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Research Article

Cephalexin Microspheres for Dairy Mastitis: Effect of Preparation Method and Surfactant Type on Physicochemical Properties of the Microspheres

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Abstract. The aim of this study was to evaluate the effects of preparation method and the type of surfactant on the properties of cephalexin (CPX) microspheres in order to obtain delivery systems suitable for the treatment of dairy mastitis. Microspheres were obtained using various preparation conditions and their physicochemical characteristics such as size, loading efficiency, morphology, and drug crystallinity were investigated. Antibacterial activity of microspheres from the optimum preparation condition was also studied. CPX microspheres were prepared by two different W/O/W emulsion solvent evaporation methods using PLGA as a matrix forming polymer. Several types of surfactants including nonionic, cationic, and anionic at different concentrations were used for preparation of the particles. The type and concentration of surfactant did neither affect the size nor morphology of the microspheres but showed a pronounced effect on the CPX encapsulation efficiency. It was found that Tween 80 showed the highest drug encapsulation efficiency (66.5%). Results from X-ray diffraction diffractograms and differential scanning calorimetry thermograms indicated that CPX entrapped in these microparticles was amorphous. Assessment of antibacterial activity showed that the obtained CPX microspheres exhibited good inhibition with minimum inhibitory concentration and minimum bactericidal concentration values of 128 μg/mL and 2,048 mg/mL against *Staphylococcus aureus* ATCC 25923, 512 μg/mL and 4,096 mg/mL against *Escherichia coli* ATCC 25922, respectively.

KEY WORDS: cephalexin; hydrophilic drug; microspheres; PLGA; surfactant.

INTRODUCTION

Cephalexin (CPX), a member of the first generation of cephalosporins, possesses strong antibacterial activity against both Gram-positive and Gram-negative bacteria. It is widely applied in the treatment of bacterial infections both in humans and animals. It is also effective in the treatment of group A beta-hemolytic streptococcal throat infections (1). Moreover, it has been shown to be highly efficient against various pathogenic microorganisms in dairy cow mastitis (2) which nowadays cause a serious problem in many countries because of its negative effect on milk production.

Intramammary antimicrobial therapy immediately after the last lactation is the most effective and widely used in dairy mastitis control therapy. However, this therapy does not prevent an infection in the late dry and prepartum period because the antimicrobial agents used have persistent activity only in the early dry period (3). Hence, there is a need to prolong the antibiotic therapy until the expected time.

Microspheres of biodegradable polymers have been widely studied as drug delivery systems (4,5). They are useful for prolonged drug release and for the targeting of drugs to specific infection sites. Besides their ability to control drug release, they have been reported to reduce drug-associated adverse effects, protect the compound from inactivation before reaching its site of action, increase the intracellular penetration, and enhance the pharmacological activity (6). In particular, poly(lactic-*co*-glycolic acid) (PLGA) has received tremendous interest for the development of controlled drug delivery systems due to its excellent biocompatibility and biodegradability (7,8). For this reason, PLGA microspheres as antibiotic delivery systems are under investigation as alternatives for the existing dry cow antibiotic formulations.

In our previous study (9), we prepared CPX-loaded PLGA microspheres by using a double-emulsion solvent evaporation technique. However, the loading efficiency of CPX in the microspheres obtained was rather low (<19%). Therefore, further experiments for improving drug loading efficiency have to be performed. It is expected that altering the preparation method or some process parameters will cause a variation in drug loading efficiency. Prior *et al.* (10) reported that gentamicin sulfate microspheres prepared by spray drying showed a higher drug-loading efficiency and lower size distribution than those prepared by the solvent evaporation method. Furthermore, drug-loading efficiency has been reported to be affected by the polymer concentration, stirring rate of the emulsion, initial drug loading,

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phase volume ratio, and surfactant for stabilization of the primary emulsion (11–14). In the W/O/W emulsion process, the type of surface-active substance added is of importance to create a stable emulsion that in turn will affect the drug-loading efficiency (15,16). Previous reports have been demonstrated that surfactants in the primary emulsion improve drug-loading efficiency. To mention, the addition of Triton X 100 and Igepal CA-630 increased the loading efficiency of fibronectin. It has also been reported that Tween 20 increased the entrapment of beta-lactoglobulin, while large losses of protein were observed with no surfactant added (17,18). However, no reports have been published so far in which the effect of surfactants with different characteristics added to the primary emulsion on size and entrapment of CPX in PLGA microspheres. The aim of the present work was to evaluate the effect of the preparation method and surfactant type on the properties of the CPX microspheres emphasizing on enhancement of drug loading efficiency. According to this purpose, microspheres characteristic such as size, morphology of microspheres, as well as drug-loading efficiency were investigated. Moreover, physical characteristics, e.g., crystallinity and thermal behavior of drug in the microspheres as well as the antimicrobial activity of a selected microsphere formulation were also investigated.

MATERIALS AND METHODS

Materials

PLGA, with a copolymer ratio of dl-lactide/glycolide of 60/40 and an inherent viscosity of 0.5 dL/g, was purchased from PURAC Biochem (Gorichem, The Netherlands). Polyvinyl alcohol (PVA) with 86–89% hydrolysis degree and molecular mass range from 30,000–70,000 g/mol was obtained from Sigma–Aldrich (St. Louis, MO, USA). CPX was a generous gift of Siam Pharmaceutical Co. Ltd (Bangkok, Thailand). Sodium lauryl sulfate was from Farmitalia Carlo Erba (Barcelona, Spain). Benzalkonium chloride was obtained from Fluka (Buchs, Switzerland). Triton X100 was from Panreac (Barcelona, Spain). Pluronic F68 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Tween 80 was purchased from Acros Organics (New Jersey, USA). Muller–Hinton broth (MHB) was purchased from Difco (Augsburg, Germany). Glacial acetic acid was from Merck (Darmstadt, Germany). Dichloromethane, chloroform, acetone, ethyl acetate, and methanol were from Fisher Chemicals (Loughborough, UK). These reagents were of analytical grade except methanol that was HPLC grade. Other chemicals were of highest grade available.

Preparation of CPX Microspheres

The preparation method of CPX microspheres was based on the double-emulsion solvent evaporation technique as described by Bodmeier and McGinity (7) and Iwata and McGinity (19) with some modifications. Two fabrication methods were used to prepare microspheres with the same amount of CPX.

Method A was based on the preparation described in our previous report (9). In short, CPX (5 mg) was dissolved in 0.5 mL water forming the first aqueous phase W1. The organic phase was formed by 5 mL of 5% *w/v* PLGA solution in chloroform/acetone at a ratio of 3:2. These solutions were emulsified using Polytron® (10,000 rpm, 1 min) to form the primary W1/O emulsion. This W1/O emulsion was then poured in to 10 ml of 2% PVA in distilled water and homogenized in Polytron® (10,000 rpm, 1 min). The resulting W1/O/W2 emulsion was stirred at 700 rpm for 18 h at room temperature to allow the chloroform/acetone to evaporate and form microspheres. When the effect of pH of internal water phase was studied, 0.5 M HCl was used instead of water to dissolve CPX. Furthermore, 50 mg of CPX was added to the primary water phase to study the effect of the amount of drug added to the formulation on the CPX encapsulation efficiency.

Method B: The double-emulsion method with ultrasonication was performed. In detail, CPX (50 mg) was dissolved in 0.375 mL of 0.5 M HCl containing different types and concentrations of surfactants. Subsequently, the drug solution was added to 2.5 mL of 10% *w/v* PLGA in dichloromethane and emulsified using an ultrasonic probe at 40% duty cycle under cooling for 3 min to yield a stable W/O emulsion. Next, this W/O primary emulsion was added to 25 mL of a 1% PVA aqueous solution and further emulsified for 1 min at a stress-mixing speed of Polytron® at 5,000 rpm. The organic solvent was allowed to evaporate at 40°C for 10 min. The obtained microspheres were collected by centrifugation at 15,000 rpm for 10 min, washed twice with deionized water, and then freeze-dried.

The preparation method that resulted in the highest encapsulation efficiency was selected for further study of the effect of surfactants in the primary water phase on the physicochemical properties of the microspheres. Different types and concentrations of surfactant including anionic (sodium lauryl sulfate), cationic (benzalkonium chloride), and nonionic surfactant (Tween 80, Pluronic F68, Triton X100) concentration range between 0.01% and 0.2% *w/v* were added in primary water phase.

Particle Size Measurement

The lyophilized microspheres were resuspended in deionized water before measuring the particle size by using particle sizing systems AccuSizer Model 780 (Santa Barbara, CA, USA). Particle size is expressed as a volume weight mean diameter (in μm ± SD).

Determination of Drug Loading

After formation, the microsphere suspension was centrifuged for 10 min at 15,000 rpm, and the supernatant was analyzed for CPX by HPLC with UV detection at 260 nm. The chromatographic method was carried out isocratically. The mobile phase consisted of 1.25% acetic acid in water/methanol (75:25), and the flow rate was set at 1 mL/min. Separation was achieved by using an Inersil® C18 (250 mm × 4.6 mm, 5 μm) analytical column connected to Inersil® C18 (50 mm × 4.6 mm, 5 μm) guard column. The column temperature was 40°C. Calibration curves were obtained over a concentration range of 0.004 to 0.5 mg/mL. The injected volume was 10 μL. The amount of encapsulated CPX in the microspheres was calculated by the difference between the amount of CPX added to the microsphere forming solution and

Table I. Effect of Preparation Method, Amount of Drug Added, and pH of Internal Water Phase on Characteristic of CPX Microspheres

Method	Amount of drug added (mg)	Internal water phase	Mean diameter (μm)	% Loading efficiency (%LE)	% Loading capacity (%LC)
A	5	Water	3.2±0.2	19.1±0.5	0.4
A	5	0.5 M HCl	3.2±0.7	20.6±2.4	0.4
A	50	0.5 M HCl	3.9±1.0	24.2±0.7	4.7
B	50	0.5 M HCl	26.7±2.6	58.6±1.9	9.8

the measured non-entrapped CPX in the external phase after microsphere formation. The percentage of drug encapsulation efficiency or drug loading efficiency (% LE) and drug-loading capacity (% LC) were calculated as the following:

$$\%\mathrm{LE} = \frac{\text{Initial drug added} - \text{Free drug}}{\text{Initial drug added}} \times 100$$

$$\%\mathrm{LC} = \frac{\text{Initial drug added} - \text{Free drug}}{\text{Weight of particles}} \times 100$$

Scanning Electron Microscopy

The morphology of the microspheres was investigated by scanning electron microscopy (SEM). Approximately 1 to 2 mg of lyophilized microspheres was evenly sprinkled onto a carbon adhesive disk mounted onto an aluminum stub. Samples were sputter-coated (Edwards Sputter-coater S150B) with gold for 3 min and subsequently viewed using a JEOL JSM-5410LV SEM (JEOL, Tokyo, Japan) operating at 10 kV, 20°C, and 10–5 Torr.

X-Ray Diffraction

The X-ray diffraction (XRD) patterns of CPX, physical mixtures of CPX/PLGA at a weight ratio of 1:3 or 1:8, CPX-loaded and unloaded microspheres prepared using the conditions which yielded the highest drug loading efficiency, and PLGA were obtained by using a Siemen D500 X-ray diffractometer (BrukerAXS, Madison, USA). Diffractograms were registered at Bragg angle (2θ) of 10° to 60° at a scanning rate of 5°/min.

Table II. Effect of Surfactant on Particle Size of CPX Microspheres

Surfactant	Particle size (μm)		
	Mean	Mode	Median
No surfactant	26.7±2.6	25.3±2.9	25.2±2.6
TW	27.4±1.6	27.3±0.0	26.1±1.3
SLS	25.7±0.0	20.3±0.4	23.0±1.0
BC	24.0±1.0	23.2±1.1	22.2±1.3

The particles were prepared using Method B
TW Tween 80, *SLS* sodium lauryl sulfate, *BC* benzalkonium chloride

Differential Scanning Calorimetry

Samples of CPX, physical mixtures of CPX/PLGA at a weight ratio of 1:3 or 1:8, CPX-loaded and unloaded microspheres prepared using the conditions which yielded the highest drug loading efficiency, and PLGA were subjected to Perkin Elmer DSC7 (Norwolk, CT, USA). A 2- to 3-mg sample was sealed in an aluminum pan. The samples were heated from 30°C to 250°C under nitrogen gas flow of 20 mL/min at a heating rate of 10°C/min. The temperature was calibrated with pure indium, with a melting point of 156.6°C. An empty pan was used as a reference.

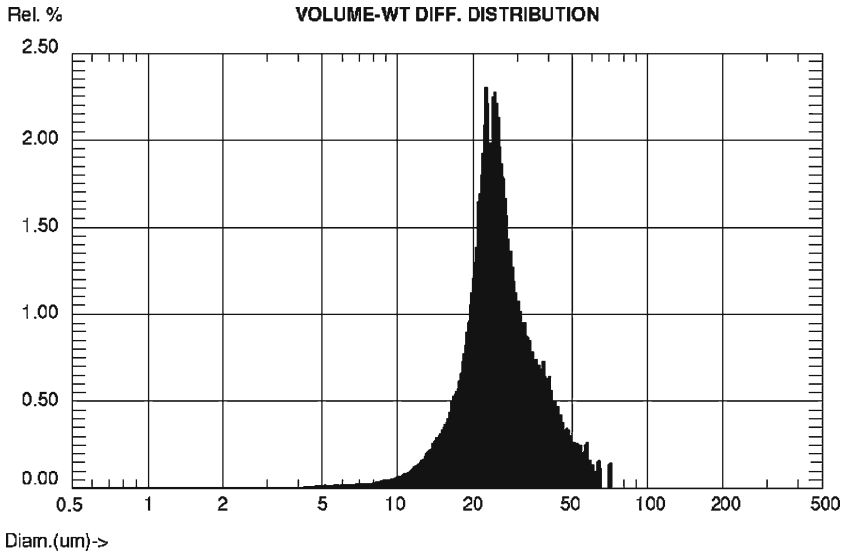
Microbiological Tests

The antimicrobial activity of the CPX microspheres was studied in comparison with that of the free drug. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by using a broth dilution technique against selected strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Briefly, CPX containing solution and suspension at drug concentration of 10 mg/mL and 16 mg/mL, respectively, were serially diluted in MHB to obtain drug, concentration ranging from 2 to 2,048 μg/mL and from 2 to 8,192 μg/mL, respectively. The dilutions were prepared in triplicate and inoculated with 1×10^6 cfu/mL microorganisms. The test tubes were incubated for 24 h at 37°C and subsequently optically checked for bacteria. The lowest concentration that completely inhibited bacterial growth was recorded as MIC. Formulations that inhibited bacterial growth were further investigated onto Muller–Hinton agar seeded with *S. aureus* and *E. coli* and incubated at 37°C for 24 h for investigation of the MBC.

RESULTS AND DISCUSSION

Effect of Preparation Method on Particle Size and CPX Loading Efficiency

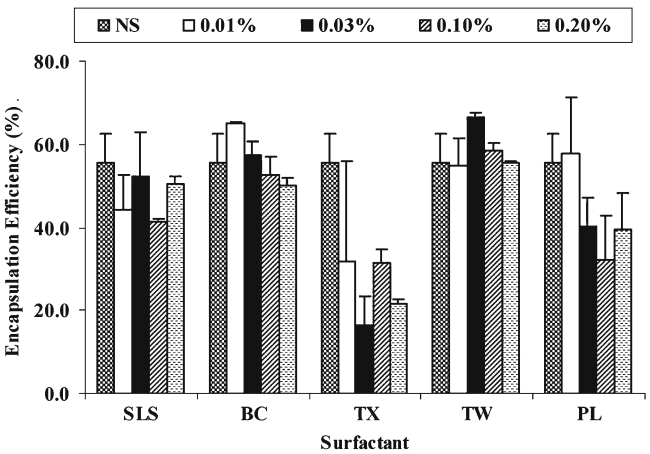
Table I shows that the CPX loading efficiency and mean particle diameter of the microspheres were affected by the microsphere preparation method. Microspheres prepared with water as an internal phase yielded particles with a size of 3.2±0.2 μm and a CPX loading efficiency of 19.1%. Replacement of the internal phase with 0.5 M in order to increase the CPX solubility resulted in microspheres with the same size and loading efficiency. Using preparation method A, the loading efficiency and particle size were not affected by the amount of drug dissolved in the 0.5 M HCl inner phase. As a consequence the loading capacity of the particles


Rel. %
VOLUME-WT DIFF. DISTRIBUTION
2.50
2.00
1.50
1.00
0.50
0.00
0.5
1
2
5
10
20
50
100
200
500
Diam.(um)->
Fig. 1. Volume weight diameter of microspheres prepared with method B

prepared with 50 mg of drug was about 10 times higher than those prepared with 5 mg drug. The loading efficiency and size of the microspheres prepared with method B were substantially higher than those of the particles prepared with method A. In Method B, microspheres were produced with 10% *w/v* PLGA while microspheres obtained by method A were produced by 5% *w/v* PLGA. It has been demonstrated before that the drug encapsulation efficiency increases with increasing PLGA concentration. Likely at lower polymer concentration, the particles have a high porosity resulting in a low drug-loading efficiency (20). The high loading efficiency obtained with method B may also be explained by the fact that ultrasonication induced smaller and more homogeneous droplets of inner phase W1 than that produced by high-speed homogenization of method A. When using high-speed homogenization, the bigger droplets present in the internal aqueous phase W1 of the first emulsion W1/O have a higher probability, because of their bigger size to fuse with the external aqueous dispersion medium W2, resulting in a lower loading efficiency. Finally, the solvent evaporation in method B was faster than that of method A. Therefore, the particles solidify more rapidly using method B, which in turn reduces the time that droplets of the inner phase fuse with the outer phase which then results in a higher drug encapsulation efficiency (13).

The mean diameters of particles prepared with method B are bigger than that of the particles prepared with method A. This can be explained by the lower stirring speed applied to prepare the secondary emulsion in method B as well as the higher polymer concentration used (9).

Because of the high encapsulation efficiency, Method B was selected to study the effect of surfactants on CPX microspheres.


NS
0.01%
0.03%
0.10%
0.20%
80.0
60.0
40.0
20.0
0.0
Encapsulation Efficiency (%)
SLS
BC
TX
TW
PL
Surfactant
Fig. 2. Encapsulation efficiency of CPX microspheres with different concentration of sodium lauryl sulfate (*SLS*), benzalkonium chloride (*BC*), Triton X100 (*TX*), Tween 80 (*TW*), and Pluronic F68 (*PL*). The particles were prepared by method B

Influence of Surfactants on Particle Size and CPX Loading Efficiency

The influence of type and concentration of surfactants used to stabilize the primary emulsion on microsphere size and drug encapsulation efficiency was investigated. Table II gives the average sizes of the CPX microspheres prepared using different surfactants in comparison with those without surfactant. It is shown that the mean size of the microspheres was not affected by the type and concentration of surfactant used for the primary emulsion and varied between 24 and 27 μm. Figure 1 shows a representative size distribution of microspheres prepared by method B. It was observed that the obtained microspheres were unimodal with rather narrow size distribution. The distribution is typical for PLGA microspheres prepared using a solvent evaporation technique (21). It has been reported that the encapsulation of hydrophilic compounds in polymeric microspheres is influenced by several factors, among which the use of surfactants (22). Our results (Fig. 2) demonstrate that surfactants played an important role on CPX encapsulation. In the absence of surfactant, the drug encapsulation was 58.7%. The addition of Triton X100 or sodium lauryl sulfate yielded CPX microspheres with obviously lower drug encapsulation efficiencies, particularly at higher surfactant concentrations. The microspheres with 0.01% *w/v* benzalkonium chloride and Pluronic

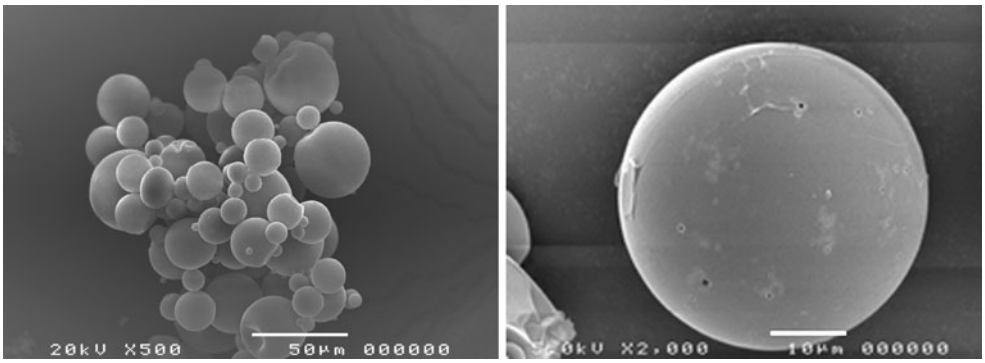


Fig. 3. Scanning electron micrographs of CPX microspheres

F68 showed high CPX encapsulation in comparison with those without surfactant but lower than those prepared using Tween 80. The drug encapsulation efficiency was found to be highest (66.5%) for the microspheres using the nonionic Tween 80 at concentration of 0.03% *w/v*. These results are in agreement with those of previous reports which described the effect of Tween on the encapsulation of beta-lactoglobulin and *Brucella abortus* in PLGA microparticles (17,23). The stability of the primary emulsion is one of the key factors for the efficient entrapment of hydrophilic drugs in polymeric microspheres (24). A high loading of hydrophilic drug can be expected from a stable primary emulsion. Our results indicate that Tween 80 is the most suitable surfactant for preparing stable CPX primary emulsions and at a surfactant concentration of 0.03% yielded CPX-loaded microspheres with the highest drug entrapment. These CPX microspheres were therefore further evaluated for their morphology and biological action. Moreover, as the physical state of drug existing in the microspheres could affect its biological activity XRD and differential scanning calorimetry (DSC) analysis was performed.

Morphological Characterization

The morphology of the CPX-loaded microspheres prepared with 0.03% Tween 80 is shown in Fig. 3. The obtained microspheres had a spherical shape and smooth surface, with some small pores. These pores can be attributed to the migration of inner aqueous droplets to the microsphere surface during emulsification (25).

X-Ray Diffraction Studies

CPX is a crystalline compound. To investigate in which form, crystalline or amorphous, CPX is loaded in the PLGA particles XRD analysis was performed. The result was demonstrated in Fig. 4. The XRD patterns of CPX showed several diffraction peaks corresponding to a crystalline

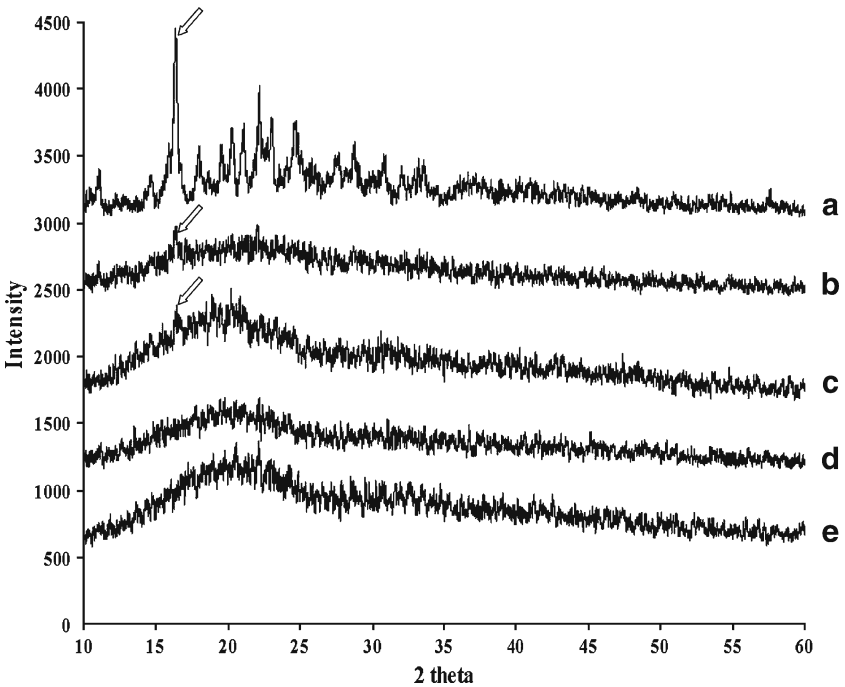


Fig. 4. X-ray diffraction patterns of intact CPX **a**, CPX-PLGA (1:3) physical mixture **b**, CPX-PLGA (1:8) physical mixture **c**, CPX-loaded microspheres **d**, and unloaded microspheres **e**, crystalline identical peak of the drug (CPX) located at the 2 theta of approximately 16.3 (*arrows*)

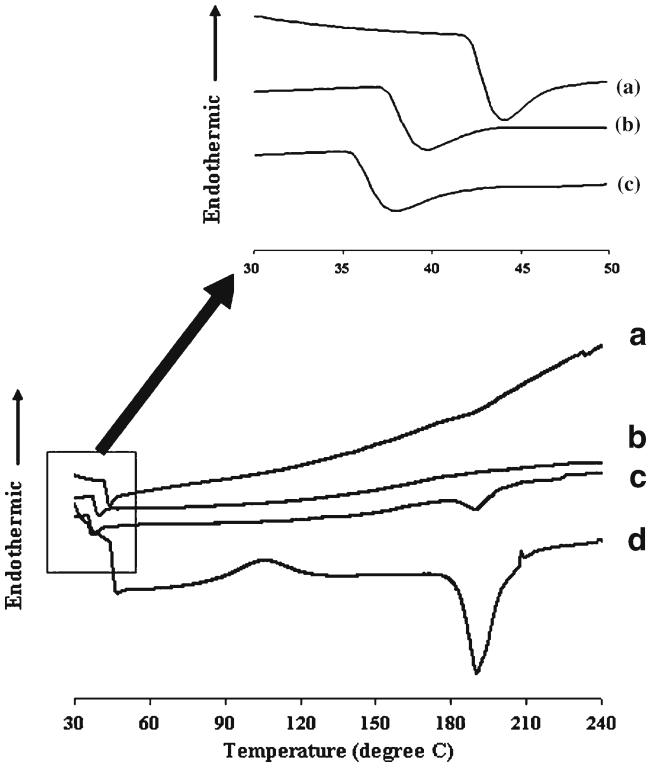


Fig. 5. DSC Thermograms of CPX-loaded microspheres **a**, PLGA **b**, CPX-PLGA (1:3) physical mixture **c**, and intact CPX **d**

structure of the drug. The XRD diffractogram of unloaded microspheres (Fig. 4e) showed a halo pattern confirming that PLGA is amorphous. Physical mixtures of CPX and PLGA at a weight ratio of drug to polymer of 1:3 and 1:8 (Fig. 4b and c, respectively) showed that CPX was in its crystalline state, although the diffraction intensity at the 2θ of 16.3 (pointed by an arrow) decreased as compared to that of the free drug. The XRD of CPX-loaded PLGA (Fig. 4d) exhibited a halo pattern similar to that of unloaded particles, suggesting that CPX is entrapped in the microspheres in an amorphous state. Likely dispersion of CPX in the PLGA matrix prevents crystallization of the drug.

Thermal Behavior of the Microspheres

DSC was used for further study of the thermal behavior of CPX-loaded PLGA microspheres. The result as thermograms was exhibited in Fig. 5. In the thermogram of CPX (Fig. 5d), an endothermic peak around 100–110°C was observed which can be ascribed to dehydration of the CPX monohydrate. Further, a strong exothermic decomposition peak was observed at around 190°C (26). The thermogram of PLGA shown in Fig. 5b reveals a glass transition temperature around 35–37°C which is in agreement with literature (25). The thermogram of physical mixtures of CPX and PLGA (weight ratio of 1:3) exhibited a glass transition which can be ascribed to PLGA and an exothermic peak due decomposition of CPX. In the thermogram of CPX microspheres, the glass transition temperature of this system was observed at a higher temperature (42–44°C) than that of PLGA. This increase in T_g indicates that CPX and PLGA interact with each other leading to a decrease in mobility of the PLGA chains. In fact, CPX acts as an anti-plasticizer for PLGA. Obviously, this interaction also prevented crystallization of CPX in the polymer matrix as discussed in previous section on XRD analysis of the CPX microspheres.

Antimicrobial Activity of the Microspheres

The CPX-PLGA microspheres were investigated for their antimicrobiological activities against *S. aureus* and *E. coli* as representative Gram-positive and Gram-negative pathogens causing mastitis. The results presented as MIC and MBC values are shown in Tables III and IV, respectively. CPX exhibited MIC values against *S. aureus* and *E. coli* of 4 and 32 μg/mL, respectively, and MBC values against respective pathogens of 2048 μg/mL. The MIC and MBC values of CPX microspheres were higher than those of the free CPX. However, considering for the actual drug content of the microspheres, the activity in the loaded microspheres was found to be similar or even better than that of the free drug.

CONCLUSION

In this paper, we demonstrated the effect of preparation method as well as the type of surfactant used in the inner water phase of W/O/W emulsion on the physicochemical properties of CPX microspheres. It was found that the size and drug encapsulation efficiency were dependent on method preparation. It was found that CPX was dispersed in its amorphous state in the microspheres. Finally, it was shown that the CPX-loaded PLGA microspheres had excellent antimicrobiological activities.

Table III. MIC of Free CPX in Comparison with CPX Microspheres

Bacterial Strains	MIC (μg/mL)		
	Free CPX	CPX[a] microspheres	CPX[b] microspheres
S. aureus ATCC 25923	4	128	5.3±0.9
E. coli ATCC 25922	32	512	21.3±3.5

Formulation prepared by Method B with 0.03% *w/v* Tween 80 in primary emulsion; CPX loading was 65%
[a] μg of microsphere
[b] μg of CPX

Table IV. MBC of Free CPX in Comparison with CPX Microspheres

Bacterial strains	MBC (μg/mL)		
	Free CPX	CPX[a] microspheres	CPX[b] microspheres
S. aureus ATCC 25923	2,048	2,048	85.0±13.8
E. coli ATCC 25922	2,048	4,096	170.0±27.6

Formulation prepared by method B with 0.03% *w/v* Tween 80 in primary emulsion; CPX loading was 65%
[a] μg of microsphere
[b] μg of CPX

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