, the dried product was dispersed in distilled water to remove unreacted dextran, with this procedure being repeated three times following lyophilized it. The molecular weight (M_w) of DexLG copolymer was calculated from elemental analysis as described previously (Jeong et al., 2006). The degree of substitution (DS) of PLGA chains grafted per dextran was estimated by subtracting the determined M_w of dextran from the M_w of DexLG graft copolymers and dividing by M_w of PLGA (Jeong et al., 2006).

2.2. Preparation of core-shell type polymeric micelle of DexLG copolymer

The DexLG polymeric micelle was prepared as reported previously with slight modification (Jeong et al., 2006). AmpB and DexLG copolymer was dissolved in 7 ml of DMSO. This solution was dropped into 20 ml of deionized water to form polymeric micelle. This solution was slowly dropped into 20 ml of deionized water for 10 min and then stirred 5 min additionally. This solution was introduced into dialysis tube (MWCO 8000 g/mol) and dialyzed against deionized water for 24 h to remove solvent. The deionized water was exchanged intervals of 1 h for 3 h and then exchanged 3 h intervals for 21 h. The dialyzed solution was filtered with 1.0 μm syringe filter to sterilize it and then lyophilized or analyzed.

Empty polymeric micelle of DexLG copolymer were prepared by same procedure described above with the exception of AmpB.

For evaluation of drug contents and loading efficiency, 5 mg of AmpB-encapsulated DexLG polymeric micelle were dissolved in 10 ml of DMSO and diluted it with DMSO. AmpB concentration was evaluated using an UV-spectrophotometer (UV spectrophotometer 1201, Shimadzu Co., Japan) at 388 nm. Empty polymeric micelle of DexLG copolymer was used as a blank test.

$$\text{Drug contents} = \left[\frac{(\text{drug weight in the polymeric micelle})}{(\text{weight of polymeric micelle})}\right] \times 100$$

$$\text{Loading efficiency} = \left[\frac{(\text{Residual drug in the polymeric micelle})}{(\text{initial feeding amount of drug})}\right] \times 100$$

2.3. Analysis

The morphology of the polymeric micelle was observed using a transmission electron microscope (TEM, JEOL JEM-2000 FX II, Japan). A drop of polymeric micelle suspension containing phosphotungstic acid (0.05%, w/w) was placed onto a carbon film coated on a copper grid for TEM. Observation was done at 80 kV.

Particle size of polymeric micelle was measured with a dynamic laser scattering spectrophotometer (DLS-7000, Otsuka Electonics Co., Japan). A sample solution prepared by dialysis method was used for particle size measurement (concentration: 0.1 wt.%).

For analysis critical micelle concentration (CMC) of DexLG copolymer, fluorescence spectroscopy was employed and CMC was determined as previously described (Jeong et al., 2006). To prepare sample solutions, a known amount of pyrene in acetone was added to each of a series of 20 ml vials, and the acetone was evaporated. The final concentration of pyrene was 6.0×10^{-7} M.

To each vial, 10 ml of various concentrations of the nanoparticle suspensions was added, and then heated for 3 h at 65 °C. Equilibration of the pyrene and the DexLG nanoparticles were achieved by allowing the solutions to cool overnight at room temperature. The fluorescence excitation spectra were measured at emission wavelength of 390 nm. Excitation and emission bandwidths were 1.5 nm and 1.5 nm, respectively.

Elemental analysis was employed to estimate degree of substitution of PLGA using a Perkin-Elmer CHNS.

2.4. Drug release study

The release experiment was carried out *in vitro* as follows: 5 mg of lyophilized polymeric micelle of DexLG copolymer was reconstituted into 5 ml of PBS and then introduced into dialysis tube (MWCO: 12,000 g/mol). The dialysis tubes were placed in 200 ml bottle with 95 ml of PBS, and the media stirred at 100 rpm and 37 °C. At specific time intervals, the medium was taken for analysis of drug concentration. After that whole media was replaced with fresh PBS to prevent drug saturation. The medium was diluted 10–100 times with DMSO and the concentration of the AmpB released was determined using an UV-spectrophotometer (UV spectrophotometer 1201, Shimadzu Co., Japan) at 388 nm. The properties of UV spectrum of amphotericin B in DMSO was not changed at the range between 0.1 μg/ml and 10 μg/ml. Therefore, we diluted the release medium with DMSO to this range for drug concentration estimation using UV spectrophotometer.

2.5. Minimal inhibitory concentration (MIC) of AmpB-encapsulated polymeric micelle

Candida albicans (KCTC 7270) was supplied from Korean Collection for Type Cultures (KCTC). Cells of *C. albicans* were grown at 30 °C in YM broth (Yeast extract 3.0 g, Malt extract 3.0 g, Peptone 5.0 g, Dextrose 10.0 g/L). AmpB was dissolved in DMSO and diluted further with phosphate-buffered saline (PBS, pH 7.2) to give a final concentration of 10 μg/ml. AmpB-encapsulted polymeric micelle was dissolved in distilled water and adjusted to the same final concentration (10 μg/ml). The fungal cells were seeded on 96-well microtiter plate (Greiner, Nurtingen, Germany) in YM broth at a density of 1×10^5 cells (100 μl per well). One hundred microliters of the serially diluted drugs were added to each well and the cell suspension was incubated for 24 h at 30 °C. The inhibition of growth was determined by measuring the absorbance at 595 nm using a microtitration ELISA reader (Molecular Devices Emax, CA, USA). The lowest concentration that completely

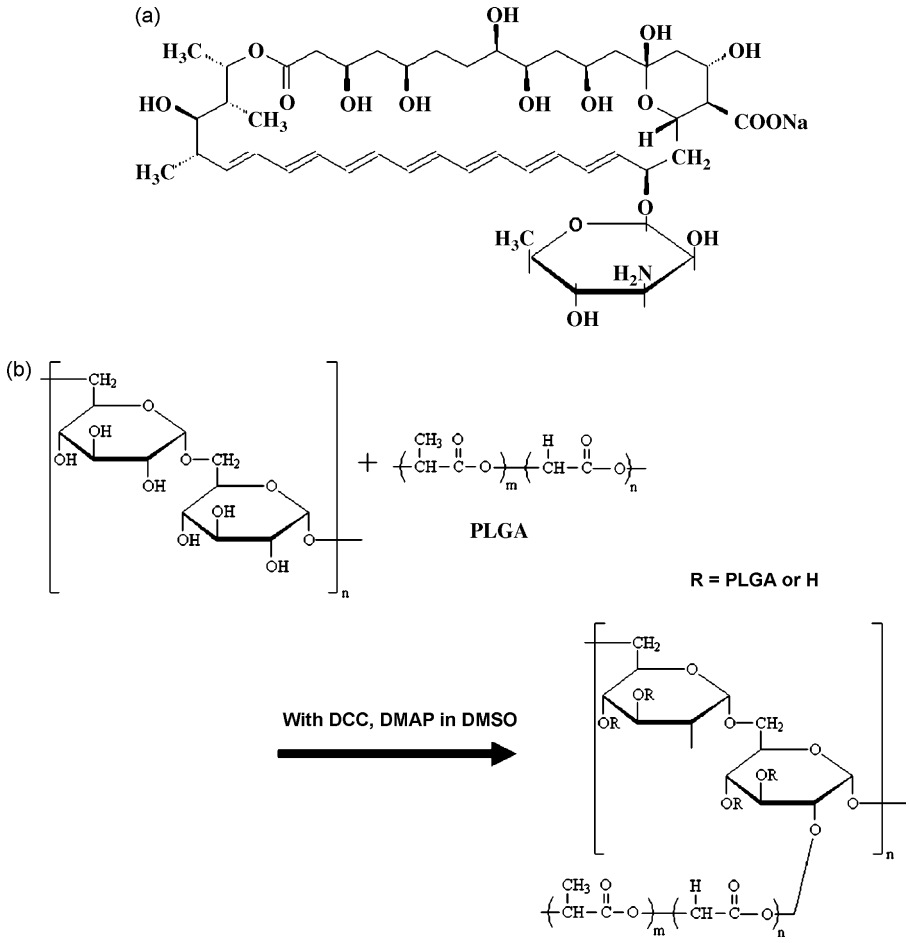

Fig. 1. Chemical structure of AmpB (a) and synthesis scheme of DexLG copolymer (b).

Table 1
Characterization of PLGA-g-dextran copolymer

	M_w[a]	DS[b]	CAC (g/L)[c]	Particle size (nm)		
				Intensity	Weight	Number
DexLG-1	85,700	1.7	0.01	32.1 ± 7.9	30.1 ± 8.1	25.8 ± 9.8
DexLG-2	96,400	3.8	0.006	31.2 ± 9.7	30.7 ± 15.5	30.1 ± 14.6
DexLG-3	108,600	6.2	0.0052	41.7 ± 13.5	41.0 ± 18.5	40.2 ± 17.4

[a] Number-average molecular weight (M_w) of DexLG was calculated from elemental analysis.
[b] The degree of substitution (DS) of PLGA chains grafted per pullulan was estimated by subtracting the determined M_w of dextran from the M_w of DexLG graft copolymers and dividing by M_w of PLGA.
[c] CAC was estimated from fluorescence spectroscopy measurements as explained in Section 2.

inhibited growth of the fungal cells was defined as the minimal inhibitory concentration (MIC). The MICs were the average of measurements in three independent assays.

3. Results and discussion

3.1. Characterization of core-shell type polymeric micelle of DexLG copolymer

We previously reported that DexLG copolymer can formed core-shell type nanoparticles by self-assembling process (Jeong et al., 2006) around particle sizes of 100 nm. Dextran is extensively investigated as a biomateriala and anti-tumor drug delivery carriers due to their water solubility, prolong the half-lives of antitumor drug, biocompatibility, immunoneutrality, and passive targeting to tumor (Ichinose et al., 2000; Mehvar, 2003). However, dextran is required to be modified hydrophobic for these kinds of application (Van Dijk-Wolthuis et al., 1995; Jeong et al., 2006; Jung et al., 2005). We modified dextran using PLGA copolymer as a hydrophobic domain as shown in Fig. 1, i.e. PLGA act as a drug incorporation site with biodegradability and dextran main chain act as a hydrophilic outershell of the polymeric micelle. The composition and molecular weight of DexLG copolymer was summarized in Table 1. The core-shell type polymeric micelle of DexLG copolymer were prepared by nanoprecipitation-dialysis method, i.e. DexLG in organic solvent was nanoprecipitated into deionized water and then dialysis procedure was employed to remove organic solvent. Even if direct dialysis method (Jeong et al., 2006; Jung et al., 2005) is also feasible method to make polymeric micelle, nanoprecipitation-dialysis method was resulted smaller particle size than direct-dialysis method. As shown in Table 1, particle size of DexLG polymeric micelle did not exceed 100 nm at all composition. Particle size of polymeric micelle of DexLG copolymer was increased according to the increase of PLGA content in the copolymer. Fig. 2 showed TEM images of DexLG polymeric micelle. As shown in Fig. 2(a), DexLG copolymer is able to form spherical polymeric micelle in the aqueous solution with particle size around 20–50 nm and particle size was almost similar to the particle size measurements.

We choose AmpB as an antimicrobial agent and encapsulated into core-shell type polymeric micelle of DexLG copolymer. The drug contents and loading efficiency was shown in Table 2. As shown in Table 2, drug contents and loading efficiency were increased according to the PLGA content in the copolymer composition. When initial drug feeding amount was increased, drug contents was increased but loading efficiency was decreased. Especially, loading efficiency was higher than 80% at all com-

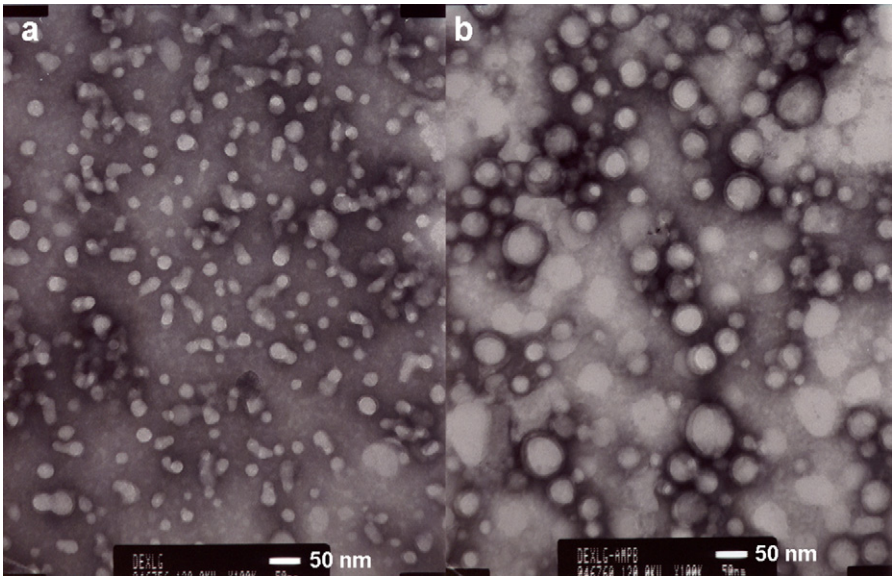

Fig. 2. TEM photographs of empty polymeric micelle (a) and AmpB-encapsulated polymeric micelle of DexLG-2 copolymer (b).

Table 2
Characterization of core-shell type nanoparticles of DexLG copolymer

DexLG/AmpB weight ratio (mg/mg)	Drug contents (%, w/w)	Loading efficiency (%, w/w)	Particle size (nm)		
			Intensity	Volume	Number
DexLG-1					
45/5	8.9	87.9	50.2 ± 20.5	49.1 ± 25.7	47.8 ± 23.7
DexLG-2					
47.5/2.5	4.8	95.7	40.8 ± 11.5	40.1 ± 21.5	38.6 ± 22.1
45/5	9.0	89.3	62.8 ± 20.1	60.9 ± 10.9	60.1 ± 12.7
40/10	18.9	93.3	124.5 ± 80.8	113.7 ± 72.5	99.5 ± 56.1
DexLG-3					
45/5	9.7	96.6	75.7 ± 23.7	72.9 ± 12.8	70.8 ± 17.5

position. Particle size was increased according to the increase of drug contents and PLGA contents in the copolymer composition, but their average size was not exceeded 200 nm. These results showed that DexLG can form polymeric micelle about 30–150 nm in their particle size. Small particle size less than 200 nm is more desirable for long blood circulation of drug and then passive drug targeting (Allemann et al., 1993). Most of AmpB-encapsulated polymeric micelle has sub-200 nm sizes.

Generally, block and graft copolymers have amphiphilic characteristics in an aqueous environment and self-assembling properties (Gref et al., 1994; Jeong et al., 1998; Jung et al., 2005). Polymeric micelle has intrinsic structures, i.e. hydrophobic domain of copolymer may compose hydrophobic inner-core of polymeric micelle while hydrophilic domain composed hydrated outershell of polymeric micelle. Hydrophobic core of polymeric micelle act as a drug-containing site since hydrophobic inner-core is useful to incorporate hydrophobic drug through hydrophobic interaction. As shown in Fig. 3, ^{1}H NMR was used to approve core-shell structure of polymeric micelle. As shown in Fig. 3(a), AmpB dissolved in DMSO has intrinsic peaks at ^{1}H NMR. Fig. 3(c) showed that intrinsic AmpB peaks was disappeared at polymeric micelle at D_2O. However, polymeric micelle at DMSO showed specific peaks both of DexLG copolymer and AmpB, indicating that AmpB was encapsulated into hydrophobic inner-core of polymeric micelle of DexLG copolymer and DexLG copolymer can form polymeric micelle in the aqueous phase. Furthermore, polymeric micelle formed critical micelle concentration (CMC) using pyrene as the hydrophobic probe as shown in Table 1. The fluorescence intensity of pyrene at fluorescence excitation spectra was found to increase with increasing concentration of DexLG copolymer, which points to the self-assembly of the DexLG copolymer in water (data was not shown). In addition, a red shift was observed in the excitation spectra with increasing DexLG copolymer. It is thought that pyrene is preferentially solubilized into the core part of the polymeric micelle. When the intensity ratio of I_{337}/I_{334} versus log c of DexLG copolymer for the pyrene excitation spectra was plotted, CMC was observed between flat region at extremely low concentration and sigmoid change in the crossover region. This result indicates that the signal change in the crossover region could be related to the CMC value of DexLG copolymer (Fig. 4).

Figs. 5 and 6 showed the drug release kinetics of polymeric micelle of DexLG copolymer *in vitro*. Fig. 5 showed the effect of drug contents on the release kinetics of AmpB from DexLG

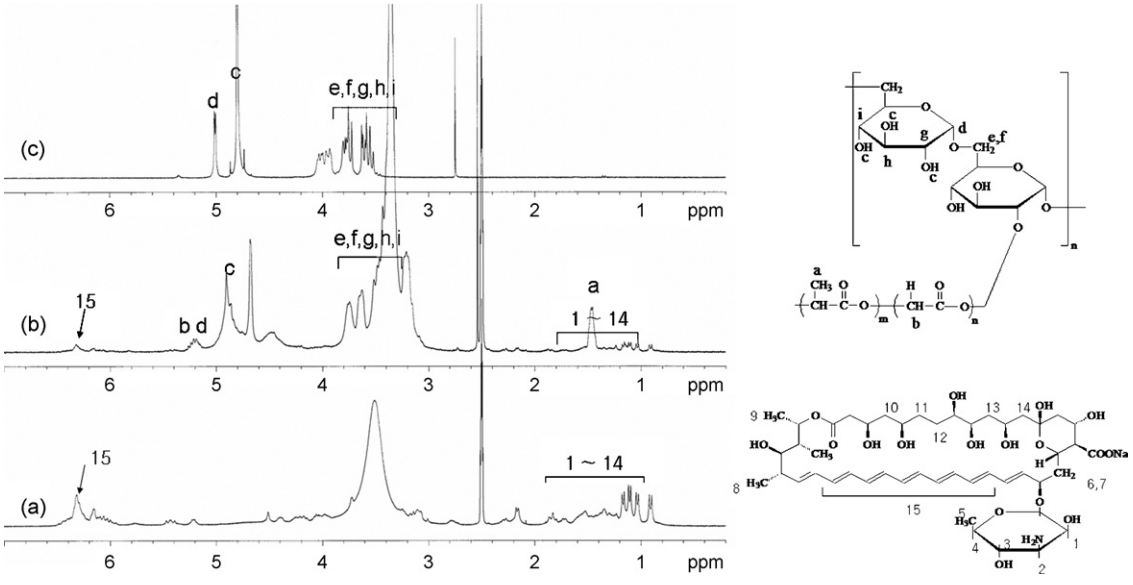

Fig. 3. ^{1}H NMR spectra of core-shell type polymeric micelle of AmpB in DMSO (a); AmpB-encapsulated polymeric micelle in DMSO (b) and D_2O (c).

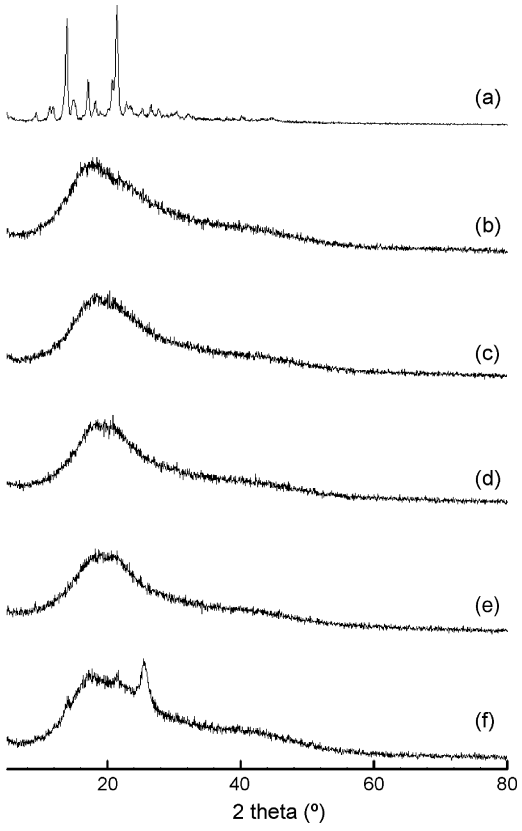

Fig. 4. X-ray powder diffraction of AmpB-encapsulated polymeric micelle. AmpB (a); DexLG-2 empty polymeric micelle (b); AmpB-encapsulated polymeric micelle, Drug contents (4.8%, w/w) (c), (9.0%, w/w) (d), (18.9%, w/w) (e); physical mixture of AmpB and DexLG-2 empty polymeric micelle (f).

polymeric micelle. As shown in Fig. 5, drug release rate was significantly changed according to the drug contents, i.e. the higher the drug contents the slower the release rate. Furthermore, increased particle size according to the increased drug contents may affected to the decreased release rate of drug. Generally, hydrophobic drug can be aggregated in the nanospheres at higher drug contents (Gref et al., 1994). Aggregated hydrophobic drug in the hydrophobic PLGA core might be slowly dissolved to

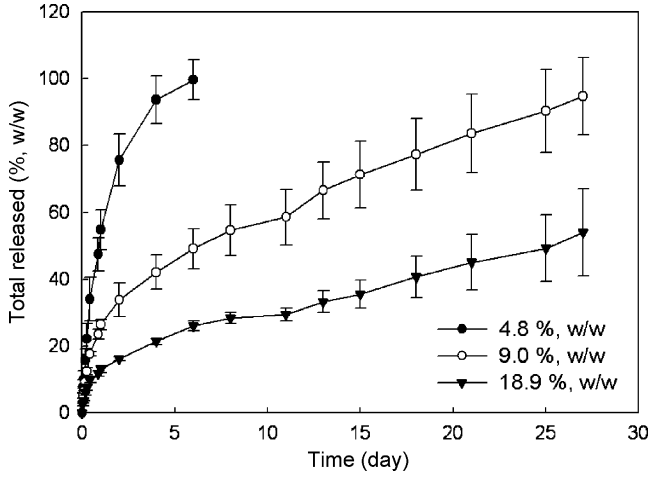

Fig. 5. The effect of drug contents on the drug release from DexLG polymeric micelle.

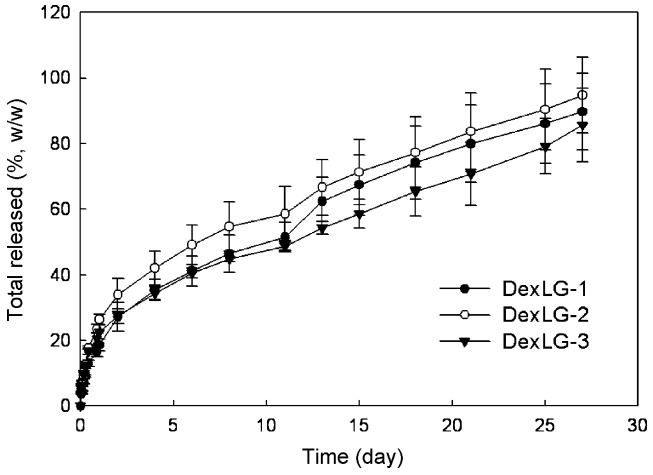

Fig. 6. The effect of copolymer composition on the drug release from DexLG polymeric micelle.

Table 3
The effect of free AmpB and AmpB-encapsulated nanoparticles on the MIC against *C. albicans*

	MIC (μg/ml, $n=3$)
Free AmpB	0.625
AmpB-encapsulated nanoparticle[a]	0.625–1.25
Empty nanoparticle[a]	≥10.0

[a] AmpB-encapsulated (Drug contents = 4.8%, w/w) or empty DexLG-2 nanoparticles was used for this test.

aqueous phase and released slowly from polymeric micelle. Fig. 6 showed the effect of copolymer composition on the drug release from polymeric micelle of DexLG copolymer. As shown in Fig. 6, drug release rate was decreased according to the increased content of PLGA in the copolymer composition. Also, increased drug content according to the increased PLGA content might be affected to the decreased release rate of drug.

3.2. Antimicrobial effect of AmpB-encapsulated DexLG polymeric micelle

To evaluate the antimicrobial activity of AmpB-encapsulated DexLG polymeric micelle, MIC of AmpB itself, AmpB-incorporated polymeric micelle of DexLG-2 copolymer, empty polymeric micelle of DexLG-2 copolymer was tested with *C. albicans* as shown in Table 3. As shown in Table 3, MIC of AmpB-incorporated polymeric micelle was almost similar value of AmpB itself while empty polymeric micelle has higher MIC value, indicating that AmpB-incorporated polymeric micelle has similar antimicrobial potential with AmpB but DexLG copolymer did not affect to the antimicrobial activity of polymeric micelle.

From these results, AmpB-incorporated polymeric micelle of DexLG copolymer can be used as an antimicrobial drug carrier.

4. Conclusion

AmpB-encapsulated polymeric micelle of DexLG copolymer was characterized *in vitro*. The average particle size of

AmpB-encpasulated DexLG polymeric micelles was around 30–150 nm while particle size of empty polymeric micelles was around 20–70 nm according to the copolymer composition. The morphology of AmpB-encapsulated polymeric micelle of DexLG copolymer was spherical shapes at transmission electron microscopy (TEM) observation. At ^{1}H NMR study, specific peaks of AmpB and DexLG copolymer was obtained at DMSO but specific peaks characterized to AmpB and PLGA was disappeared at D_2O environment. These results indicated that AmpB was encapsulated into the micellar core of polymeric micelle. XRD results also support these results, indicating that specific crystal peaks of AmpB and broad peaks of DexLG copolymer were obtained but specific peaks of AmpB was disappeared at polymeric micelles while physical mixture of AmpB/empty polymeric micelles showed both specific peaks. Drug release rate was decreased according to the increase of drug contents and increase of PLGA component of DexLG copolymer. At the minimal inhibition concentration (MIC) study using *C. albicans*, AmpB-encapsulated polymeric micelle showed almost similar effectives on the growth inhibition of microorganisms. These showed that AmpB-encapsulated polymeric micelle of dexLG copolymer can be considered to potential antimicrobial agent carriers.

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