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Norfloxacin이 담지된 Poly(ε-caprolactone)/ Poly(ethylene glycol) 이중블록공중합체 미셀의 제조

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Norfloxacin-Incorporated Polymeric Micelle Composed of Poly(ε-caprolactone)/Poly(ethylene glycol) Diblock Copolymer

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초록: 이 연구에서 norfloxacin(NFX)이 담지된 poly(ε-caprolactone)/poly(ethylene glycol)(PCL/PEG, abbreviated as CE) 이중블록공중합체로 구성된 고분자 미셀을 제조하였다. 입자크기는 PCL블록길이에 따라 60~200 nm사이였다. 임계회합농도는 소수성 PCL 블록길이가 증가함에 따라 감소하는 경향을 보였다. ^{1}H-NMR 연구에서 PCL 블록은 내핵, PEG는 외피를 형성한 미셀구조로 형성되었음을 확인하였다. 약물의 방출은 약 2일간 지속되었으며 PCL 블록길이와 약물함량이 증가함에 따라 감소하는 경향을 보였다. 항미생물 성능 실험에서 고분자 미셀은 기존의 NFX와 비슷한 독성을 보였다.

Abstract: We prepared norfloxacin (NFX)-incorporated polymeric micelle using poly(ε-caprolactone)/poly(ethylene glycol) (PCL/PEG, CE) diblock copolymers. Particle size was from 60 to 200 nm according to the PCL block length. Their critical association concentration (CAC) was decreased according to the increase of PCL block length. ^{1}H-NMR study showed core-shell type micelle structures of CE diblock copolymers in the aqueous environment. Drug release from polymeric micelle was continued over 2 days. Duration of drug release was varied according to the PCL block length and drug contents. At antimicrobial activity test, polymeric micelle showed almost similar cytotoxicity compared to NFX itself.

Keywords: polymeric micelle, norfloxacin, biodegradable polymer, antimicrobial agent, diblock copolymer.

Introduction

Polymer micelles[1,2] or core-shell type nanoparticles[3,4] using amphiphilic block copolymers were extensively investigated for the last decade. Amphiphilic block or graft copolymers can form micellar structures due to their surfactant behaviours in aqueous environment, i.e. hydrophobic domain forms inner-core of the micelle and hydrophilic domain forms outershell of the micelle. Each domain of the block copolymers has separate functions, i.e. hydrophobic block acts as a drug incorporation site through hydrophobic interaction with hydrophobic drugs and hydrophilic domain which composes outershell of the polymeric micelle, may protect from the attack of protein absorption or reticuloendothelial system (RES). Due to the these peculiar structures, polymeric micelle has some advantages such as reduced toxic side effects of anti-chemotherapeutic agents, passive targeting, solubilization of hydrophobic drugs, stable storage of drugs, long blood circulation, and thermal stability.[2,5]

Norfloxacin, a fluoroquinolone antibiotics, has broad spectrum against Gram-positive and Gram-negative bacteria, which are widely used to various infectious disease caused by bacteria, such as gonorrhea, prostate, and urinary tract infections.[6-8] The antibacterial action of norfloxacin involves the inhibition of bacterial DNA gyrase which selectively inhibits bacterial DNA

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synthesis.[9-12] However, its limited aqueous solubility is one of the drawbacks for clinical application.[13,14] Fluoroquinolones such as norfloxacin and ciprofloxacin exist as a zwitterionic form in aqueous solution due to the interaction between the nitrogen group of piperazine ring and the carboxylic acid group. Then, this interaction induces the limited aqueous solubility around pH 7.[15,16] This limited aqueous solubility at neutral pH frequently prevents the clinical application of fluoroquinolones.

In this study, we synthesized CE diblock copolymers to prepare NFX-incorporated polymeric micelle. PCL is a non-toxic biodegradable polymer with hydrophobic characteristics. Poly(ethylene glycol) (PEG) is a non-immunogenic and non-toxic water-soluble polymer, has the ability to increase bioavailability of the drug at *in vivo*.[17] The particle size, drug loading capacity, and physicochemical properties of NFX encapsulated polymeric micelles of CE diblock copolymers were investigated and the antimicrobial activity was also evaluated in this study.

Experimental

Materials. Methoxy PEG(MW=5000) (MPEG), dialysis tube (molecular weight cut-off (MWCO)=2000, 12000), norfloxacin (NFX), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Co. Ltd. USA. ε-Caprolactone, acetone, methanol, dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, and tetrahydrofuran (THF) were purchased from Aldrich Chem. Co. Ltd, USA. All other chemicals and reagents were used as extra reagent grade in all of the experiments.

Synthesis of PCL/PEG (CE) Diblock Copolymer. CE diblock copolymers were synthesized by a non-catalyzed ring-opening polymerization of ε-caprolactone in the presence of MPEG as shown in Figure 1(a).[18] MPEG and ε-caprolactone were mixed in a round-bottomed flask under vacuum. The mixture was cooled and degassed with pump. The round-bottomed flask was sealed off and placed in an oil bath at 180 ℃. After the polymerization was completed, the resulting product was cooled at room temperature and then dissolved in DCM. The solution was precipitated into an excess amount of cold ethanol and filtered to remove the unreacted MPEG homopolymer and ε-caprolactone monomers. The precipitates were washed with diethylether three times and then dried in a vacuum oven for 3 days.

Measurement of Fluorescence Spectroscopy. To measure the critical association concentration (CAC) of CE diblock copolymer using fluorescence spectroscopy, polymeric micelle of CE diblock copolymer without drug was prepared as follows; 40 mg of CE block copolymer was dissolved in 4 mL of acetone and dropped into 10 mL of deionized water under reduced pressure. Residual solvent was removed using a rotary evaporator and then dialyzed using molecular cut-off 2000 g/mol dialysis tube (Sigma Chem. Co. USA) against 1 L×3 of distilled water for 3 h and then 3-4 h during 2 days. Resultant solution was adjusted to the various concentrations of block copolymers.

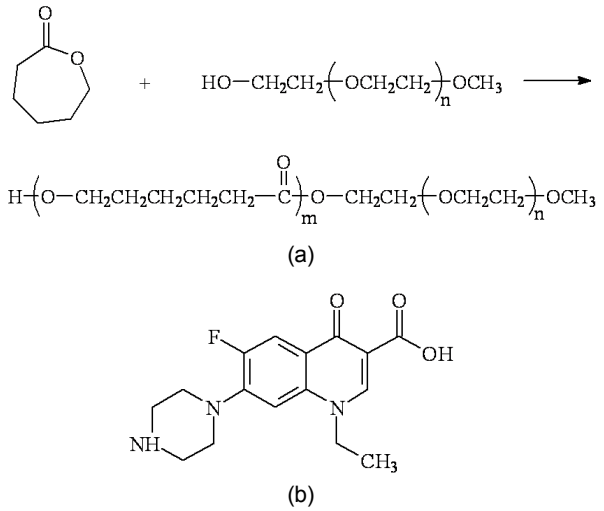


Figure 1. Synthesis scheme of CE diblock copolymer and chemical structure of NFX.

CAC of the CE diblock copolymers was estimated to prove the potential of micellar structure formation under aqueous environment by the measurement of fluorescence spectroscopy (Shimadzu F-7000 spectrofluorometer, Shimadzu Co. Ltd., Tokyo, Japan) using pyrene as a probe.[17] To get sample solutions, a known amount of pyrene in acetone was added to each of a series of 20 mL vials and the acetone evaporated. The amount was adjusted to give a pyrene concentration in the final solution of either 6.0×10^{-7} M. 10 mL of various solutions were added to each vial and then heated for 3 h at 65 ℃ to equilibrate the pyrene and the micelles and left to cool overnight at room temperature. Emission wavelength was 390 nm for excitation spectra. Excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

Preparation of Polymeric Micelle Containing NFX. Polymeric micelles of CE diblock copolymer containing NFX prepared by nanoprecipitation technique were as follows: 40 mg of CE diblock copolymer and 2~10 mg of NFX dissolved in 4 mL of acetone were poured into 10 mL of deionized water. Solvent was evaporated using a rotary evaporator for at least 30 min. Resulting solution was dialyzed using dialysis membrane for 9 h at room temperature. During the dialysis procedure, the dialyzed solution was exchanged every 1 h.

After that, the dialyzed solution was harvested and the volume of solution was adjusted to 20 mL (i.e. 40 mg polymer/20 mL of water). This solution was used for analysis or lyophilization for 2 days. All procedures for polymeric micelle preparation were carried out at darkened condition to avoid drug degradation by light.

To measure drug contents and loading efficiency, 5 mg of lyophilized nanoparticles were dissolved in acetone and drug concentration was measured at 322 nm with UV spectrophotometer (UV-1200, Shimadzu Co., Ltd., Japan). 5 mg of empty polymeric micelles were dissolved in acetone for blank test to verify interference of CE diblock copolymer.

$$\text{Drug contents} = \frac{\text{Amount of NFX in the nanopartides}}{\text{Weight of nanopartides}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of NFX in the nanoparticles}}{\text{Feeding amount of NFX}} \times 100$$

^{1}H Nuclear Magnetic Resonance Spectrometer (NMR) Measurement. In order to estimate the copolymer compositions and the molecular weights of the PCL blocks, the ^{1}H-NMR spectra of the copolymers were measured in $CDCl_3$ using a 300 MHz NMR spectrometer (FT-NMR, Bruker AC-300F, 300 MHz). As the number-average molecular weight of PEG (5000) is known, one can estimate the number-average molecular weights of the PCL block and the copolymer composition as calculated from the peak intensities in the spectrum assigned to both polymers.

^{1}H-NMR spectra were employed to approve the core-shell structure of the polymeric micelle of CE block copolymer using $CDCl_3$ and D_2O. The concentration of the polymeric micelle was 1.0 wt% in $CDCl_3$ and 0.5 wt% in D_2O.

Measurement of Particle Size. Size of polymeric micelle was measured with an ELS-8000 electrophoretic LS spectrophotometer (NICOMP 380 ZLS zetapotential/particle sizer, Otsuka electronics Inc., Japan) equipped with a He-Ne laser beam at a wavelength of 632.8 nm at 25 ℃ (scattering angle of 90°). This sample solution prepared was used for the particle size measurement (concentration: 1.0 mg/mL).

TEM Observation. For the TEM observations, a drop of polymeric micelle suspension was placed onto a carbon film coated on a copper grid. The observation was done using a JEOL JEM-2000 FX Ⅱ at 80 kV.

X-ray Powder Diffraction (XRD) Measurement. X-ray powder diffractograms were obtained with a Rigaku D/Max-1200 (Rigaku) using Ni filtered CuKα radiation (40 kV, 20 mA) to determine the crystallinity of drug. All experiments were performed at room temperature.

Drug Release Studies. Drug release study from polymeric micelles are performed as follows: 5 mg of NFX-incorporated polymeric micelles were reconstituted into 2 mL of phosphate buffered saline (PBS, 0.1 M, pH 7.4) and this solution was introduced into dialysis tube (M.W. cut-off: 2000). Dialysis tube was introduced into a bottle with 198 mL of PBS (0.1 M, pH 7.4). Release test was performed at 37 ℃ with stirring rate of 50 rpm. The whole release medium was exchanged with fresh medium at predetermined time to maintain the sink condition. Released amount of drug was monitored at 322 nm with UV spectrophotometer (UV-1200, Shimadzu Co., Ltd., Japan).

For comparison, equivalent amount of NFX was dissolved in PBS (supplemented with sodium dodecyl sulfate (2%, w/v)). After that, this solution was introduced into dialysis membrane and release experiment was performed similar to polymeric micelle.

Antimicrobial Test *in vitro*. 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. Treatment of drug or polymeric micelle was for 1 day. After that, bacterial growth was determined by reading optical density at 600 nm (UV-spectrophotometer 1201, Shimadzu Co. Ltd. Japan).

Results and Discussion

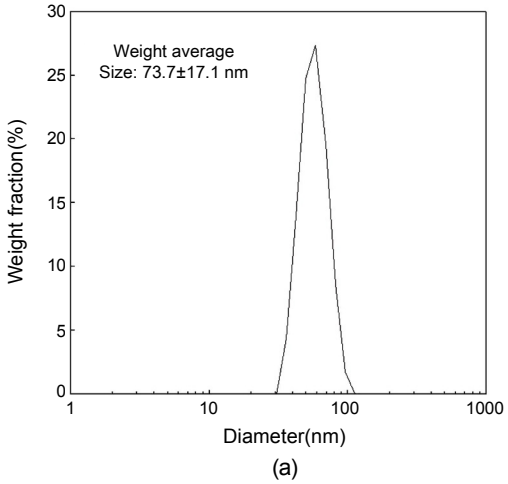
Characterization of Polymeric Micelles of CE Diblock Copolymers. CE diblock copolymers were synthesized by ring-opening polymerization of ε-caprolactone monomer in the presence of MPEG. An active hydrogen atom at one end of MPEG chains acts as an initiator and induces a selective acyl-oxygen cleavage of ε-caprolactone as shown in Figure 1. Different molecular weight of CE diblock copolymers were synthesized by changing the molar ratio of MPEG/ε-caprolactone monomer. Their M.W. and composition were calculated from ^{1}H-NMR spectra. The unit ratio of MPEG and ε-caprolactone was obtained from peak intensities of the methylene proton of MPEG chain (3.7 ppm) and methylene proton (4.13 ppm) in ε-caprolactone units (data not shown) and estimated M.W. of CE diblock copolymer were shown in Table 1.

Figure 2 showed typical particle size distribution and morphology of empty polymeric micelle of CE-2 diblock co-

Table 1. Characterization of CE Diblock Copolymers and Their Polymeric Micelles

	$\overline{M}_n$ of PCL	Total $\overline{M}_n$	Particle size(nm)			CAC (g/L)
			Intensity ave.	Weight ave.	Number ave.	
CE−1	1350	6350	70.1±12.3	65.6±10.1	61.0±9.8	0.015
CE−2	3120	8120	81.3±14.7	73.7±17.1	70.6±12.8	0.0034
CE−3	4370	9370	92.5±19.2	83.6±16.5	78.9±13.8	0.0023

* $\overline{M}_n$; Number average molecular weight.
*CAC: Critical association concentration.



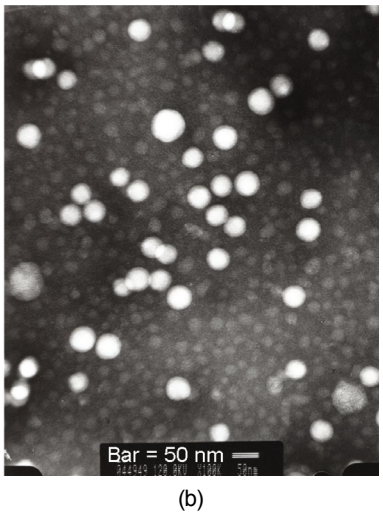


Figure 2. Typical particle size distribution (a) and TEM photograph (b) of polymeric micelle of CE−2 diblock copolymer.

polymer. As shown in Figure 2, polymeric micelle of CE diblock copolymer showed narrow size−distribution and its morphology observed by TEM was spherical shapes. These results indicated that CE diblock copolymers can form spherical nanoparticles in the aqueous environment. Their average particle size was summarized in Table 1. As shown in Table 1, polymeric micelle of CE diblock copolymers has

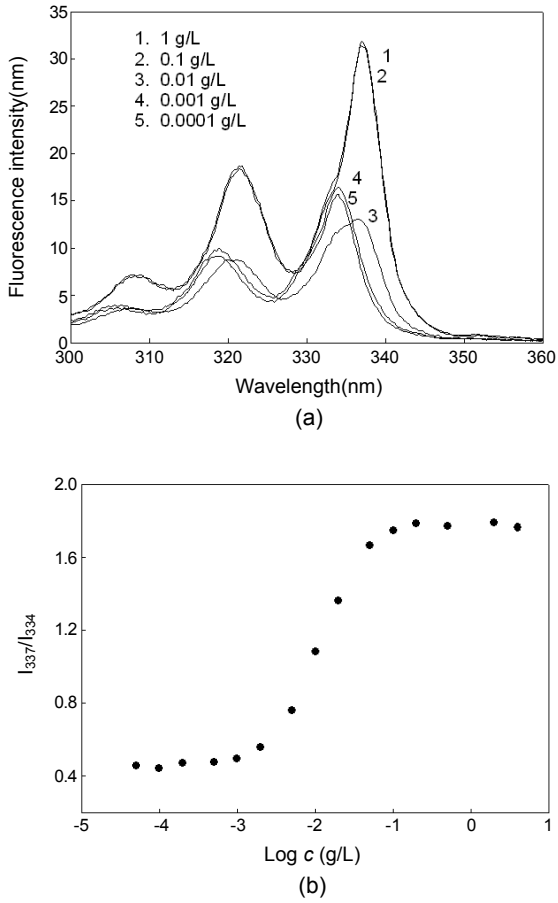


Figure 3. Fluorescence excitation spectra of pyrene/CE−3 against concentration of CE−3 in distilled water (Emission wavelength: 390.0 nm) (a) and plots of the intensity ratio of I_{337}/I_{334} from pyrene excitation spectra vs. log c for block copolymer against concentration of CE in distilled water (b).

less than 100 nm in average particle sizes and their particle size was increased according to the increase of M. W. of copolymers.

CE diblock copolymer can form polymeric micelles at aqueous environment at critical concentration due to their amphiphilic properties. The critical micelle concentration of CE diblock copolymer was measured by a fluorescence probe technique using pyrene as a hydrophobic probe at various concentrations as shown in Figure 3. Pyrene will be preferentially partitioned into hydrophobic environment with a concurrent change of the photophysical properties of the molecules.[19,20] It was reported that polymeric micelle of polystyrene (PS)/PEO block copolymers formed in water was confirmed using a fluorescence technique with pyrene as a hydrophobic probe.[19] At this report, the CMC was determined from the fluorescence and excitation spectra, as pyrene partitions between aqueous and micellar environments. Figure 3 shows fluorescence excitation spectra of pyrene

(6.0×10^{-7} M) in the presence CE block copolymer at various concentrations. In the excitation spectrum, a red shift was observed with increasing concentration of CE block copolymer. A red shift of pyrene in the excitation spectrum was observed in the study of micelle formation of PS-PEO block copolymers.[19] The (0,0) bands in the pyrene excitation spectra were examined and compared with the intensity ratio I_{337}/I_{334}. This ratio takes the value characteristic of pyrene, in water at low concentrations and the value of pyrene, entirely in the hydrophobic domain. A plot of I_{337}/I_{334} versus log c is shown in Figure 3(b). A flat region in the low concentration and sigmoidal region in the crossover region was noted and CAC value of 0.0034 g/L was obtained. Furthermore, the increase of PCL block length as a hydrophobic domain induced the decrease of CAC value as shown in Table 1.

Characterization of NFX-Incorporated Polymeric Micelles of CE Diblock Copolymers. Further evidence of core-shell structure and drug loading in the core of the CE polymeric micelle were studied with ^{1}H-NMR in $CDCl_3$ and D_2O as shown in Figure 4. Specific peaks of NFX were recorded as shown in Figure 4(a). In $CDCl_3$, the characteristic peak of the methyl protons of the PCL segment was shown to be about 1.4, 1.65, 2.3 and 4.13 ppm, respectively. The protons of the ethylene glycol of the PEG segment was also shown in 3.6 ~3.7 ppm in that solvent as shown in Figure 4(b). NFX-incorporated polymeric micelle in $CDCl_3$ showed all of the peaks of NFX, PCL block, and PEG block. However, when it was in D_2O, NFX-incorporated polymeric micelle of CE block copolymer showed specific peaks of MPEG at 3.0~4.0 ppm only and peaks of NFX/PCL was disappeared, indicating that CE block copolymers formed core-shell type micellar structure and NFX is incorporated into the inner-core of the polymeric micelle. Since solid phase is not recorded at NMR spectra, hydrophobic inner-core composed of PCL block and NFX present as a solid state at aqueous solution and peaks of them was not appeared at D_2O. These results approved that CE block copolymer can form polymeric micelle in aqueous solution and incorporate hydrophobic drugs in the inner-core. Kwon *et al.* also reported that poly(β-benzyl *L*-aspartate)(PBLA)/PEO diblock copolymer has a rigid PBLA core.[1] In their results, the peaks of 7.4 and 5.2 ppm did not completely disappear and this result suggested that PBLA/PEO diblock copolymer micelles may have a relatively less rigid core compared to our polymeric

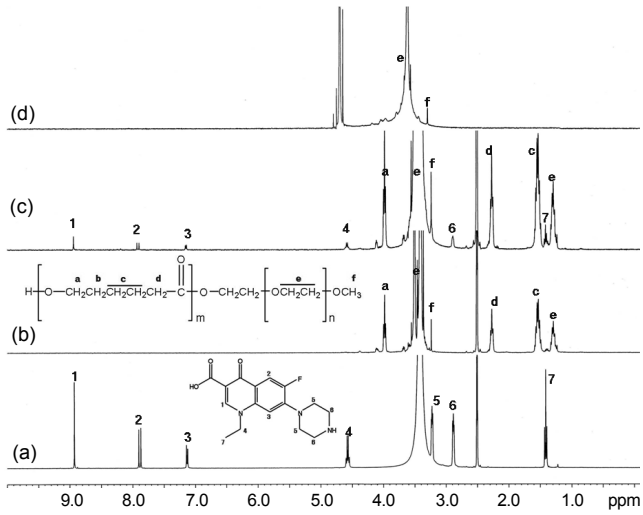


Figure 4. ^{1}H-NMR spectra of polymeric micelles. NFX in DMSO (a); Empty polymeric micelle in DMSO (b); NFX-incorporated polymeric micelle in DMSO (c); NFX-incorporated polymeric micelle in D_2O (d).

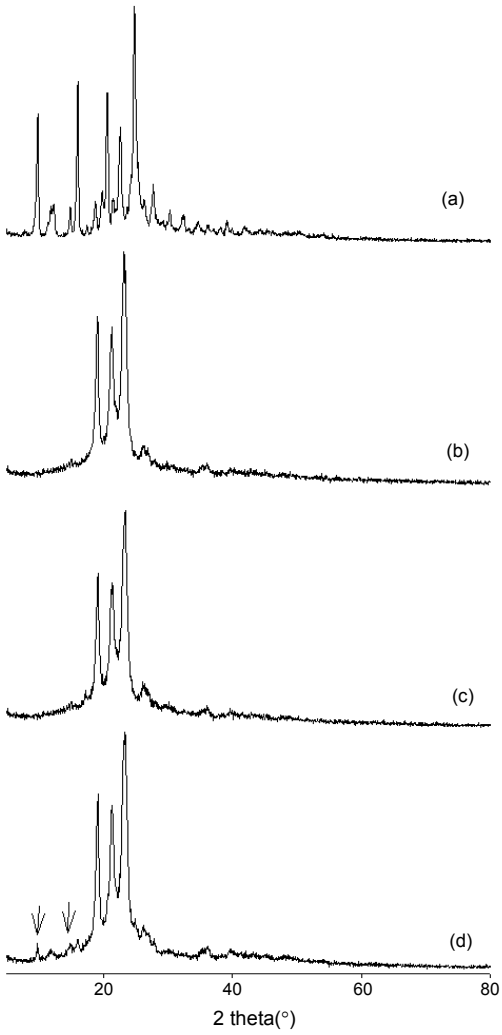


Figure 5. X-ray powder diffraction patterns of polymeric micelles. NFX (a); Empty polymeric micelles (b); NFX-incorporated polymeric micelle (CE-2-2) (c); Empty polymeric micelles/NFX physical mixture (weight ratio of empty polymeric micelle/NFX=9.5/0.5) (d).

micelles.[20]

XRD spectra were recorded to investigate the physicochemical characteristics of NFX-incorporated CE polymeric micelles. Figure 5 shows the XRD scans of CE-2 polymeric micelle with or whithout NFX. NFX showed specific sharp crystalline peaks at XRD measurement as shown in Figure 5(a). Empty polymeric micelle of CE-2 block copolymer revealed specific peaks at 10~40 degree. When NFX was incorporated into CE polymeric micelles, the specific drug crystal peaks were not observed in the XRD patterns (Figure 5(c)) while physical mixture of NFX/empty polymeric micelles showed both of peaks, indicating that drug crystallites exhibited sharp specific crystal peaks when existing as drug crystals but drug existed as molecular dispersion inside the polymeric micelles.[3,4,21] On the other hand, small crystal peaks of drug were observed at the high drug entrapment (Figure 5(d)). The reason of these results was due to that the drug is aggregated inside the polymeric micelle and drug crystals formed in the core of the polymeric micelle.

Drug Release Characteristics from Core-Shell Type Nanoparticles of CE Diblock Copolymer. Drug loading capacity of polymeric micelle was evaluated under various experimental conditions. As shown in Table 2, drug contents and loading efficiency increased with increase of the M. W. of PCL block and feeding amount of NFX. The increase of PCL block length may induce higher hydrophobic interaction with hydrophobic drugs and then loading efficiency should be increased. Also, particle size increased at larger PCL block length. When initial drug concentration increased, the drug contents and particle size was increased.

NFX release from polymeric micelle was studied at *in vitro* as shown in Figure 6. Figure 6(a) showed the effect of drug contents on the NFX release rate from polymeric micelles. As shown in Figure 6(a), increase of drug contents resulted in decreased drug release rate. Increase of drug content might induce aggregation of drugs in the inner-core of the polymeric micelle and this fact may induce delayed release of drug. Delayed release of hydrophobic drugs from polymer nanoparticles or polymeric micelles has been reported by several authors.[1-4,21] Generally, crystallization of hydrophobic drug occurred inside the nanoparticles and, especially, at higher drug loading contents, a phase separation occurs, leading to the crystallization of part of the drug in the nanoparticles. Therefore, crystallized hydrophobic drugs inside the polymeric micelles are released more slowly at higher drug contents while molecular dispersion inside the polymeric micelles at lower drug contents induce faster release rate of drug. NFX was continually released *in vitro* for 2~4 days according to the drug contents. Furthermore, the effect of PCL block length of CE block copolymer resulted in decrease of release rate. Increased drug contents at higher PCL block length must be another reason for decreased release rate. Furthermore, since larger PCL block length induce increased hydrophobic interaction with hydrophobic drug, drug inside the polymeric micelle might has relatively weak hydrophobic interaction and this fact might induce in faster release rate of drug.

Table 2. Drug Loading Characteristics and Particle Size of Core-Shell Type Nanoparticles of CE Diblock Copolymers

	Polymer/ Drug (mg/mg)	Drug contents (%, w/w)	Loading efficiency (%, w/w)	Particle size (nm)		
				Intensity ave.	Weight ave.	Number ave.
CE-1	40/5	5.4	46.0	138.2±36.3	120.9±17.2	109.6±12.9
CE-2-1	40/2	2.9	60.0	132.1±22.4	120.1±20.6	112.0±17.8
CE-2-2	40/5	6.1	52.0	159.8±±40.3	150.7±41.7	141.2±36.2
CE-2-3	40/10	10.7	48.0	182.6±38.2	173.2±30.6	170.9±28.6
CE-3	40/5	7.0	60.0	176.3±±45.8	171.2±38.7	166.0±40.1

Antimicrobial Activity. The resistance of microorganisms against antibiotics is one of the major problems for clinical application.[22,23] It is known that some antibiotics is difficult to uptake by macrophages. Otherwise, some antibiotics is easy to penetrate into cells but inactive in the acidic environment of endosomes. Furthermore, bacteria remained in the cell can relapsed secondary infection. From these point

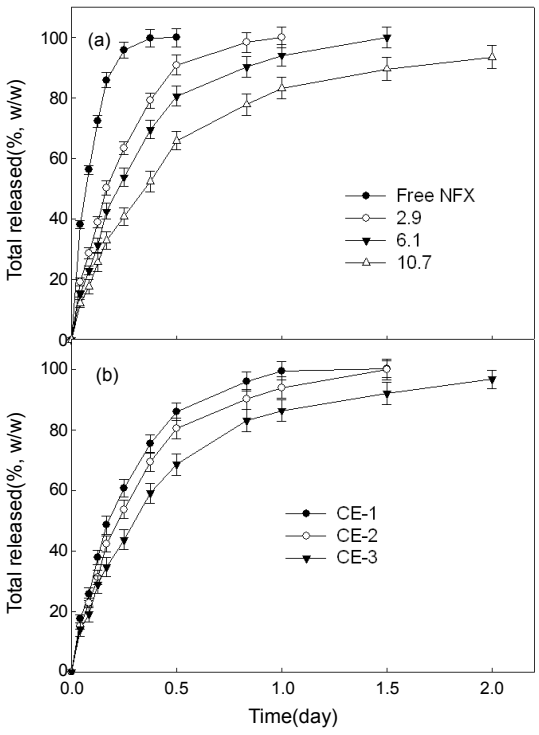


Figure 6. NFX release from polymeric micelle of CE diblock copolymers. The effect of M.W. of diblock copolymer (a); drug contents (b).

Table 3. The Effect of NFX-incorporated Polymeric Micelle of CE Block Copolymer (CE 2-2) on the Bacteria Growth Inhibition

	IC_{50}(μg/mL, n=3)
NFX	0.34±0.01
NFX-polymeric micelle	0.35±0.02
Empty polymeric micelle	24.1±0.2

* Treatment of drug or polymeric micelle was 1 day.

of view, liposomes or nanoparticles is regarded as a ideal solution for this problem.[23,24] Couvreur *et al.*[24] reported that antibiotic-incorporated nanoparticles are effective to treat infected cells by bacteria. They reported that nanoparticles is efficiently endocytosed into the cells, released the drug in the endosomes and then killed the bacteria. Therefore, nanoparticles are known to effective to treat bacterial infection and prevent secondary infection. Antibacterial activity of NFX-incorporated polymeric micelle was evaluated *in vitro* using *E. coli*. Growth inhibition was expressed as IC_{50}. As shown in Table 3, NFX-incorporated polymeric micelle showed almost similar antibacterial activity against *E. coli* bacterial cells compared to free NFX while empty polymeric micelle has significantly higher value of IC_{50}. These results indicated that antibacterial capacity of NFX was not affected from the encapsulation process using block copolymer.

From these results, NFX-incorporated polymeric micelle is considered as good candidate for antibacterial drug delivery system.

Conclusions

NFX-incorporated polymeric micelles composed of CE diblock copolymer were fabricated by solvent evaporation method. Their particle sizes were around 60 to 200 nm according to the polymer M. W. and drug contents. Their CAC was evaluated by fluorescence spectroscopy, i.e. CAC of CE diblock copolymers was decreased according to the increase of hydrophobic PCL block length. ^{1}H-NMR study showed micellar structure of CE diblock copolymers in the aqueous environment, i.e. PCL block composed of hydrophobic inner core of the polymeric micelle and PEG composed of hydrated outershell. XRD results showed that NFX was encapsulated in the core of the polymeric micelle. Drug was released from polymeric micelle for 2~4 days and varied according to the polymer. At antimicrobial activity test, NFX-incorporated polymeric micelle showed almost similar cytotoxicity compared to NFX itself while empty polymeric micelle showed low cytotoxicity.

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