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O-Carboxymethylchitosan-based novel gatifloxacin delivery system

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Abstract

The increase in the treatment efficacy due to the enhanced permeability and retention properties and the decrease in the minimum inhibitory concentration (MIC) are the vital challenges related to the administration of the antibiotic drugs. In the present paper, we describe a novel delivery system of gatifloxacin (GFLX) from *O*-carboxymethylchitosan (OCMCS). GFLX is a fourth-generation fluoroguinolone, which has shown promise with excellent activity against both Gram-positive cocci and Gram-negative bacteria both in vitro and in vivo. OCMCS is a biocompatible amphiphilic derivative of chitosan. GFLX could be entrapped into OCMCS by the interaction between OCMCS and GFLX, which was characterized by fluorescence spectrum, transmission electron microscope, and dynamic light scattering techniques. The release behaviors of GFLX from this proposed delivery system in phosphate-buffered saline (PBS) solution at 37 °C were studied by fluorescence spectroscopy. The MIC of OCMCS formulation was evaluated. The results demonstrate that the release of GFLX from OCMCS formulation is slower than that from GFLX solution. In vitro bacteria antiproliferative activity assay confirms that the MIC of OCMCS formulation against Gram-negative bacteria is fourfold lower than the system without OCMCS. However, it seems that OCMCS has insignificant effect against Gram-positive bacteria. These results suggest that OCMCS matrix has obvious "transmission effect" on Gram-negative bacteria.

Keywords: *O*-Carboxymethylchitosan; Gatifloxacin; The Minimum inhibitory concentration; *Staphylococcus*; *Escherichia coli*

1. Introduction

Recently, a number of novel delivery systems have been developed including the entrapment of drugs, proteins, or genes in small vesicles or within polymeric matrices (Stolnik, Illum, & Davis, 1995). Colloid delivery system is one of the most promising delivery systems because it may reduce the unnecessary toxic side effect and improve the therapeutic effect (Kreuter, 1994). Colloid delivery systems include nanoparticles, liposomes, microemulsions, polymeric self-assemblies, and so on. Among them, the nanoparticles assembled from amphiphilic synthesized copolymers (Bae, Huh, Kim, & Park, 2000; Discher & Eisenberg, 2002; Gan et al., 1999; Ge, Hu, & Jiang, 2002; Rosler, Vandermeulen, & Klok, 2001) and the self-aggregates of natural polymers have been widely applied to the field of biotechnology and pharmaceutics (Lee & Jo, 1998; Lee, Jo, Kwon, Kim, & Jeong, 1998a, Lee, Kwon, Kim, Jo, & Jeong, 1998b; Shantha & Harding, 2002).

Chitosan is the second most plentiful biomass and is already known as a biocompatible, biodegradable, and non-toxic natural polymer (Dureja, Tiwary, & Gupta, 2001). It has been found a number of applications in drug delivery including that of adsorption enhancer of hydrophilic macromolecular drugs (Artursson, Lindmark, Davis, & Illum, 1994). When protonated (pH < 6.5), it is able to increase the paracellular permeability of peptide drugs across mucosal epithelia (Senel et al., 2000). To overcome chitosan's limited solubility and effectiveness as absorption enhancer at neutral pH values such as those found in the intestinal tract, chitosan derivatives have been developed. For example, *N*-carboxymethylated chitosan was found to

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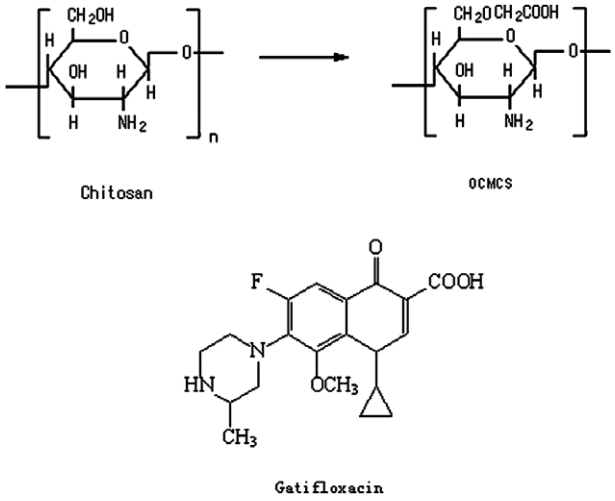
increase the permeation and adsorption of low-molecular-weight heparin, an anionic polysaccharide across intestinal epithelia. *O*-Carboxymethylated chitosan (OCMCS) is another kind of water-soluble chitosan derivative, with its *O*-hydroxyl group of each monomer substituted by a carboxymethylic group through the ether-bond formation (Scheme 1), and has been proved to have biocompatibility (Cai, Yao, Li, Yang, & Li, 2001; Zhu, Zhang, & Zhang, 2004) as well as the inducing dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), the main component of cell membrane, vesicles fusion (Zhu, Chan-Park, Dai, & Li, 2005; Zhu, Fang, Chan-Park, & Chan, 2005). These novel properties of OCMCS motivate our great interests to further investigate the OCMCS as a matrix for antibiotic drugs.

It is well known that the second-generation fluoroquinolones such as ciprofloxacin and ofloxacin have been found widespread acceptance for the treatment of bacteria infections. Their excellent activities against most of the frequently encountered Gram-positive and -negative pathogens have made these drugs the initial choice for the treatment of bacteria keratitis (Leibowitz, 1991; O'Brien, Maguire, Fink, Alfonso, & McDonnell, 1995). Unfortunately, their widespread use has led to the emergence of resistance in many bacteria species (Chaudhry et al., 1999; Goldstein, Kowalski, & Gordon, 1999; Jones, Beach, Pfaffer, & Doem, 1998). Newer fluoroquinolones have been recently introduced to overcome this problem.

GFLX, a fourth-generation fluoroguinolone, has shown promise with excellent in vitro activity against most pathogens responsible for bacteria infections (Kaliamurthy, Jesudasan, Geraldine, Kalavathy, & Thomas, 2005; Kowalski et al., 2003; Mather, Karenchak, Romanowski, & Kowalski, 2002). GFLX had a significantly better action against Gram-positive cocci both in vitro and in vivo when compared with ciprofloxacin. In view of these organisms being the leading cause of keratitis worldwide, GFLX may be a preferred alternative to ciprofloxacin as the first-line monotherapy in bacteria keratitis (Parmar et al., 2006).



Scheme 1. Chemical structures of OCMCS and GFLX.

Kaliamurthy et al. (2005) found that the MIC of GFLX for all Gram-positive cocci was significantly lower than for all Gram-negative bacteria. They also found that GFLX had the lower MIC against streptococcus pneumoniae. Parmar et al. (2006) found that pneumococcal ulcers responded much better to gatifloxacin as compared with ciprofloxacin (88.9% healing rate in GAT group compared with 50% in the CIP group; $p = .007$). However, GFLX demonstrated a similar activity to ciprofloxacin in vitro against Gram-negative bacteria.

The newer generation of fluoroquinolones is likely to play an important role in the treatment of bacteria keratitis in the future because of an increasing resistance to the second-generation fluoroquinolones that are in current use. In vitro studies on isolates from bacteria infections of the eye have shown an encouraging response to gatifloxacin, including those organisms resistant to ciprofloxacin (Mather et al., 2002; Tungsiripat, Sarayba, & Kaufman, 2003). However, these results have not yet been validated by clinical trials on human eyes. In vitro results may not always coincide with clinical response because the latter depends on tissue penetration of the antibiotic and the host response to infection (Parmar et al., 2006).

To enhance the penetration of the antibiotic, local drug delivery systems consisting of biocompatible and biopermeable OCMCS was developed in this study. Hitherto, there have been no published studies on GFLX delivery system. Our results demonstrate that OCMCS and GFLX can form nanoparticles. The MIC of OCMCS formulations was significantly low against Gram-negative bacteria (*Escherichia coli*) but similar against Gram-positive cocci (*Staphylococcus*) in comparison with that of GFLX solution. This finding is important because this delivery system could enhance the treatment effect of GFLX against Gram-negative bacteria. As a result, it will broaden the applications of GFLX to the Gram-negative bacteria.

2. Experimental

2.1. Materials

Chitosan powder (average molecular weight, 6.7×10^5 g/mol; degree of deacetylation, 90%) was obtained from Lianyungang Biologicals, Inc. (China). GFLX was purchased from Sigma–Aldrich Company. The other materials were of commercial grade and were used without further purification.

2.2. Synthesis of OCMCS

The synthesis of OCMCS was according to our previous report (Zhu et al., 2005), in detail: 2 g chitosan was immersed into 25 ml 50 wt% NaOH solution for 24 h in order to swell and alkalize the polymer. The alkalized chitosan was crushed into a filtration cake and then transferred into a flask. Monochloroacetic acid (5 g) was dissolved in isopropanol (25 ml), and added drop-by-drop into the

alkalized chitosan inside the flask for 20 min. The reaction was carried for 8 h at room temperature. At the end of reaction, the mixture was filtered to remove the solvent. The filtrate was dissolved in 100 ml water, and then 2.5 M HCl was added to adjust the pH to 7. With centrifugation of the above solution for removal of the precipitate, and then addition of 150 ml anhydrous ethanol to the super-solution, the product precipitated from the solution. The solid was filtered and rinsed by anhydrous ethanol for three times and vacuum-dried under room temperature. The molar fraction of carboxymethylated groups and amino groups on OCMCS molecules was found to be about 100 and 75%, respectively. The molecular structure of OCMCS was shown in Scheme 1.

2.3. Loading GFLX into of OCMCS

Mixed OCMCS and GFLX stock solutions by stirred in a desired molar ratio of OCMCS to GFLX for at least 24 h. The obtained transparent solution was centrifuged at 16,000 rpm for 30 min. The supernatant, which contained uncapsulated GFLX and OCMCS matrix, was discard and replaced with deionized distilled water. The particulate sediment was re-dispersed with the aid of an ultrasonic bath and then for the following characterization and experiments.

2.4. Characterizations

2.4.1. Fluorescence spectroscopy

Stock solution of 5 mg/ml OCMCS in water was prepared under gentle stirring for at least 10 h. To obtain samples for fluorescence spectroscopy, aliquots of OCMCS stock solution were mixed with aliquots of a 10^{-3} M of GFLX aqueous solution and stored at rest for 24 h. The fluorescence spectra were measured by Shimadzu RF 5301 spectrometer equipped with a quartz-fluorescence cell at room temperature. All measurements were made at the front surfaces of the samples at 45° position with an excitation wavelength of 330 nm and the slit widths for excitation and emission were both kept at 1.5 mm. The emission intensities at 484 nm were recorded for all the solutions measured.

2.4.2. Transmission electron microscopy (TEM)

TEM was used to observe the morphology of the OCMCS/GFLX delivery system. The specimens were prepared by dropping the sample solution onto a copper grid. The grid was held horizontally for 20 s to allow the molecular aggregates to settle and then at 45° to allow excess fluid to drain for 10 s. The grid was returned to the horizontal position, and one drop of 2% phosphotungstic acid was added to give a negative stain. The grid was then allowed to stand for 30 s to 1 min before excess staining solution was removed by draining as above. The specimens were air-dried and examined using a (TE CHAI-12) (Philips) transmission electron microscope at an accelerating voltage of 80.

2.4.3. Dynamic laser light scattering (DLS)

DLS was carried out using a Brookhaven Zetaplus laser light scattering system. The light source is a He–Ne laser with a wavelength of 671 nm. Cumulant was used to analyze the time correlation function and to obtain the particle size. Before the light scattering experiments, all the sample solutions were filtered using a 0.45 μm syringe filter to remove dust.

2.5. In vitro release study

The vitro release study was conducted in the way reported by Xiong, Tam, and Gan (2005), briefly: 10 ml of GFLX solution and OCMCS formulation were put in the dialysis membrane (molecular weight cut-off 12,000–14,000 Da). The sealed dialysis membrane placed in phosphate-buffered saline (PBS) (PBS, pH 7.4, 0.01 M) aqueous solution (250 ml). The whole solution was then placed in a shaking water bath at 37 °C for the drug release study. At predetermined time intervals released GFLX solution (3 ml) outside the dialysis membrane was withdrawn and measured at wavelength of 484 nm by fluorescence spectrophotometer to determine the concentration of GFLX. Fresh PBS solution (3 ml) was added to replenish the sample that was removed in order to maintain a constant volume. The error in the concentration profile attributed to this method of dilution is around 3%. The drug release experiments were performed in triplicate.

2.6. In vitro bacteria antiproliferative activity

The *E. coli* and *Staphylococcus* bacteria were grown in Luria–Bertani (LB) medium at 37 °C under a 5% CO_2 containing atmosphere. Approximately, 6.62×10^6 *E. coli*/ml and 3.33×10^6 *Staphylococcus* bacteria/ml were seeded on 24-well plates. Thereafter, bacteria was incubated for 12 h in OCMCS solution, drug solution, and OCMCS containing drug solution, respectively, while keeping equivalent concentrations of either drug or excipient. All solutions or OCMCS formulations were containing 0.015–40 mg/ml drug, blank OCMCS were prepared with equivalent masses of excipients compared to drug-loaded OCMCS. The cell concentration in the liquid culture was determined by the absorbance at 630 nm (Shimadzu UV-160 spectrophotometer), and agar plate counts were used to calibrate the absorbance. All measurements were carried out in triplicate.

3. Results and discussion

Because OCMCS is a kind of amphiphilic chitosan derivative, it can form aggregates in aqueous solution mainly driven by the intermolecular hydrogen bond and hydrophobic interaction, with a critical aggregation concentration (cac) of ~0.05 mg/ml (Zhu et al., 2005). The glucose backbones and hydrophobic groups, such as acetyl, form the hydrophobic domains, and the dissociated

carboxylic groups as well as the hydrophilic groups, such as hydroxyl and amino groups, constitute the hydrophilic ones (Zhu et al., 2005). This unique structure of OCMCS aggregate and its fusion function of DPPC vesicles (Zhu et al., 2005) motivate our interest to utilize OCMCS as the matrix to load GFLX.

3.1. Static fluorescence spectrum

Scheme 1 shows the molecular structure of OCMCS and GFLX. From Scheme 1, it can be seen that the glucose backbones and acetyl groups of OCMCS are hydrophobic, and the dissociated carboxylic groups as well as the hydroxyl, amino, and carboxyl groups are the hydrophilic groups. Therefore, there should produce hydrophobic interactions and H-bond between OCMCS chains and GFLX molecules. Fig. 1 shows the static fluorescence spectra of GFLX as a function of OCMCS concentration. As can be seen from Fig. 1, the fluorescence intensity is strongly dependant on the OCMCS concentration. In 0.025 mg/ml of OCMCS solution, the peak intensity becomes weaker, while it becomes stronger in higher concentration of OCMCS solution (0.075 mg/ml) compared with that of GFLX solution with equivalent drug concentration, while the peak does not shift. However, the peak shifts bluish obviously in high concentration of 5 mg/ml OCMCS.

When the OCMCS concentration is as low as 0.025 mg/ml, which is low than its cac (0.05 mg/ml), OCMCS mainly shows hydrophilic property, which changes the microenvironment of GFLX molecules in solution, and thus resulting in the decrease of peak intensity. When the OCMCS is 0.075 mg/ml, which is higher than its cac, the hydrophobic domain forms, hydrophobic GFLX molecules are easy to enter into the hydrophobic domain of OCMCS aggregates. Thus, the peak intensity increases. When the OCMCS increases to 5 mg/ml, which is much higher than its cac. The peak shifts bluely, which may suggest that there are complex formed between OCMCS chains and GFLX molecules, driven by hydrophobic interaction and H-bond association.

Figs. 2a and b compares the fluorescence spectra of 5 and 50 μM GFLX in 5 mg/ml of OCMCS solution and distilled water, respectively. Fig. 2a shows that the peak not only has blue shift, but also its intensity increases obviously compared to equivalent drug concentration in distilled water. This result is due to the low concentration of GFLX inclines to enter into the hydrophobic domain of OCMCS aggregates, and at the same time, there are some interactions produced between OCMCS chains and GFLX molecules due to high OCMCS concentration utilized. Fig. 2b shows that the peak also has blue shift, however its intensity decreases. This result suggests that there are complexes formed at high ratio of GFLX to OCMCS (Fig. 2b) driven by strong interactions between OCMCS chains and GFLX molecules in comparison with that case of low ratio of GFLX to OCMCS (Fig. 2a). These results indicate that the fluorescence spectrum of GFLX is dependant not only on OCMCS concentration, but also on the ratio of GFLX to OCMCS.

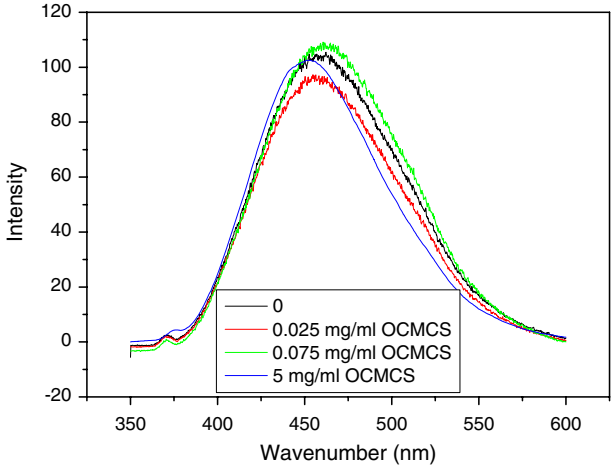


Fig. 1. The OCMCS concentration dependence of GFLX fluorescence emission spectra at 25 °C, where the GFLX concentration was set to 3×10^{-6} M.

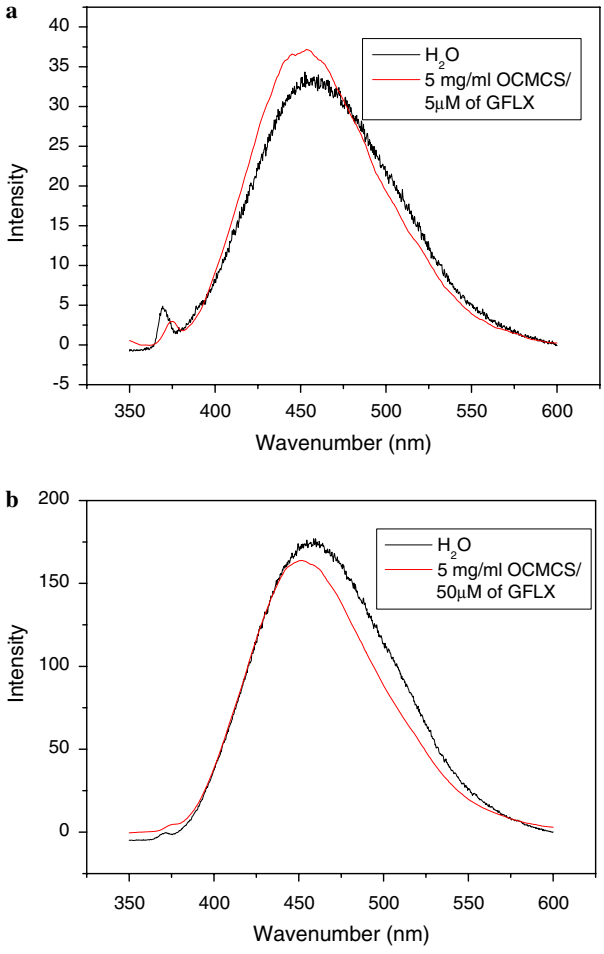


Fig. 2. The GFLX fluorescence emission spectra by varying the ratio of GFLX to OCMCS at 25 °C.

3.2. The morphology of delivery system of GFLX-loaded OCMCS

From the fluorescence spectrum analysis, it can be known that the complex of GFLX and OCMCS can be formed at high concentration of OCMCS solution and high ratio of OCMCS to GFLX. Transmission electron microscopy (TEM) technique is selected to observe the morphology of GFLX-loaded OCMCS formulation. Fig. 3 shows the morphology of OCMCS formulation, OCMCS and GFLX concentration used were 5 mg/ml and 10^{-4} M, respectively. OCMCS aggregates formed in distilled water showed very swollen microgel morphology characterized by AFM, and cannot be measured by DLS or TEM (Zhu et al., 2005). Fig. 3 shows the nanosphere morphology with 30–70 nm in diameter, which suggesting that the complex formed between OCMCS and GFLX. Fig. 3b is the magnification of Fig. 3a. From this result, it can be concluded that it is the hydrophobic GFLX molecules that promote OCMCS to assemble into relative compact nanospheres. As a result, GFLX molecules can be entrapped into OCMCS matrix. This result is consistent with the result of fluorescence spectrum analysis.

From the protocol of entrapment of GFLX into OCMCS, it can be known that the loading of GFLX is very simple without the need of high temperature, organic solvent, surfactant, and some other special experimental technology in comparison to that of other technologies of loading drugs (Andersson & Löfroth, 2003; Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997; Genta, Costantini, Asti, Conti, & Montanari, 1998; Hu et al., 2002; Onishi, Shimoda, & Machida, 1996).

DLS was used to measure the average diameter and nanoparticle distribution of OCMCS/GFLX release system. Fig. 4 shows the DLS of OCMCS/GFLX complex. The average diameter of OCMCS/GFLX complex is determined to be 61 nm, which is larger than that measured from TEM. This is reasonable because the diameter determined using DLS is the hydrodynamic radius, while TEM measured is the drying state. The particle distribution shows a monomodal particle size distribution.

3.3. The release of OCMCS/GFLX delivery system

The release kinetics of a drug from colloid release system is of scientific significance and applied importance. The fluorescence standard of GFLX in OCMCS solutions is shown in Fig. 5. From Fig. 5, the release percentage can be calculated. The release profiles are shown in Fig. 6, in which an initial burst release was observed for both GFLX solution and OCMCS formulations. The model-independent parameters were obtained from individual experimental curves and summarized in Table 1 where $t_{50\%}$, t_{eq}, and F_{eq} represent percentage release after the time at which 50% amount of the drug was released, time to achieve equilibrium, and the fraction of GFLX released at equilibrium, respectively. From Table 1, it can be seen that $t_{50\%}$ and t_{eq} of GFLX released from OCMCS matrix prolong, while F_{eq} decreases in comparison in that from GFLX solution,

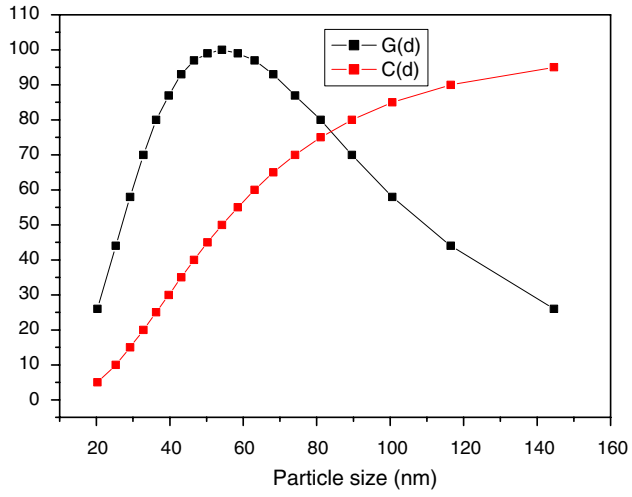


Fig. 4. Particle size distribution of OCMCS/GFLX complex from dynamic light scattering.

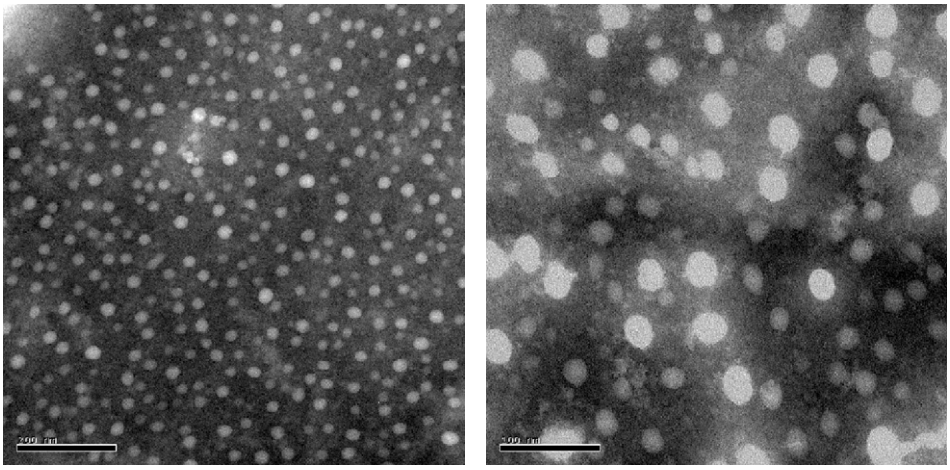


Fig. 3. TEM morphologies of OCMCS/GFLX complexes.

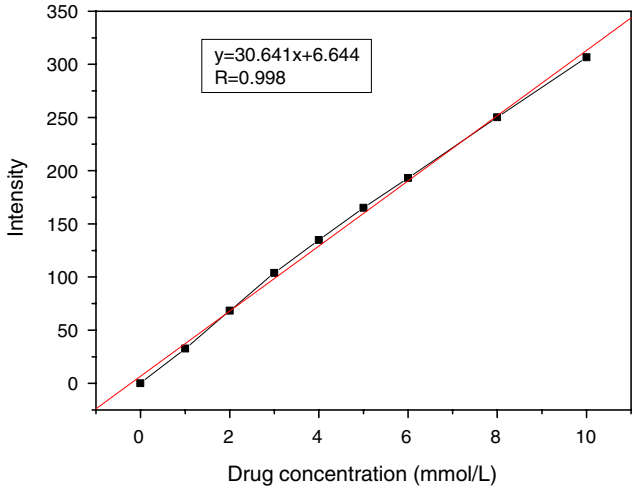


Fig. 5. GFLX concentration dependence of the fluorescence emission intensity as determined at 25 °C.

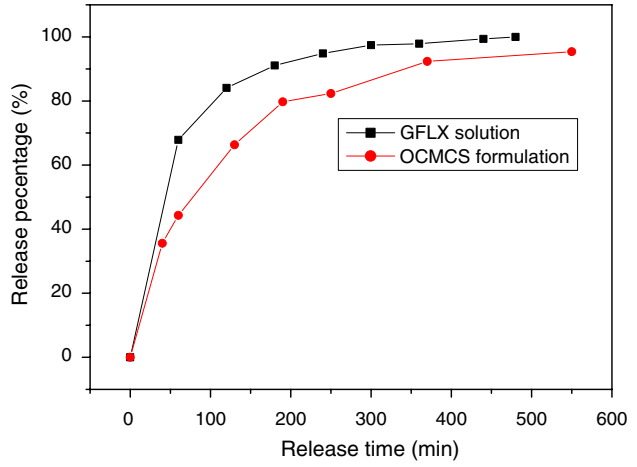


Fig. 6. The release profiles from GFLX solutions with and without OCMCS in PBS (pH 7.4, 0.01 M) at 37 °C.

Table 1
Release parameters of GFLX

GFLX	$t_{50\%}$ (min)	t_{eq} (min)	F_{eq} (%)
Water	42	300	99.5
OCMCS formulation	78	368	94.8

which is due to the interactions between OCMCS chains and GFLX molecules. However, the sustained release behavior of present delivery system is not as significant as the delivery system of lipophilic camptothecin (CPT) entrapped into OCMCS matrix (Zhu, Liu, & Ye, 2006), which is due to the different chemical structure of loaded drug into OCMCS matrix.

3.4. In vitro bacteria antiproliferative activity

Kaliamurthy et al. (2005) found that the MIC of GFLX for all Gram-positive cocci was significantly lower than for

Table 2
The effect of OCMCS concentration on the activity for *E. coli* and *Staphylococcus*

	OCMCS (mg/ml)									
	5	0.5	0.25	0.125	0.0625	0.031	0.0156	0.0078	0.0039	0.0019
Sta	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	+	+	+	+	+

Table 3
The effect of GFLX concentration on the activity for *E. coli* and *Staphylococcus*

	GFLX (mg/ml)									
	40	4	2	1	0.5	0.25	0.125	0.0625	0.0312	0.0156
Sta	-	-	-	-	-	+	+	+	+	+
E. coli	-	-	-	-	-	+	+	+	+	+

Table 4
The effect of GFLX/OCMCS concentration on the activity for *E. coli* and *Staphylococcus*

GFLX	38.4	3.84	1.92	0.96	0.48	0.24	0.12	0.06	0.03	0.015
OCMCS	5	0.5	0.25	0.125	0.0625	0.031	0.0156	0.0078	0.0039	0.0019
Sta	-	-	-	-	-	+	+	+	+	+
E. coli	-	-	-	-	-	-	-	+	+	+

all Gram-negative bacteria. They also found that gatifloxacin had the lower MIC against *Streptococcus pneumoniae*. Parmar et al. (2006) found that pneumococcal ulcers responded much better to GFLX as compared with ciprofloxacin (88.9% healing rate in GAT group compared with 50% in the CIP group; $p = .007$). However, GFLX demonstrated a similar activity to ciprofloxacin in vitro against Gram-negative bacteria. From the previous studies, it can be known that, GFLX, a fourth-generation fluoroguinolone, has shown promise with excellent in vitro activity against Gram-positive bacteria.

In this study, *E. coli* and *Staphylococcus* were selected as the candidates of Gram-negative bacteria and Gram-positive cocci, respectively. The antiproliferative activity of *E. coli* and *Staphylococcus* dependence on OCMCS concentration, GFLX concentration, and the GFLX concentration in OCMCS formulations is shown in Tables 2–4. Table 2 indicates that OCMCS has no antibacterial activity against *E. coli* and *Staphylococcus*. Table 3 reveals that both the MIC of gatifloxacin for *E. coli* and *Staphylococcus* are determined to be 0.5 mg/ml under the experimental conditions. However, Table 4 indicates that the MIC of OCMCS formulation against Gram-negative bacteria is 0.12 mg/ml, which is fourfold lower than the system without OCMCS. At the same time, it seems that OCMCS has insignificant effect against Gram-positive bacteria, where the MIC is 0.48 mg/ml ($p = .007$). These results suggest that OCMCS matrix can favor GFLX to penetrate into Gram-negative bacteria and has obvious "transmission effect" on Gram-negative bacteria. However, it has almost no effect on Gram-negative bacteria. These results are due to the different biological structure for Gram-positive and -negative bacteria. Previous studies demonstrate GFLX shows

advantages against Gram-positive bacteria, and they believed that GFLX has shown promise with excellent in vitro activity against Gram-positive bacteria. Our results demonstrate that GFLX delivery system of OCMCS formulation shows the excellent effect in vitro activity against Gram-negative bacteria. Therefore, the present novel delivery system can broaden the applications of GFLX to Gram-negative bacteria.

4. Conclusions

In summary, a novel delivery system of OCMCS entrapment of GFLX was developed. GFLX could encapsulated into OCMCS driven by H-bond and hydrophobic interaction between OCMCS chains and GFLX molecules. OCMCS/GFLX complex shows compact and sphere morphology with 30–70 nm in diameter. The MIC of OCMCS formulation against Gram-negative bacteria is fourfold lower than that of GFLX solution. However, the MIC of OCMCS formulation against Gram-positive bacteria is similar to that of GFLX solution. OCMCS matrix has obvious "transmission effect" on Gram-negative bacteria. The finding is possible to broaden the applications of GFLX to Gram-negative bacteria.

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