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Ciprofloxacin surf-plexes in sub-micron emulsions: A novel approach to improve payload efficiency and antimicrobial efficacy☆

Vikas Jain[a], Deepak Singodia[a], Girish K. Gupta[a], Durga Garg[a], G.B. Shiva Keshava[b], Rahul Shukla[a], Praveen K. Shukla[b], Prabhat R. Mishra[a,*]

[a] Pharmaceutics Division, Central Drug Research Institute (CSIR), Lucknow, India
[b] Fermentation Technology Division, Central Drug Research Institute (CSIR), Lucknow, India

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ABSTRACT

The aim of this study was to investigate antimicrobial efficacy and pharmacokinetic profile of ciprofloxacin (CFn) loaded oil-in-water (o/w) submicron emulsion (SE-CFn). This study emphasized on development of hydrophobic ion-pair complexes of CFn with sodium deoxycholate (SDC) [CFn–SDC], which was incorporated in the core of SE (SE-CFn–SDC). SE-CFn–SDC was characterized for globulet size (278 ± 12 nm), zeta potential (-25.3 ± 1 mV), viscosity (2.6 ± 0.3 cP), transmission electron microscopy (TEM), drug entrapment and for *in vitro* release profile. The entrapment efficiency (EE) was significantly improved ($\geq$80%; $p \leq 0.05$) on ion-pairing while it was merely 27.2 ± 3.1% for free CFn. The cytotoxicity studies of formulations on J774 macrophage cells showed that more than 90 ± 3% of cells were viable, even at high concentration (100 μg/ml). SE-CFn–SDC was further modified with cationic inducer chitosan (SE-CH–CFn–SDC), which showed almost twofold and fourfold enhancement in antimicrobial efficacy as compared to SE-CFn-SDC and SE-CFn, respectively when tested *in vitro* against *E. coli*, *S. aureus*, and *P. aeruginosa*. When tested in male Balb/c mice, the $AUC_{0-24\,h}$ of SE-CH–CFn–SDC (23.27 ± 2.8 h μg/ml) was found to be 1.7-fold and 5-fold higher as compared to SE-CFn-SDC (13.17 ± 0.88 h μg/ml) and CFn solution (4.70 ± 0.77 h μg/ml), respectively. The study demonstrates that surfactant based ionic complex formation incorporated in surface modified submicron emulsion is a promising approach to improve payload efficiency of poorly water soluble drugs with improved antimicrobial efficacy and pharmacokinetic profile.

1. Introduction

To deliver a therapeutic molecule through colloidal carrier in order to achieve optimum therapeutic efficacy remains a challenge for pharmaceutical scientists. CFn, a powerful broad-spectrum antibiotic is useful in the treatment of several types of infections (Blondeau, 2004). It has been reported that CFn possess an efficacy against Gram-negative as well as Gram-positive pathogens, superior to other antibiotics tested (Egger et al., 2001). Moreover, the frequency of spontaneous resistance to CFn is very low (Hwang, 2004). Delivery systems like hydrogel (Cho et al., 2003) polyelectrolyte ultrathin capsules (Bhadra et al., 2004), microspheres (Mao et al., 2005), nanoparticles (Dillen et al., 2006), and liposomes (Ditizio et al., 2002) have been developed and used parenterally or topically for prolonged drug delivery especially for ocular infection. Unfortunately, they were limited in their use by low encapsulation efficiency, rapid leakage and poor storage stability. These formulation-related problems of CFn mainly result in poor bioavailability through variety of routes (Charoo et al., 2003; Ke et al., 2001).

Ciprofloxacin surf-plexes (surfactant complex with CFn) have been prepared to improve loading efficiency into submicron emulsion (SE). It has been reported that CFn is an amphoteric quinolone antibiotic having an acidic pK_a at 6.0 and a basic pK_a at 8.8 (BioLiterature Inc., 1988) and its solubility or retention in oil (lipidic) phase is very low resulting in low entrapment efficiency (not >50%). This could be anticipated to variable trapping efficiencies, depending on physico-chemical characteristics such as the pK_a, lipophilicity, and mass of the molecule being encapsulated (Webb et al., 1988). One approach to improved retention of charged drug in oil phase is by ion pair formation (Lee, 1990) which increases the lipophilicity of charged drug molecule, both *in vitro* and *in vivo* by shielding their charges with counter ions (Pardo and Cohen, 1992). The proposed loading technique described for CFn could be advantageous from several perspectives. First, the technique increases the drug/lipid ratio due to improvement in lipophilicity, thereby allowing SE to carry and deliver larger amounts of antibiotics to infected cells. Second, it minimizes the

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* Corresponding author at: Pharmaceutics Division, Central Drug Research Institute (CDRI), Chattar Manzil Palace, Lucknow 226001, India.
Tel.: +91 522 2212411–18x4458; fax: +91 522 2223938/522 2223405.
E-mail addresses: mishrapr@hotmail.com, prabhatcdri@gmail.com (P.R. Mishra).

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potential loss of expensive drugs during the formulation processes. Third, by loading CFn ionic complex into SE the retention capacity has been dramatically improved. Many authors have reported use of ion pairs for enhanced or improved bioavailability of drug (Abdulrazik et al., 2001). Timolol bioavailability enhanced, when it was ion paired with sorbic acid (Higashiyama et al., 2004). Further, many studies have shown advantages of imparting cationic surface to SE which not only improves drug retention at infection site but also results in enhanced permeability of drug (Rabinovich-guilatt et al., 2004).

The proposed work has been taken up considering two tier objectives. The first is to prepare surf-plexes of CFn with sodium deoxycholate (SDC) to improve the loading efficiency of CFn within submicron emulsion and secondly to improve the antimicrobial efficacy of formulations by imparting cationic charge on the surface, which can be used as potential drug delivery vehicle for topical and parenteral applications.

2. Materials and methods

Soya-oil, lecithin, poloxamer 188 (Pluronic F68), chitosan, SDC, MTT, Dulbecco's modified eagle medium (DMEM) with glutamate, fetal bovine serum (FBS), antibiotic solution (penicillin/streptomycin, 0.1%, v/v) and Trypan blue solution were purchased from Sigma (MO, USA). All other ingredients were of pharmaceutical grade. CFn was generously provided by Alkem Laboratories (Mumbai, India) as a gift sample.

2.1. Preparation and optimization of ionic complexes of CFn with SDC (CFn–SDC)

The CFn–SDC was prepared by dissolving appropriate quantities of surfactant in deionised water and gradually adding it to CFn solution. The formation of ionic complex was optimized by preparing at different pH and taking different molar ratio of both the components.

2.1.1. Effect of pH

CFn in equimolar concentration was added dropwise in SDC solution at pH 3.5, 7.4 and 9.2. CFn-ionic complex formation was indicated by precipitation in aqueous phase. The tubes were centrifuged and amount of drug left uncomplexed in supernatant was measured using RP-HPLC at 277 nm as reported by Ellbogen et al. (2003) with slight modification.

2.1.2. Effect of molar ratio

The various concentration of SDC (1.03–20.70 mg/ml) were added in CFn solution at pH 3.5, keeping SDC/CFn molar ratio in the range of 0.5–10 M. The formation of water insoluble ion paired complex between CFn and SDC at different molar ratio was traced by monitoring the transmittance of solution at 500 nm using spectrophotometer (UV-1701, Shimadzu, Kyoto, Japan).

2.2. Characterization of CFn–SDC ionic complex

Following optimization the ionic complex of CFn and surfactants SDC was prepared at pH 3.5 using 1:1 molar ratio. The ionic complex prepared was characterized to ascertain the formation of complexes.

2.2.1. Mass spectra

Fast atomic bombardment (FAB) was also used to confirm the formation of complexes by recording mass spectra on FABMS (JMS SX-102) Jeol, Tokyo, Japan Mass spectrometer.

2.2.2. NMR spectra

^{1}H NMR spectra were taken on Bruker DRX-300 (Bruker, Fallanden, Switzerland) at 300 MHz by dissolving ionic complex in $CDCl_3$.

2.2.3. Partition coefficient

CDn–SDC complex was added in equal volume of aqueous/chloroform mixture in beaker with stirring for 6 h and then kept in separating funnel overnight for partitioning. After complete partition, the mixture was separated into two phases (layers), and CFn was measured using RP-HPLC.

2.3. Preparation of lipid based SEs

Different submicron lipid emulsions containing CFn (SE-CFn), ionic complex of CFn with SDC (SE-CFn–SDC) were prepared following standard procedure with minor modifications (Zurowska-Pryczkowska et al., 1999). The lipid phase was prepared by heating soya oil at 70 °C containing lecithin. The CFn–SDC (equivalent to 0.3% CFn) was added to the lipid mixture mentioned above after dissolving in chloroform. This lipid phase was added gradually to aqueous phase containing pluronic F-68 at 70 °C and magnetically stirred. The primary emulsion was prepared by stirring for 20 min using high shear mixer (Ultra Turrax T25, Janke & Kunkel, Germany) at the speed of 22,000 rpm, and subsequent emulsification was accomplished by sonication using ultrasonic probe at 20% amplitude for 5 min. To develop chitosan (CH) based cationic submicron lipid emulsion (SE-CH–CFn–SDC), the aqueous phase was prepared by dispersing CH in 5% aqueous solution of sorbitol to enable adjustment to isotonicity and an equal amount of a 2% solution of lactic acid. The pH of the resulting solution was adjusted to 6.0 to avoid any flocculation of chitosan. The final emulsion was prepared by same procedure as explained above.

2.4. Physicochemical characterization

The globule size and zeta potential were measured with the Malvern Zetasizer (Malvern, UK) while its pH was recorded at given time intervals using a pH meter (Corning pH meter 245, USA). The viscosity of sample was measured using cone and plate viscometer (Bohlin, USA). Each emulsion sample was diluted in water to an appropriate concentration before measurement at room temperature.

2.4.1. TEM

TEM was performed using negative staining with sodium phosphotungstate solution (0.2%, w/v) [JEM-1200 EX, JEOL, Tokyo, Japan].

2.4.2. Drug entrapment

The CFn loaded emulsions were centrifuged at 48,000 × *g* and 4 °C for 30 min in a Beckman Optima MAX® ultracentrifuge (Beckman Coulter, USA) in order to separate the incorporated drug from the non-incorporated drug. The supernatants were analyzed by RP-HPLC for the un-incorporated drug (A1) concentration to determine the encapsulation percentage from total amount of drug (A2). EE was calculated using the equation: $EE\ (\%) = (A2 - A1/A2) \times 100$.

2.4.3. Drug analysis

CFn concentrations in the samples were measured by reported RP-HPLC method (Samanidou et al., 2003) with slight modification. A Shimadzu HPLC (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) with an attached UV/visible detector and a stainless steel C_{18} column (2.5 mm × 4.6 mm) was used. The mobile phase consisted of a mixture of acetonitrile:0.05 mM triethylamine dibasic buffer adjusted to pH 3.5 with phosphoric acid (55:45, v/v) and the

flow rate was 1 ml/min. The injection volume was 20 μl and the detection was made at 277 nm.

2.4.4. Stability assessment

The drug content, pH and droplet size distribution were monitored over periods of time stored at 4 °C and 37 °C. The creaming and the phase separation were assessed visually at given time intervals. All other visible changes were recorded. To evaluate its mechanical and physical resistance, the emulsion was subjected to an accelerated mechanical stress and its globule size distribution was measured before and after shaking at 100 strokes per min over 48 h at room temperature.

2.4.5. In vitro drug release

In vitro release studies were carried out using bulk equilibrium reverse dialysis bag technique (Chidambaram and Burgess, 1999) at 37 °C, as previously described. A volume of 25 ml of the different SE containing CFn and its ionic complex was directly placed into 400 ml of a stirred sink solution (PBS 0.1 M, pH 7.4) where numerous dialysis bags (12Kd, Sigma, USA) containing 1 ml of the same sink solution had been previously immersed. The dialysis bags were equilibrated with the sink solution for few hours prior to experiment. At given time intervals, a dialysis bags was withdrawn from the stirred release solution and the content of the dialysis bag was assayed for CFn.

2.4.6. Cell viability studies

This was carried out using MTT assay on macrophages cell line J744. Briefly 100 μl of J774 macrophage cell suspension (containing usually 5×10^4cells/ml) was incubated at 37 °C with different formulations for a period of 12 h in 96-well tissue culture plate. After incubation, 10 μl of MTT solution (5 mg/ml) was added to each well. It was incubated for 3–4 h. The formazan crystals formed were solubilized with 200 μl of organic solvent (DMSO:ethanol, 1:1). Plates were read at 550–570 nm (L1) and 620–650 nm (L2) as reference on scanning multiwell spectrophotometer. Final optical density (OD) obtained from formazan formation can be calculated as OD = L1 − L2.

2.4.7. Antimicrobial efficacy

The antimicrobial activity of selected formulations was determined in comparison to free drug by determining the MIC values against *S. aureus*, *P. aeruginosa* and *E. coli* strains. MIC tests were performed by broth micro dilution method according to the NCCLS standards (NCCLS, 2000). Serial two-fold dilutions from 20.00 to 0.02 μg/ml of CFn, SE-CFn, SE-CFn–SDC, and SE-CH–CFn–SDC was carried out. For the experiment, bacterial strains were inoculated onto agar plates, incubated for 18 h at 35 °C, and then diluted in PBS to make its optical density equal to McFarland No. 0.5. Ten μl of bacterial culture was diluted 1:10 (approximate concentration: 10^7 cells/ml) and added to the microtitre wells containing the drug solution and incubated at 35 °C. After 2 h, 100 μl Mueller Hinton II Broth (MHB) (USA) were added to each well. The final concentration of microorganisms was 5×10^5 cfu/ml. The plates were then incubated for 18 h at 35 °C. Positive controls (growth) consisted of bacteria in broth and bacteria with empty formulation in broth. Negative controls (sterility) consisted of uninoculated broth and each of the drug/formulation dilutions in broth.

3. *In vivo* performance

In vivo performance evaluation of different submicron formulation was carried out using male Balb/c mice (4 weeks old, 18–20 g) procured from CDRI animal house. All the animal studies were approved by the CPCSEA and Institutional ethics committee of CDRI (65/06/E/Pharmaceu/IAEC). Twenty-four mice were divided into three groups comprising eight mice each. The first group received free CFn solution (10 mg/kg), second and third group received SE-CFn–SDC and SE-CH–CFn–SDC, respectively containing equivalent amount of CFn through caudal vein. At different time intervals (0, 0.25, 0.5, 2, 6, 12, and 24 h) 200 μl of blood sample was taken from the retro-orbital plexus. The collected blood samples were centrifuged at 5000 rpm for 8 min. Serum was harvested from supernatant. To 150 μl of serum, equal volume of 10% (v/v) trichloroacetic acid in water was added and mixed by vortexing for 30 s. The mixture was then centrifuged at 5000 rpm for 5 min and supernatant was filtered through 0.45 μm membrane filter, aliquot of filtrate (50 μl) was injected to HPLC column for analysis.

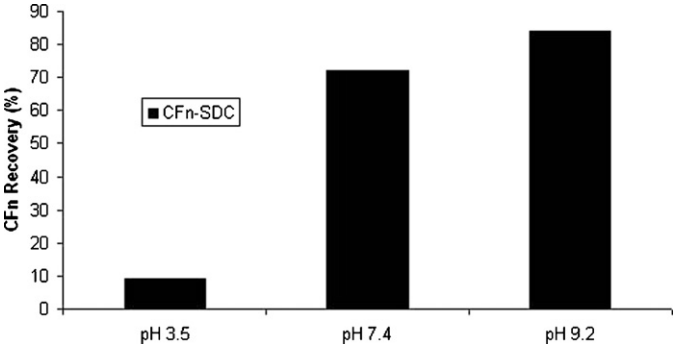

Fig. 1. Effect of different pH on ionic complex formation between ciprofloxacin and sodium deoxycholate (CFn–SDC).

4. Results and discussion

CFn exists in protonated form at acidic pH, which can form ion pair complex with negatively charged counter ions (Zhigaltsev et al., 2006). Based on this assumption, we have prepared CFn ionic complexes with another amphipathic molecule having an opposite charge like SDC, etc. It has been postulated that formation of ion pairs would increase the lipophilicity of CFn and SE would constitute an optimal carrier for these lipophilic ionic complexes. SDC was chosen for ion pairing because this is negatively charged in a wide range of pH (Wu et al., 2009).

The hydrophobic ionic complex formation being governed by the pH of solvent and ionic strength of solutes, it was mandatory to study complex formation at different pH and molar ratio of surfactant and CFn at room temperature (25 °C). The amount of CFn recovered in supernatant was less at lower pH (3.5) which gradually increases as pH of the buffer approached to neutral (7.4) or basic pH (9.2) as depicted in Fig. 1. This may be attributed to the fact that as the pH of the medium decreases, the proportion of protonated CFn increases (Srinatha et al., 2008), and thus available for complexation with negatively charged surfactant and as pH increased, the zwitterionic and anionic form of CFn becomes more dominant (Allemandi et al., 1999) which do not favor complex formation. Further complexation was therefore, carried out at acidic pH.

The value of transmittance decreases sharply as the molar ratio of surfactant/CFn increases from 0.5 to 2 which is indicative of complex formation; however, there was abrupt increase in transmittance as molar ratio exceeds 2. However, at 8 molar ratio complete clear solution was observed. This clearly indicates saturation of binding sites of CFn with negatively charged amphiphilic surfactant i.e. reduced aqueous solubility and further addition of surfactant beyond binding saturation point resulted in dissociation of hydrophobic ion pair complex in to individual micelles which are completely soluble (Fig. 2) therefore equal molar ratio (1:1) of CFn–SDC was chosen for complex formation.

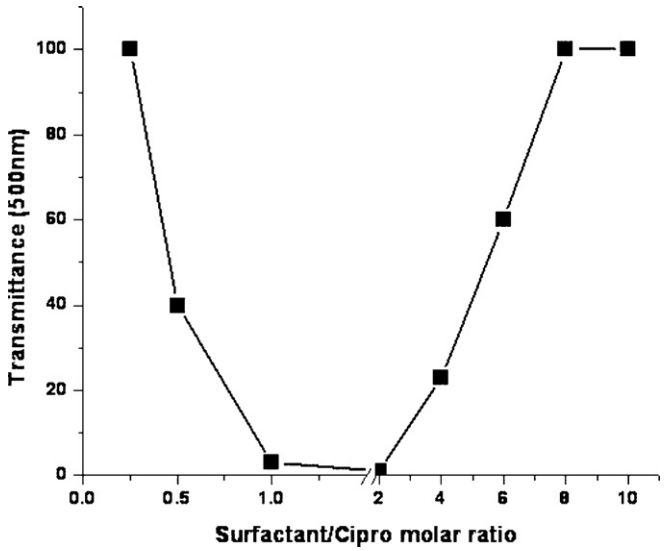

Fig. 2. Effect of Surfactant/ciprofloxacin molar ratio on % transmittance of ionic complexes at pH 3.5.

These studies, further confirm that ionic complexation occurs mainly due to electrostatic interaction between protonated amino group of CFn and carboxyl group of SDC. The formation of CFn–SDC was confirmed by mass and NMR spectroscopy. The FAB-Mass analysis shows presence of mass ion peak (M^{+1}) at 722 for CFn–SDC. NMR spectrum (Fig. 3) confirms the formation of complexes as it exhibits peaks corresponding to both the components. NMR spectrum confirms the formation of complexes as it exhibits peaks corresponding to both the components. ^{1}H NMR of CFn–SDC shows: 0.65 (S, $3CH_3$); 0.82–0.88 (m, 5H, CH_3 & CH_2); 0.93–0.98 (m, 5H, CH_3 & CH_2); 1.16–1.28 (m, 10, $5CH_2$); 1.30–1.47 (m, 10H, $5CH_2$); 1.62–1.82 (m, 8H, $4CH_2$); 3.07–3.12 (m, 4H, $2CH_2$); 3.30–3.37 (m, 6H, $3CH_2$); 3.50–3.58 (m, 4H, $2CH_2$); 3.94 (m, 1H, CH); 7.37 (d, J = 7.2 H2, 1H, ArH); 8.0 (d, J = 10 H, 1H, ArH); 8.76 (S, 1H, ArH). The electrostatic nature of this binding was confirmed by the fact that the CFn–SDC complex can be disrupted using a high concentration of NaCl (5 M, data not shown). There was significant improvement in lipophilicity of CFn on ionic complexation as partition coefficient of CFn–SDC (4.934) in chloroform was almost increased to 25 times, when compared to free CFn (0.176).

The compositions of formulation prepared are depicted in Table 1. The EE of CFn was improved significantly as depicted in Table 2 when encapsulated in the form of ionic complex. The loading efficiency improved almost four times (≥80%) in the form of ionic complex (CFn–SDC) compared to CFn loaded without complexation.

The loading efficiency of all the formulations was found to be in the order of SE-CH–CFn–SDC > SE-CFn–SDC > SE-CFn. The EE in the order of 93.7 ± 2.3 was found with SE-CH–SDC. The reason being that, chitosan acts as strong emulsifier and forms a polymer coat around oil droplet (Jumaa and Muller, 1999), which results in strong retention of hydrophobic ionic complex. It is well known that,

Table 1
Description and composition of various optimized formulations.

Excipients	Sub micron emulsion composition (%, w/w)		
	SE-CFn	SE-CFn–SDC	SE-CH–CFn–SDC
Soya oil	10.00	10.00	10.00
Pluronic (F-68) (mg)	2.5	2.5	2.5
Lecithin (mg)	1.25	1.25	1.25
Chitosan (mg)	–	–	0.5
CFn (%)	0.3	–	–
CFn–SDC[a]	–	0.3	0.3
Water	100.0	100.0	100.0

SE-CFn: submicron emulsion containing plain CFn without any charge inducer; E-CFn–SDC: submicron emulsion containing CFn–SDC ionic complex without any charge inducer; SE-CH–CFn–SDC: Submicron emulsion containing CFn–SDC ionic complex with chitosan as cationic charge inducer.

[a] CFn–SDC loaded as (1:1) molar ratio equivalent to 0.3% of ciprofloxacin.

the concentration of ionic complex (CFn–SDC) present at oil/water interface govern the encapsulation in SE formulations. The ionic complex being highly lipophilic retain in oil phase and thus contributing highest payload efficiency.

The average globule size of all the formulations showed a small mean globule size in the submicron range of 225–325 nm (Table 2), which are well suited for possible parenteral or ocular application as particle size requirement in such cases is ≤2 μm (Klang et al., 1999). TEM microphotographs, further confirm that globules size was found to be ≤1 μm (Fig. 4). Due to innate physicochemical properties of surfactants/emulsifiers, the interfacial film formed was strong enough to prevent droplet coalescence upon any physical and thermal condition. PF-68 is mandatory to get stable formulation as it is known to prevent unfavorable interactions between free fatty acids present in soya oil and cationic agents. It has been reported (Jumaa and Muller, 1998b) that PF-68 in optimized concentration is necessary to obtain a chitosan emulsion with sufficient stability.

The zeta potential of the formulations was determined in order to assess the contribution of cationic inducer on charge distribution. In blank SE formulation reversal of zeta potential (−35.8 ± 2.2 to +34.0 ± 4.2 mV) was observed when chitosan (0.5%, w/w) was added to lipid emulsions. Chitosan impart a positive charge in the formulation due to the presence of amino groups which results in reversal of charge in formulations. Similar behavior was also observed by the group of Jumaa and Muller (1998b). However, further addition of chitosan resulted in unstable formulation. The pH of SE-CH–SDC was found to be 5.65.

The SE-CFn formulations showed sustained release profile (Fig. 5) which released more than 90% of CFn within 24 h; it indicates the barrier to release is being provided by the liquid membrane of formulation. Aside from this the drug release was fairly sustained in formulations loaded with ionic complex. As evinced from Fig. 5 that SE-CFn–SDC, SE-CH–CFn–SDC, released 64 ± 3% and 41 ± 0.5% of the drug respectively within 24 h. The SE-CH–SDC shows lesser release profile among all which, could be due to the formation of polymer coating around oil droplet and increase in viscosity. Consequently, it may be due to the partitioning of the drug between the oil droplets and external aqueous medium. CFn-ionic

Table 2
Physicochemical characterization of different formulations.

Formulation	Zeta potential (mV)		Average globule size (nm)	Polydispersity	Viscosity (cP)	pH	CFn encapsulation (%)
	Blank (without CFn)	With CFn					
SE-CFn	–	–	284 ± 9	0.201 ± 0.07	2.4 ± 0.7	7.1 ± 0.4	27.2 ± 3.1
SE-CFn–SDC	−35.8 ± 2.2	−25.3 ± 1	278 ± 12	0.189 ± 0.06	2.6 ± 0.3	7.2 ± 0.4	82.1 ± 2.1
SE-CH–CFn–SDC	+34.8 ± 4.1	+28.2 ± 2	324 ± 16	0.197 ± 0.08	3.1 ± 0.7	5.8 ± 0.8	93.7 ± 2.3

Values are expressed as mean ± SD of three different batches.

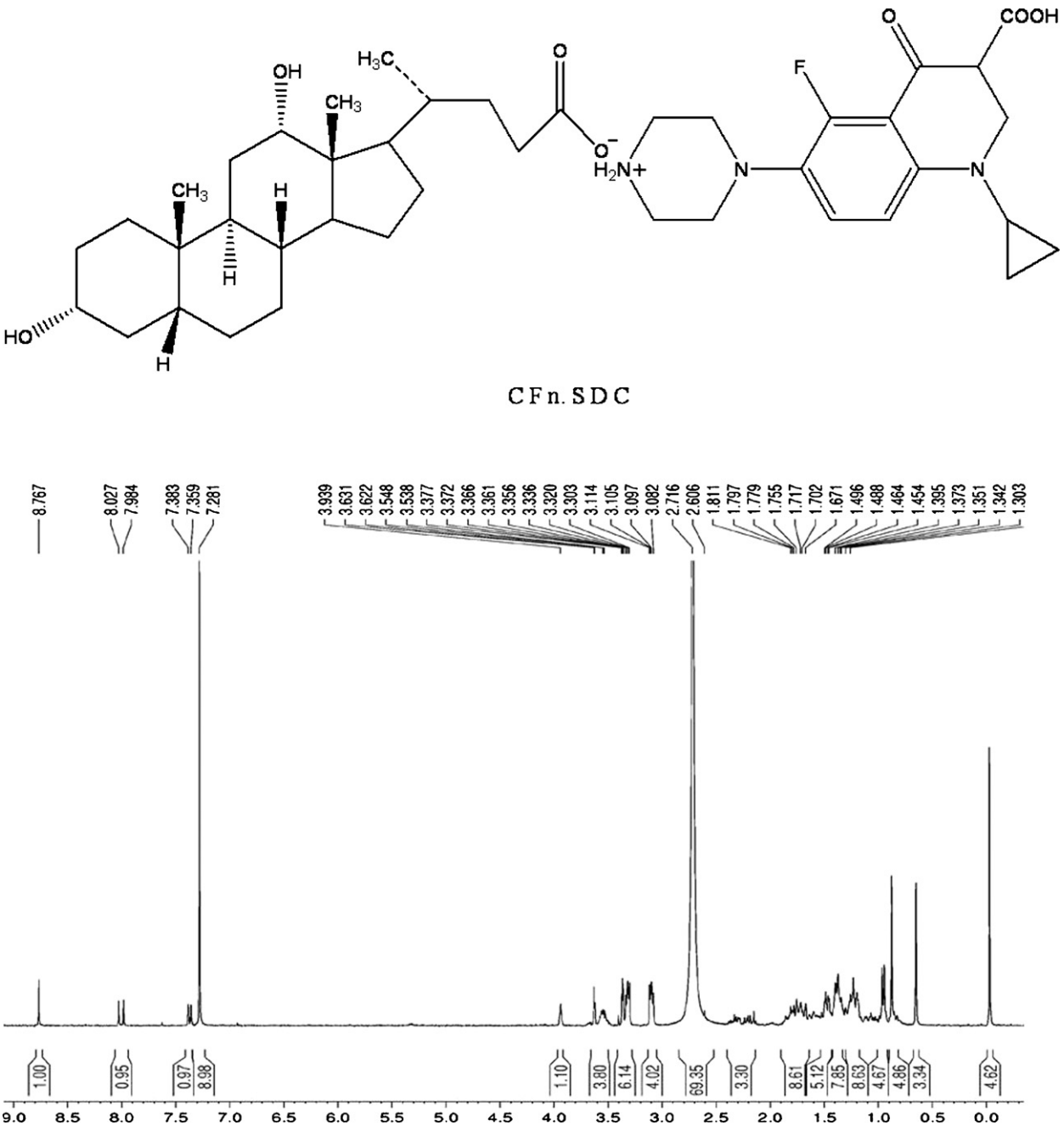

Fig. 3. ^{1}H NMR spectra of CFn–SDC ionic complex. The spectra was recorded using Bruker DRX-300 at 300 MHz by dissolving ionic complex in $CDCl_3$.

complex are lipophilic in nature, diffuse out slowly from oil phase to external aqueous phase (Tamilvanan and Benita, 2004).

The antimicrobial activity (MIC) was twice in SE-CFn–SDC compared to free CFn which may be due to detergent action of anionic surfactants SDC (Table 3). However, further improvement of antimicrobial efficacy in SE-CH–CFn–SDC could be due to electrostatic interaction between formulation and bacterial components like lipotheicoic and teichuronic acids in case of gram (+) bacteria and lipopolysaccharide in case of gram negative bacteria (Helander et al., 1997). These binding of cationic inducer with bacteria outer wall, disturb its integrity, which results in improved permeability of antibiotics.

In stability studies, during the excessive shaking (100 strokes per min over 48 h) of lipid emulsions no phase separation or creaming and change in mean droplet size is observed. The storage

Table 3
Antimicrobial efficacy of different formulations.

Formulation	MIC (nominal) (μg/ml)		
	E. coli	*S. aureus*	*P. aeruginosa*
CFn	0.195 ± 0.01	0.195 ± 0.01	0.391 ± 0.02
SE-CFn	0.195 ± 0.02	0.195 ± 0.02	0.391 ± 0.01
SE-CFn–SDC	0.0975 ± 0.003	0.0975 ± 0.003	0.195 ± 0.02
SE-CH–CFn–SDC	0.0487 ± 0.003	0.0487 ± 0.003	0.0975 ± 0.005

Values expressed as mean ± SD of three different observations ($N = 3$). MIC tests were performed by broth micro dilution method. Positive controls (growth) consisted of bacteria in broth and bacteria with empty formulation in broth. Negative controls (sterility) consisted of uninoculated broth and each of the drug/formulation dilutions in broth.

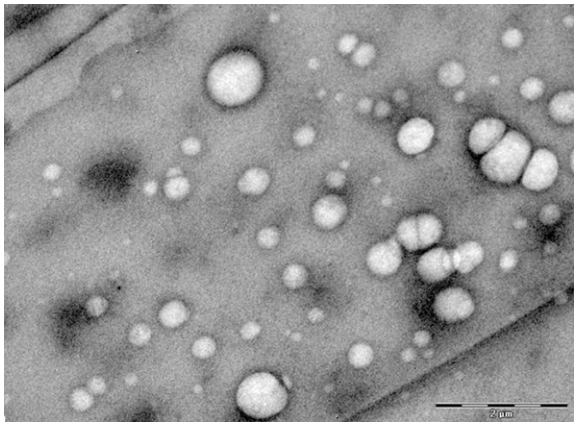

Fig. 4. Transmission electron microphotograph (TEM) of optimized SE-CH–CFn–SDC formulation.

stability of formulation was checked in terms of change in globule size, viscosity and pH of the emulsion samples stored at 4 and 37 °C over a period of 3 months. The mean droplet size did not differ markedly when these formulations were stored for period of 3 months at 37 °C.

The pH of SE-CFn–SDC was in the range of 7.2–7.4 while SE-CH–CFn–SDC has lower pH range (5.8–6.0) above which chitosan get precipitates. It has been observed that the pH of emulsions was affected by the storage temperature with time. At 37 °C, slight decrease ($P > 0.05$) in the pH value could be due to the formation of fatty acids as a result of the hydrolysis of triglyceride and phospholipid moieties (Heurtault et al., 2001). Nevertheless, the pH change in emulsions was minimal even after 3 months of storage at 4 °C. Slight variation in viscosity stored at 37 °C was not significantly indifferent ($P > 0.05$) after 3 months. These observations confirm that CFn electrostatically bound to amphiphilic surfactant does not influence the structure of the interfacial film. These complexes are distributed mainly to the oily phase and interface of the SE.

The cytotoxicity studies on J774 macrophage cell lines was carried out and it has been observed that CFn–SDC is biocompatible and does not have pronounced effect on cell viability even at higher doses (100 μg/ml), as depicted in Fig. 6.

It is anticipated that positive charge inducers may also affect cell viability due to strong interaction with cell membrane. Cytotoxicity studies in J774 macrophages cell line shows dose dependent

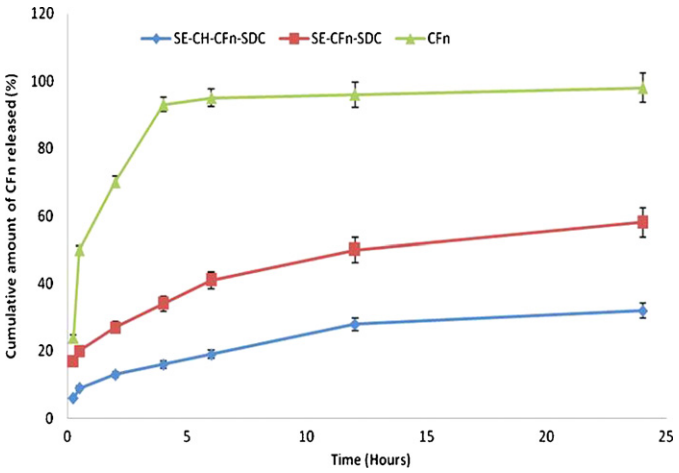

Fig. 5. *In vitro* release profile of CFn-SDC loaded different submicron emulsion formulations carried out using bulk equilibrium reverse dialysis bag technique at 37 °C. The data are represented as mean average value of three separate experiments ($N = 3$).

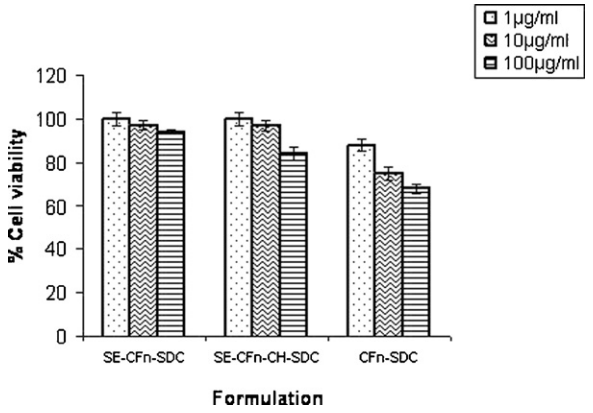

Fig. 6. Concentration dependent cell viability studies of different CFn–SDC loaded submicron emulsion formulations tested using J774 macrophages. The cell suspension containing usually 5×10^4 cells/ml, taken in 96-well tissue culture plate and incubated at 37 °C for a period of 12 h and viability was determined by MTT assay. The data are represented as mean average value of three separate experiments ($N = 3$).

cytotoxicity. However, cell viability with different positive charge emulsion was within acceptable limit (80–85%). As shown in Fig. 6, at lower concentration (1 μg/ml) all the formulations show ≥99% viability except CFn–SDC which showed nearly 80% of cell viability. However, at higher concentration (100 μg/ml) the viability was found to be in order of SE-CH–CFn–SDC > SE-CFn–SDC > CFn–SDC. SE-CH–SDC shows minimum cytotoxicity, as chitosan is reported to be non toxic cationic polymer, and its uses in different delivery system are well documented (Schulz et al., 1998).

Fig. 7 represents the blood concentration time profiles of CFn solution, SE-CFn–SDC and SE-CH–CFn–SDC after i.v. injection. The plasma concentration time profile was adequately described by a two-compartment open model (based on the Akaike, r^2 and positive and negative residuals) following intravenous administration, as has already been reported by Munoz et al. (1996). The correlation coefficient (r^2) of the fit of the curve was 0.98. The pharmacokinetic parameters were determined by analyzing the data by Winnolin software (Table 4). Pharmacokinetic parameters of CFn solution, SE-CFn–SDC and SE-CH–CFn–SDC emulsion differed significantly. The initial plasma drug concentration was 9.40 μg/ml for CFn, which rapidly declined to 1.95 μg/ml within 0.25 h. This sharp

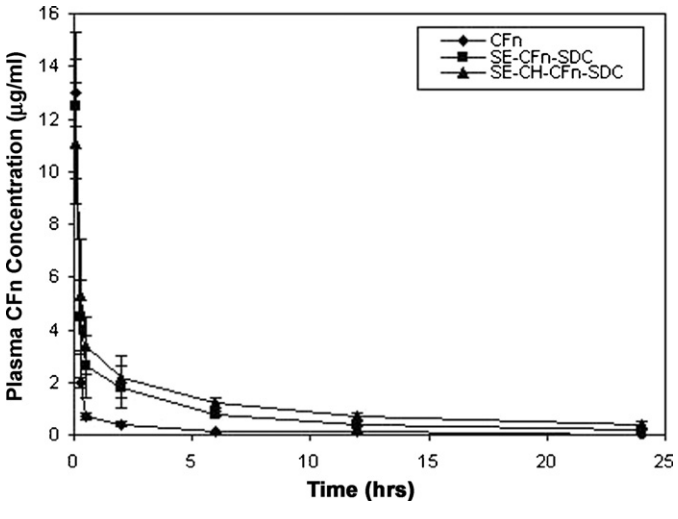

Fig. 7. Plasma concentration time profile of different SE formulations after intravenous administration containing CFn equivalent to 10 mg/kg. CFn-aqueous solution of free drug; SE-CFn–SDC-submicron emulsion containing CFn–SDC ionic complex and SE-CH–CFn–SDC-cationic submicron emulsion containing CFn–SDC ionic complex. Each value is average of four determinations ($N = 4$); mean ± SD.

Table 4
Pharmacokinetic parameter of intravenously injected CFn as single dose (10 mg/kg) delivered in solution and SE-CFn–SDC submicron emulsion.

Kinetic parameter	CFn	SE-CFn–SDC	SE-CH–CFn–SDC
A (μg/ml)	34.20 ± 3.3	10.79 ± 1.2	10.58 ± 1.8
B (μg/ml)	1.94 ± 0.25	4.05 ± 0.45	2.71 ± 0.81
α (1/h)	31.00 ± 3.0	18.87 ± 1.45	6.1 ± 1.4
β (1/h)	0.67 ± 0.05	0.41 ± 0.07	0.13 ± 0.03
$t_{1/2}$ (α) (h)	0.03 ± 0.001	0.02 ± 0.002	0.11 ± 0.02
$t_{1/2}$ (β) (h)	1.02 ± 0.11	1.65 ± 0.18	5.51 ± 1.2
$AUC_{0-24\,h}$(h μg/ml)	4.70 ± 0.77	13.17 ± 0.88	23.27 ± 2.8
MRT (h)	0.93 ± 0.23	1.77 ± 0.17	6.59 ± 1.7
V_d (ml/gm)	396.66 ± 12.00	269.37 ± 17.90	537.4 ± 43.2
Cl (ml/min)	425.15 ± 11.21	151.82 ± 14.37	85.91 ± 12.1

A, zero time intercept of distribution slope in the two-compartment model; B, zero time intercept of elimination slope in the two-compartment model; α, distribution rate constant; β, elimination rate constant; $t_{1/2}\alpha$, distribution half-life; $t_{1/2}\beta$, elimination half-life; AUC, total area under the concentration time–curve; AUMC, total area under the first moment concentration time–curve; MRT, mean residence time; V_d, apparent volume of distribution; Cl, clearance of drug. CFn, ciprofloxacin solution; SE-CFn–SDC, Ciprofloxacin sodium deoxycholate ionic complex loaded submicron emulsion; SE-CH–CFn–SDC, cationic ciprofloxacin sodium deoxycholate ionic complex loaded submicron emulsion.

fall in the serum concentrations can be attributed to high distribution rate constant (α, 31.00 h^{-1}). The elimination rate constant (β) was found as 0.67 h^{-1}. Similarly, SE-CFn–SDC and SE-CH–CFn–SDC experienced an initial rapid but slow clearance after 2 h of injection. SE-CFn–SDC and SE-CH–CFn–SDC significantly decreased the elimination rate constant (kel/β) and increase the mean residence time (MRT) of CFn in blood. The MRT of SE-CFn–SDC was drastically enhanced to 6.59 h from 1.77 h and 0.93 h for SE-CFn–SDC and CFn solution respectively. This data indicates that chitosan incorporated SE is able to retain CFn for longer duration.

The plasma concentration of CFn administered through SE-CFn–SDC and SE-CH–CFn–SDC remained significantly higher than CFn solution at all time points and was detectable up to 24 h. However, the $AUC_{0-24\,h}$ of SE-CH–CFn–SDC (23.27 ± 2.8 h μg/ml) was found to be 1.7-fold and 5-fold higher as compared to SE-CFn–SDC (13.17 ± 0.88 h μg/ml) and CFn solution (4.70 ± 0.77 h μg/ml) respectively which may be due to lower clearance and kel/β. The clearance of CFn delivered through SE-CH–CFn–SDC was found to be 85.91 ± 12.1 ml/min, which is much lower than CFn solution (425.15 ± 11.21 ml/min). The CFn, in general, have a large volume of distribution and infiltrate in all organs and cells (Bugyei et al., 1999). CFn is reported to undergo minimal metabolism into any of the active metabolites, therefore clearance is mainly attributed to urinary excretion (Campoli-Richards et al., 1988).

The plasma concentration of 0.1 μg/ml was considered to be therapeutically adequate, corresponding to MIC of CFn against sensitive bacteria (Prescott and Yielding, 1990). The therapeutic concentration was maintained only up to 6 h after i.v. injection in case of CFn solution, whereas maintained up to 24 h in case of both SE-CFn–SDC and SE-CH–CFn–SDC. This observation is of therapeutic relevance as fluoroquinolones exhibit concentration-dependent killing, i.e. as the concentration of the drug increases from two to eight times the MIC, the rate and extent of bacterial killing increases proportionately (Drusano et al., 1993).

5. Conclusion

Hydrophobic ion pair complex of CFn with SDC is suitable technique for enhanced loading efficiency (≥80%) within SE. The developed formulations were stable with desired physiochemical profile. It has been shown that the incorporation of chitosan as cationic charge inducers was crucial for the improved antimicrobial efficacy against *E. coli*, *S. aureus* and *P. aeruginosa*. When tested in male Balb/c mice, the concentration of CFn was maintained well above MIC level for 24 h suggesting that this formulation hold promise for improved antimicrobial efficacy. This CFn–SDC containing formulation with high pay load of CFn and improved antimicrobial activity can be a promising delivery system for topical as well as parenteral administration.

Competing interests

None declared.

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