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Ciprofloxacin-encapsulated poly(DL-lactide-*co*-glycolide) nanoparticles and its antibacterial activity

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Abstract

The aim of this study was to prepare ciprofloxacin HCl (CIP)-encapsulated poly(DL-lactide-*co*-glycolide) (PLGA) copolymer nanoparticles and its antibacterial potential was evaluated with pathogenic bacteria, *Escherichia coli* (*E. coli*), *in vitro* and *in vivo*. CIP-encapsulated nanoparticles of PLGA were prepared by multiple emulsion solvent evaporation method. PLGA nanoparticles showed spherical shapes with particle sizes around 100–300 nm. Loading efficiency was lower than 50% (w/w) because of water-solubility properties of CIP. At drug release study, CIP showed initial burst effect for 12 h and then continuously released for 2 weeks. At *in vitro* antibacterial activity test, CIP-encapsulated nanoparticles showed relatively lower antibacterial activity compared to free CIP due to the sustained release characteristics of nanoparticles. However, CIP-encapsulated PLGA nanoparticles (doses: 25 mg CIP/kg of mice) effectively inhibited the growth of bacteria due to the sustained release characteristics of nanoparticles, while free CIP was less effective on the inhibition of bacterial growth. These results indicated that CIP-encapsulated PLGA nanoparticles have superior effectiveness to inhibit the growth of bacteria *in vivo*.

Keywords: Ciprofloxacin HCl; Sustained release; Poly(DL-lactide-*co*-glycolide); Nanoparticles; Animal infection model

1. Introduction

A lot of antibiotics including fluoroquinolones have thus been used to treat bacterial infectious disease of humans (Emmerson, 1989). The fluoroquinolones are well-established broad-spectrum antibiotics with potent bactericidal activity against clinically important pathogens responsible for a variety of infections, including urinary tract infections (UTI), gastrointestinal (GI) infections, respiratory tract infections, sexually transmitted diseases, skin infections, and chronic osteomyelitis (Appelbaum and Hunter, 2000; Stratton, 1992). Among them, ciprofloxacin (CIP) is one of the most widely available fluoroquinolone antibiotics and has potent bactericidal activity against a broad range of clinically relevant Gram-negative and Gram-positive pathogens. It has been effectively used to treat a variety of bacterial infections, including UTI, sexually transmitted infections, gastrointestinal infections, as well as skin and bone infections (Davis et al., 1996; Stratton, 1992). Oral CIP still has been available only as conventional, immediate-release tablets that required twice daily at doses of 250 mg. Once-daily extended-release ciprofloxacin (500 mg dose) was available (Henry et al., 2002). Many patients with UTI and infectious disease required alternative antimicrobial therapy (Naber et al., 2001; Warren et al., 1999) such as drug targeting colloidal carriers (Fontana et al., 1998; Page-Clisson et al., 1998a) and sustained release microspheres (Ma et al., 2005; Ramchandani and Robinson, 1998) to reduce inconvenience of patient and increase therapeutic efficacy. Sustained release formulation, nanoparticles, and preformed microcapsules were reported to solve these problem due to the alteration of its tissue distribution, improving the drug efficacy, reducing the drug toxicity, and prolong the half-lives in blood (Ma et al., 2005; Page-Clisson et al., 1998a;

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Talan et al., 2004). Sustained release formulation of CIP will provide a new treatment option to clinicians that may enhance patient adherence/convenience and thereby enhances clinical success rates. Nanoparticles has effectiveness to treat infection by *Salmonella typhimurium* (Page-Clisson et al., 1998b) and infection by *Helicobacter pylori* (Fontana et al., 2001), and can protect antibiotics from degradation at acid environment (Fontana et al., 1998).

In this study, we prepared CIP-encapsulated nanoparticles using PLGA to treat urinary tract infection. The bacterial aetiology of UTI is well-established, with at least 80% of community-acquired, uncomplicated cases being caused by *E. coli* and other various uropathogens (Barnett and Stephens, 1997; Fihn, 2003; Hooton, 2003; Mourani et al., 2005; Warren et al., 1999). Furthermore, the resistance of uropathogens to ciprofloxacin has remained very low, while its resistance to ampicillin and trimethoprim/sulphamethoxazole has been dramatically increased. Physicochemical properties of CIP-encapsulated PLGA microspheres were investigated and their *in vitro*/*in vivo* antibacterial activities were evaluated using uropathogen.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-*co*-glycolide) (RESOMER RG 504) (PLGA) was purchased from Boeringher Ingelheim Co. Inc., Germany. Ciprofloxacin HCl (CIP) was gift from Jeil Pharm. Co., Korea. Poly(vinyl alcohol) (PVA, MW 15,000 g/mol) was purchased from Fluka Chem., Germany. Dichloromethane (DCM) as a HPLC grade was purchased from Aldrich Chem. Co. Inc., USA. Dialysis membrane with molecular weight cut-off (MWCO) size of 12,000 Da was purchased from Sigma Chem., USA and MWCO of 10,000 Da (Spectra/Por molecular porous membrane tubing) was purchased from Spectrum Lab. Inc., USA. All other chemicals were used as HPLC grade or extra pure grade.

2.2. Preparation of CIP-encapsulated PLGA nanoparticles

PLGA copolymer was dissolved in 7 ml of DCM and CIP was dissolved in 2 ml of deionized water. CIP aqueous solution was dropped to PLGA/DCM solution by sonification using ultrasonicator (40 W, 1 min, Vibracell VCX 400, Sonica & Materials Inc., USA) (first W/O emulsion). After that, this W/O emulsion was poured into 10 ml of aqueous phase (1% (w/v) PVA) and homogenized with high-pressure homogenizer (High-pressure homogenizer, R5-12.38, SMT Co., Japan) sonicated once more (40 W, 1 min) at 600 bar for 5 cycles per minute to make W/O/W emulsion. This emulsion was poured into 40 ml of PVA aqueous solution (0.4% w/v) and stirred at 1000 rpm with top-loading stirrer (Direct Driven Digital Stirrer SS-11D, Young HANA Tech. Co. Ltd., Korea) for 1 h. After that, CIP-encapsulated nanoparticles were harvested by ultracentrifugation (15,000 rpm, Supra 30 K, Vacuum High Speed Centrifuge, Hanil Science Industrial Co. Ltd., Korea). To wash nanoparticles, harvested solid was distributed to the distilled water and harvested again by centrifugation. This washing procedure was repeated for three times. Harvested nanoparticles were analyzed or lyophilized for 3 days.

2.3. Determination of drug contents and loading efficiency

To determine drug contents, 5 mg of nanoparticles were dissolved in 5 ml of DCM and then 5 ml of deionized water was added. This mixture was stirred magnetically for 6 h and then 1 ml of water phase was taken to measure drug concentration. Absorbance of aqueous phase was measured at 277 nm using UV-spectrophotometer (UV-spectrophotometer 1601, Shimadzu Co. Ltd., Japan). Drug contents and loading efficiency were as follows:

$$\text{drug contents} = \left[\frac{\text{drug weight in nanoparticles}}{\text{total weight of nanoparticles}}\right] \times 100$$

$$\text{loading efficiency} = \left[\frac{\text{drug remained in the nanoparticles}}{\text{feeding weight of drug}}\right] \times 100$$

2.4. X-ray powder diffractogram (XRD) measurement

XRD was obtained with a Rigaku D/Max-1200 (Rigaku) using Ni-filtered Cu Kα radiation (40 kV, 20 mA) to determine the crystallinity of drug. All experiments were performed at room temperature. The conditions of powder XRD measurement was as follows:

- Data type = binary; goniometer = 1; attachment = 1; scan mode = continuous.
- Mode 2 (R/T) = reflection; scan axis = $2\theta/\theta$.
- Start angle = 10.000; stop angle = 80.000; scan speed = 5.000; sampling interval = 0.050; θ angle = 5.000; 2θ angle = 10.000; fixed time = 0.01; full scale = 1000; counting unit = CPS; target = Cu.
- Wave length Ka1 = 1.540510; wave length Ka2 = 1.544330; wave length Ka = 1.541780; wave length Kb = 1.392170; 40.0 kV; 20.0 mA.

2.5. Transmission electron microscopy (TEM) observation

A drop of nanoparticle suspension containing 0.03% (w/v) of phosphotungstic acid was placed on a TEM copper grid coated with carbon film and dried at room temperature. Observation was performed at 80 kV with JEM-2000 FX II (JEOL, Japan).

2.6. Particle size measurement

The size of the nanoparticles was measured by dynamic light scattering (DLS) instruments. The DLS measurement was carried out using an ELS-8000 electro phoretic LS spectrophotometer (Otsuka Electronics Co., Japan) equipped with a He–Ne laser operating at 632.8 nm at 25 °C and a fixed scattering angle of 90°. The concentration of nanoparticles was adjusted to

1.0 mg/ml. The hydrodynamic diameters of the nanoparticles were calculated by the Stokes–Einstein equation and the polydispersity factors represented as μ_2/Γ^2 were evaluated from the cumulant method (μ_2: second cumulant of the decay function; Γ^2: average characteristic line width)

2.7. Drug release study

To study drug release, 10 mg of nanoparticles in 5 ml of phosphate buffered saline (PBS, pH 7.4, 0.1 M) were introduced into dialysis tube (MWCO: 12,000 g/mol). This dialysis tube was put into 200 ml glass bottle with 95 ml of PBS. Release test was performed at 37 °C with stirring rate of 100 rpm. The release medium was exchanged everyday to maintain the sink condition. The exchange of medium was varied according to the series of sample to prevent saturation of drug in the release medium. At predetermined time schedule, release medium was taken and concentration of released drug was measured with UV-spectrophotometer (UV-1601, Shamdzu Co. Ltd., Japan) at 277 nm (Ramchandani and Robinson, 1998). All experiments were triplicate.

2.8. Antibiotic preparations and susceptibility testing of PLGA nanoparticles encapsulating CIP at in vitro and in vivo

Stock solutions were prepared and dilutions were made according to the Clinical Laboratory Standards Institute-CLSI (formerly NCCLS) M7-A6 method (15 National Committee for Clinical Laboratory Standards 2003). *Escherichia coli* (*E. coli*) were provided by Korea Centers for Disease Control and Prevention (KCDC). Following subcultures from frozen stock, antimicrobial agents or nanoparticles were added to solutions of microorganisms at various concentrations. All experiments were performed in triplicate on separate days. Bacterial growth was determined by reading optical density at 600 nm (UV-spectrophotometer 1201, Shimadzu Co. Ltd., Japan) after overnight culture.

For *in vivo* antibacterial activity test (Na and Lee, 2006), *E. coli* was cultured at 37 °C in Luria-Bertani (LB) broth. To mimic isolated bacterial growth *in vivo*, *E. coli* (1×10^6/ml) was sealed in dialysis membrane (MW 10,000 Da, Spectra/Por molecular porous membrane tubing). To implant the dialysis membrane unit, ICR mice were anesthetized with ketamine (100 mg/kg). The dialysis membrane unit was surgically implanted into the peritoneal cavity. The free CIP and CIP-encapsulated nanoparticles were subcutaneously introduced 4 h after the implant on the dorsal side of the mice. The doses of CIP was 25 mg/kg of mice. Distilled water (DW) was treated for control. After 3 days, the mice were sacrificed to enumerate the bacterial count in the dialysis membrane unit. The cells were enumerated by plating on HI agar plate.

2.9. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were determined by a micro-dilution test with LB broth for MICs and LB broth and LB agar for MBC (Wagenlehner et al., 2006). The inoculum of LB broth was 1×10^6 colony-forming units (CFU)/ml. The MIC was defined as the lowest concentration inhibiting visible growth after incubation at 37 °C for 18 h in ambient air. The MBC was determined in a second step according to the guidelines recommended by the National Committee for Clinical Laboratory Standards. In brief, cultures were grown to mid-logarithmic phase for the final inoculum, which was confirmed by actual count and was added to the wells using a multipoint inoculator (0.0015 ml). The subcultured broth was spotted with a multipoint inoculator on LB agar and incubated for 24 h at 37 °C. The number of colonies subsequently grown was used to determine the lethal endpoint. Bactericidal activity was defined as a >99.9% (>3 log) reduction in the numbers of CFU.

3. Results and discussion

3.1. Characterization of ciprofloxacin-encapsulated PLGA nanoparticles

Since CIP, which is water-soluble drug, is not soluble in common solvents such as dimethylsulphoxide, dimethylformamide, chloroform, DCM, acetone, tetrahydrofuran and acetonitrile, CIP-encapsulated nanoparticles were prepared by multiple emulsion solvent evaporation method. CIP was dissolved in deionized water and emulsified with PLGA/DCM solution to make first W/O emulsion. To make nanoparticles, W/O/W emulsion was break down by high-pressure homogenizer and emulsified again by pouring W/O/W emulsion into PVA aqueous solution. Fig. 1 shows TEM observation of CIP-encapsulated PLGA nanoparticles. CIP-encapsulated nanoparticles showed spherical shapes with particle size around 100 nm (Fig. 1(a), 180-20) and 200–300 nm (Fig. 1(b), 360-40). The higher the feeding amount of PLGA and drug increased the particles size. As shown in Table 1, mean size of CIP-encapsulated nanoparticles analyzed by DLS was 131 nm (180-20) and 353 nm (360-40), indicating that particle size analysis was almost similar to TEM observation. Fig. 2 shows typical particle size distribution of

Table 1
Characterization of CIP-encapsulated PLGA nanoparticles

	Weight ratio of PLGA/CIP (% (w/v) in solvent/% (w/v) in water)	Drug contents (%, w/w)		Loading efficiency (%, w/w)	Particle size (nm)	Polydispersity index
		Theoretical	Experimental			
180-20	2.57/1.0	10.0	4.5	42.4	130	0.185
360-40	5.14/2.0	10.0	5.0	47.4	353	0.107

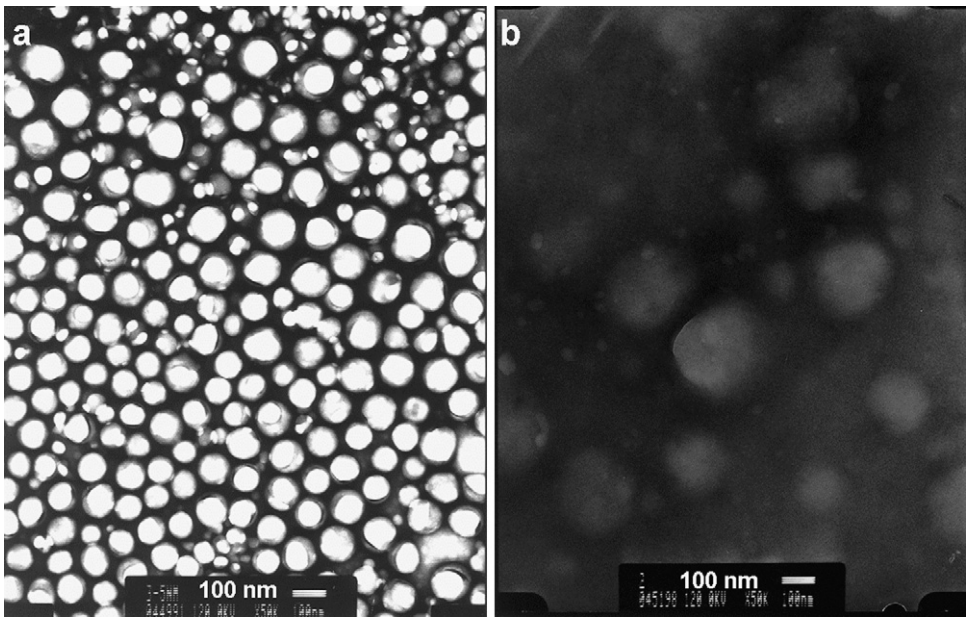

Fig. 1. TEM photographs of CIP-encapsulated PLGA nanoparticles: 180-20 (a) and 360-40 (b). For observation of TEM, nanoparticles were negatively stained with phosphotungstic acid (0.1%, w/w) and concentration of nanoparticle solution was 0.1 mg/ml as a basis of PLGA weight.

CIP-encapsulated PLGA nanoparticles (360-40). The size distribution of CIP-encapsulated PLGA nanoparticles was relatively broad and their mean size was 353 nm. Table 1 showed drug contents and loading efficiency. As shown in Table 1, loading efficiency was 42.4 (180-20) and 47.4 (360-40), indicating that the higher the feeding amount of drug and polymer increased the drug contents and loading efficiency. Especially, loading efficiency of PLGA nanoparticles did not exceed 50.0%, w/w. These results might be due to that CIP is a water-soluble drug and CIP was liberated during the emulsification or solvent evaporation process. To study the characteristics of drug in the nanoparticles, XRD patterns were measured using free CIP, empty nanoparticles, CIP-encapsulated nanoparticles, and physical mixtures (free CIP + empty nanoparticles). As shown in Fig. 3, CIP showed its specific crystal peaks around 20° and empty nanoparticles has broad peaks around 10°–60°.

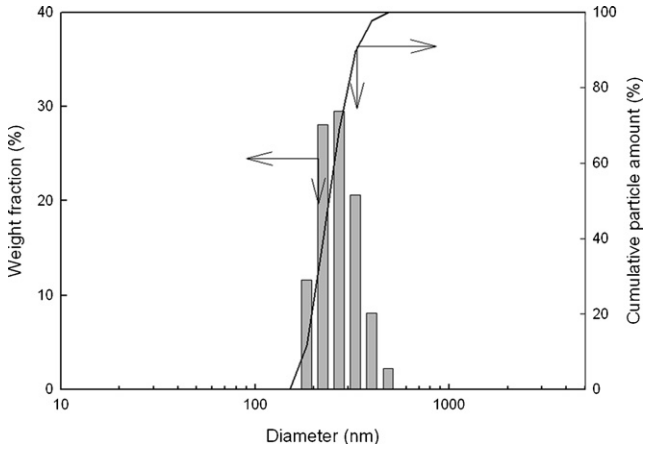

Fig. 2. Particle size distribution of CIP-encapsulated PLGA nanoparticles (360-40). Particle size distribution was expressed as percentage of weight fraction and their percentage of cumulative particle amount.

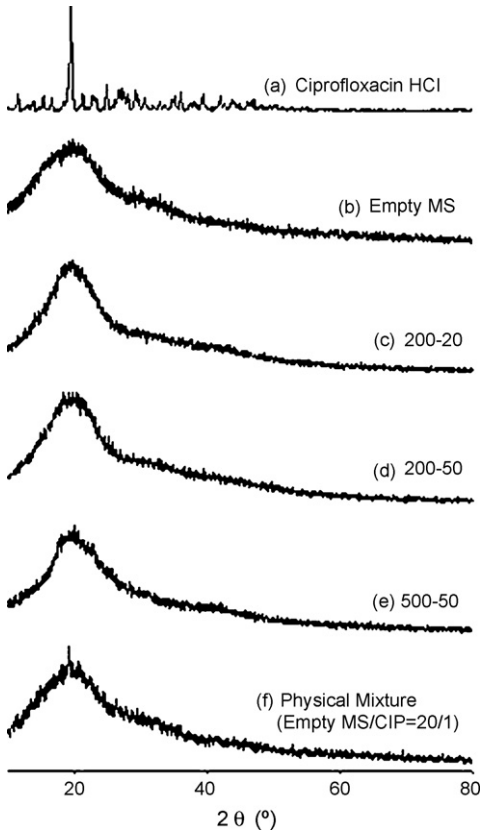

Fig. 3. XRD powder diffractograms of CIP (a), empty nanoparticles (b), CIP-encapsulated nanoparticles (180-20) (c), CIP-encapsulated nanoparticles (360-40) (d), and physical mixtures of empty nanoparticles/CIP (weight ratio of CIP/empty NP = 1/20).

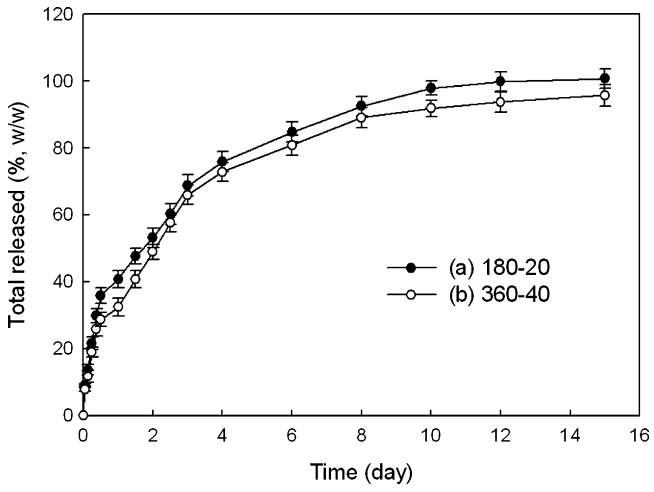

Fig. 4. Drug release from PLGA nanoparticles. Drug release test was performed at 37 °C, 100 rpm using dialysis method.

Especially, CIP-encapsulated nanoparticles at all formulations showed similar peak characteristics to empty nanoparticles, while physical mixture of free CIP and empty nanoparticles showed both sharp crystal peaks of CIP (at 20°) and broad peak of empty microspheres.

3.2. Drug release from PLGA nanoparticles

Fig. 4 shows the drug release kinetics of CIP-encapsulated nanoparticles. To test drug release, CIP-encapsulated PLGA nanoparticles were introduced into dialysis bag and outer aqueous phase was frequently exchanged to everyday. As shown in Fig. 4, CIP was continuously released over 2 weeks and burst release of CIP was observed for initial 12 h at both formulations. Although 360-40 showed slower release of drug than 180-20, drug release from PLGA nanoparticles was not significantly changed according to the formulations. These results indicated that the differences of particle size and drug contents did not significantly affect the release kinetics of nanoparticles. CIP release from nanoparticles was maintained for 2 weeks. Page-Clisson et al. (1998a) reported that CIP was released over 60% for 2 days from polyethylbutylcyanoacrylate. Other report on CIP-encapsulated PLGA nanoparticles showed about 15–60% (w/w) released for 1 day (Dillen et al., 2004). Other types of formulation such as CIP-incorporated PLGA implant showed extended released profile for 18 weeks (Ramchandani and Robinson, 1998).

3.3. Antibacterial activity of ciprofloxacin and ciprofloxacin-encapsulated PLGA nanoparticles

MIC of *E. coli* was 0.05 μg/ml and MBC was 0.2 μg/ml. 99.9% of *E. coli* was suppressed at 0.2 μg/ml of ciprofloxacin concentration. Even though it was treated by ciprofloxacin at 0.2 μg/ml concentration for 24 h, *E. coli* was multiplicated again when it was transferred to fresh broth. Therefore, sustained release formulation is required to treat bacterial infection such as urinary tract infections (UTI), which has not effective-immune system to remove bacteria. Our isolated system (*E. coli* in dialysis membrane) to test UTI was similar to the state of real UTI, i.e. infected bacteria is separated from effective-immune system and removed only by antibiotics such as ciprofloxacin. This UTI model system is similar to real UTI system.

To test antibacterial capacity of CIP-encapsulated PLGA nanoparticles, free CIP and CIP-encapsulated nanoparticles were treated to *E. coli* at *in vitro* and *in vivo* since *E. coli* is one of the major source of urinary tract infection (Barnett and Stephens, 1997). In case of CIP-encapsulated PLGA nanoparticles, formulation of 180-20 was used to study antibacterial activity. To test antibacterial activity, contents of free CIP and CIP-encapsulated nanoparticles (180-20) were adjusted to various equivalent concentration of CIP between 0.0005 μg/ml and 1.0 μg/ml. Fig. 5 shows *in vitro* antibacterial activity of free CIP and CIP-encapsulated nanoparticles against *E. coli*. At antibacterial test, free CIP itself showed most effectiveness at all treatment. Relative bacterial growth was evaluated by measurement of absorbance (ABS) using UV-spectrophotometer and expressed as an OD values. OD values were significantly decreased till 0.05 μg/ml of CIP concentration and then plateau region in OD values was observed till 1.0 μg/ml of CIP concentration. PBS or empty nanoparticles did not significantly affect the growth of *E. coli*, indicating that empty nanoparticles do not have any cytotoxicities against bacteria. Furthermore, physical mixtures of free CIP and empty nanoparticles showed almost similar cytotoxicity with free CIP, also indicating that the component of nanoparticles (i.e. PLGA copolymer) does not affect the antibacterial activity of free CIP. Antibacterial activity of CIP-encapsulated nanoparticles showed relatively less cytotoxic than that of free CIP. These results might be due to the sustained release characteristics of CIP-encapsulated nanoparticles, indicating that released CIP from nanoparticles may inhibit bacterial growth and CIP concentration in outerphase of nanoparticles must be lower than free CIP.

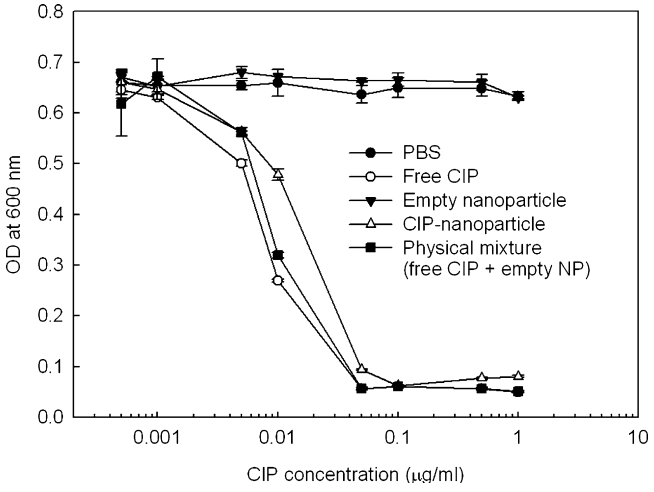

Fig. 5. *In vitro* antibacterial activity of free CIP and CIP-encapsulated PLGA nanoparticles against *E. coli*. For antibacterial activity, *E. coli* was treated with PBS as a control; empty NP: empty nanoparticles as a placebo; CIP-NP: CIP-encapsulated PLGA nanoparticles (360-40); empty NP + CIP: physical mixtures of empty nanoparticle/CIP for comparison. (OD = absorbance at 600 nm of UV-spectrophotometer measurement).

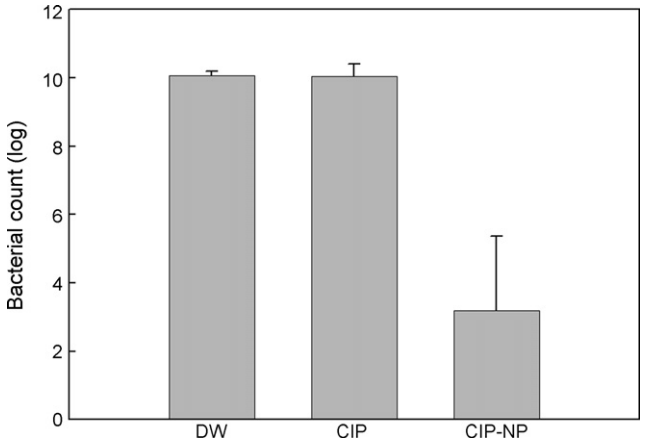

Fig. 6. *In vivo* antibacterial activity of CIP-encapsulated nanoparticles. For antibacterial test, *E. coli* (1×10^6/ml) was sealed in dialysis membrane and implanted into the peritoneal cavity of ICR mice. The free CIP and CIP-encapsulated nanoparticles (doses: 25 mg/kg) were subcutaneously introduced 4 h after the implant on the dorsal side of the mice. Mice were treated with distilled water (DW), free CIP, and CIP-encapsulated nanoparticles (CIP-NP), respectively. The bacterial number was mean of four mice.

To test antibacterial potential of CIP-encapsulated nanoparticles, UTI animal model was prepared as reported previously (Na and Lee, 2006), i.e. *E. coli* was introduced into dialysis membrane tubing and CIP can penetrate into dialysis membrane while *E. coli* cannot escape from dialysis tubing, indicating that *E. coli* in dialysis tubing can be used as model of local infection. Fig. 6 shows antibacterial activity of CIP and CIP-encapsulated nanoparticles against infected mouse model. As shown in Fig. 6, microorganisms in dialysis membrane tubing were rapidly increased. Number of microorganisms was significantly increased when PBS were administered. When free CIP treated to the mouse infection model, the number of microorganisms was not significantly changed compared to PBS (control). These results suggested that free CIP might be rapidly wash-out from the mouse body and administration of free CIP is hardly inhibited the growth of bacteria. Interestingly, CIP-encapsulated nanoparticles significantly inhibited the growth of bacteria as shown in Fig. 6. These results indicated that CIP was continuously release from nanoparticles and released drug can inhibit the growth of bacteria for a longer period rather than free CIP. These results suggested that CIP-encapsulated nanoparticles were successfully prepared and has at least similar or superior antibacterial activity compared to free CIP.

4. Conclusion

CIP-encapsulated PLGA nanoparticles was prepared by emulsion–solvent evaporation method. Antibacterial activities of free CIP and CIP-encapsulated PLGA nanoparticles were evaluated with *E. coli in vitro* and *in vivo*. PLGA nanoparticles has spherical shapes with particle sizes of 100–300 nm. Loading efficiency was lower than 50% (w/w) because of water-solubility properties of CIP. At drug release study, CIP showed initial burst effect for 12 h and then continuously released for 2 weeks. At *in vitro* antibacterial activity test, CIP-encapsulated nanoparticles showed relatively lower antibacterial activity compared to free CIP due to the sustained release characteristics of nanoparticles. However, CIP-encapsulated PLGA nanoparticles effectively inhibited the growth of bacteria due to the sustained release characteristics of nanoparticles, while free CIP did not significantly changed the growth of bacteria. These results indicated that CIP-encapsulated PLGA nanoparticles are superior effectiveness to inhibit the growth of bacteria *in vivo*.

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