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Preparation and *in vitro* evaluation of chitosan-based films for the sustained delivery of enrofloxacin

Ali Rassouli^a, Sakineh Khanamani Falahatipour^b, Yalda Hosseinzadeh Ardakani^c, Hamid Akbari Javar^c,
Katayoun Kiani^a, Taghi Zahraee Salehi^d

^a Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

^b Physiology and Pharmacology Research Centre, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

^c Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

^d Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Keywords

Enrofloxacin, chitosan, β -glycerophosphate, sustained release, triple-layer film

These findings suggest that the triple-layer chitosan/ β -GP films could be effective to deliver ENR for a long period.

Abstract

The implantable drug products are developed mainly to sustain the drug release. This study was conducted to formulate and evaluate cross-linked films of chitosan/ β -glycerophosphate (β -GP) for the sustained delivery of enrofloxacin (ENR). Two types of formulations, single-layer (F1 and F2) and triple-layer (F3 and F4) films, were prepared. *In vitro* drug release, kinetic modelling, Fourier transform infrared spectroscopy (FTIR) spectra, morphological and microbiological studies were performed. Drug release from F1 and F2 continued up to 5 hours but from F3 and F4, it was extended over 96 and 168 hours, respectively. The cumulative drug release for F1, F2, F3 and F4 were 72.6, 70.1, 90.5 and 82.4%, respectively. The inhibition zones of bacterial growth by using positive controls and single layer films were significantly greater than those of triple-layer films ($p < 0.05$), indicating sustained drug release pattern of the multi-layer films.

Abbreviations

ENR: Enrofloxacin;
FTIR: Fourier transform infrared spectroscopy;
 β -GP: β -glycerophosphate

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Tel: +98 51 3880 3742 Fax: +98 51 3876 3852
Web: ijvst.um.ac.ir Email: ijvst@um.ac.ir

Corresponding author:

Ali Rassouli
Department of Pharmacology, Faculty of Veterinary Medicine,
University of Tehran, Tehran, Iran.

E-mail: arasooli@ut.ac.ir
Tel: +98 912 583-3863
Fax: +98 021 6693-3222,

Introduction

Novel drug delivery systems are going to be developed to optimize the therapeutic properties of drug products and make them to be safer, more effective, and reliable. Advantages of the implantable drug-delivery systems may include improved efficiency, reduced dosage, reduced side effects, on-spot delivery, relatively linear drug delivery for longer periods of time, and as a result, maintaining the plasma drug levels continuously in a therapeutic range. The major driving forces for the development of innovative veterinary sustained release products include the reductions in the duration of drug therapy, the frequency of drug dosing, and the imposed stress to the animals [1, 2].

Hydrogels are cross-linked, three dimensional networks of linear hydrophilic polymers capable of absorbing large amounts of water while remaining insoluble [3]. Hydrogels can be formulated in a variety of physical forms, including slabs, microparticles, nanoparticles, and films. When a drug is incorporated into a polymer solution, it becomes entrapped within polymer matrix upon its solidification, and the drug release occurs over time as the polymer degrades gradually in the body [4].

Chitosan is a natural cationic copolymer derived from chitin [5]. It is highly interesting for pharmaceutical applications because of its high solubilization capacity, lack of toxicity, biocompatibility, biodegradability, low cost, high mechanical strength and good film-forming ability [6]. Recently, the development of crosslinking methods has been attempted to improve structural and mechanical stabilities and to decrease the rate of drug release by employing natural or synthetic reagents such as glutaraldehyde [7].

ENR is a broad spectrum antimicrobial agent that has been developed as a drug for animal use. It has the maximal lipid solubility among fluoroquinolones and this property promotes its penetration into biological tissues [8]. ENR is currently FDA-approved for treatment of pets and farm animals in the United States. [9]. The focus of the present study was to prepare and evaluate the physicochemical and antibacterial properties of a chitosan/ β -GP system as implantable films for controlled delivery of ENR.

Results

In vitro drug release

The cumulative drug release (%) from the film formulations as a function of time were shown in Fig-

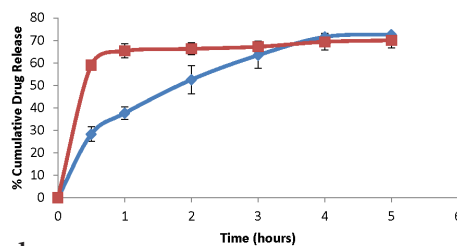


Figure 1
In-vitro cumulative drug release of enrofloxacin (%) from single layer films (F1 and F2). Each data represents mean \pm SD (n=3).

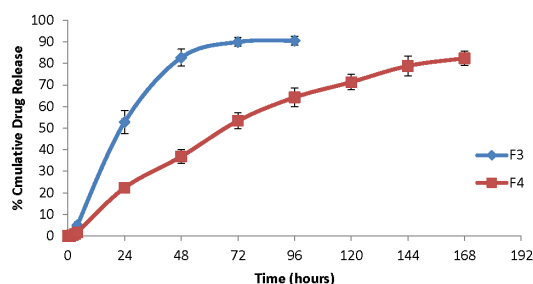


Figure 2
In-vitro cumulative drug release of enrofloxacin (%) from triple layer films (F3 and F4). Each data represents mean \pm SD (n=3).

ures 1 and 2. The cumulative drug release (%) of F1, F2, F3 and F4 formulations were 72.6, 70.1, 90.5 and 82.4, respectively. Formulations F1 and F2 released the drug for 5 h but drug release from F3 and F4 extended over 96 and 168h, respectively. Formulations F1 and F2 started the release of ENR within the first hour of the experiment. The release of formulation F1 in 0.5, 1 and 2 h were significantly lower than those of F2 ($p < 0.05$). Formulation F3 and F4 started the release of ENR in 2 and 3 h, respectively. The burst effect had been controlled in formulation F3 and F4 by changing the preparation method. The release of formulation F4 in 24, 48, 72 and 96 h was significantly lower than those of F3 at these time points ($p < 0.05$).

In vitro drug release kinetic model

The model fitting for the release profile of formulations was shown in table 1. Based on the higher regression values (r^2), the best fit model was Korsmeyer-Peppas for formulation F1 and F2, whereas Higuchi model for F3 and F4 formulations. The values of "n" were calculated from the drug release data ($< 70\%$). The obtained values of formulation F1 and F2 were between 0 and 0.5, indicating that the release pattern of ENR was correlated to Fickian diffusion. These values for formulations F3 and F4 were > 1 , indicating that the drug release was by super case II transport

Table 1.
In vitro drug release kinetics parameters of different enrofloxacin films

Film code	Zero order	First order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas	
	r ²	r ²	r ²	r ²	n	r ²
F1	0.933	0.970	0.9822	0.960	0.45	0.9929
F2	0.782	0.819	0.8572	0.807	0.067	0.9073
F3	0.887	0.959	0.9726	0.939	3.43	0.7970
F4	0.960	0.704	0.9920	0.711	2.70	0.8755

[10].

Fourier transform infrared spectroscopy (FTIR) findings

The FTIR spectra of ENR, chitosan, β -GP, chitosan/ β -GP and formulation F1 are shown in figure 3.

The FTIR studies showed no chemical interaction between ENR and excipients used in the study.

The ENR has two characteristic absorption peaks, 1736 cm⁻¹ and 1628 cm⁻¹; the first is the C=O vibration absorption peak from carboxylic acid oxygen, and the second is assigned to keto C=O peak

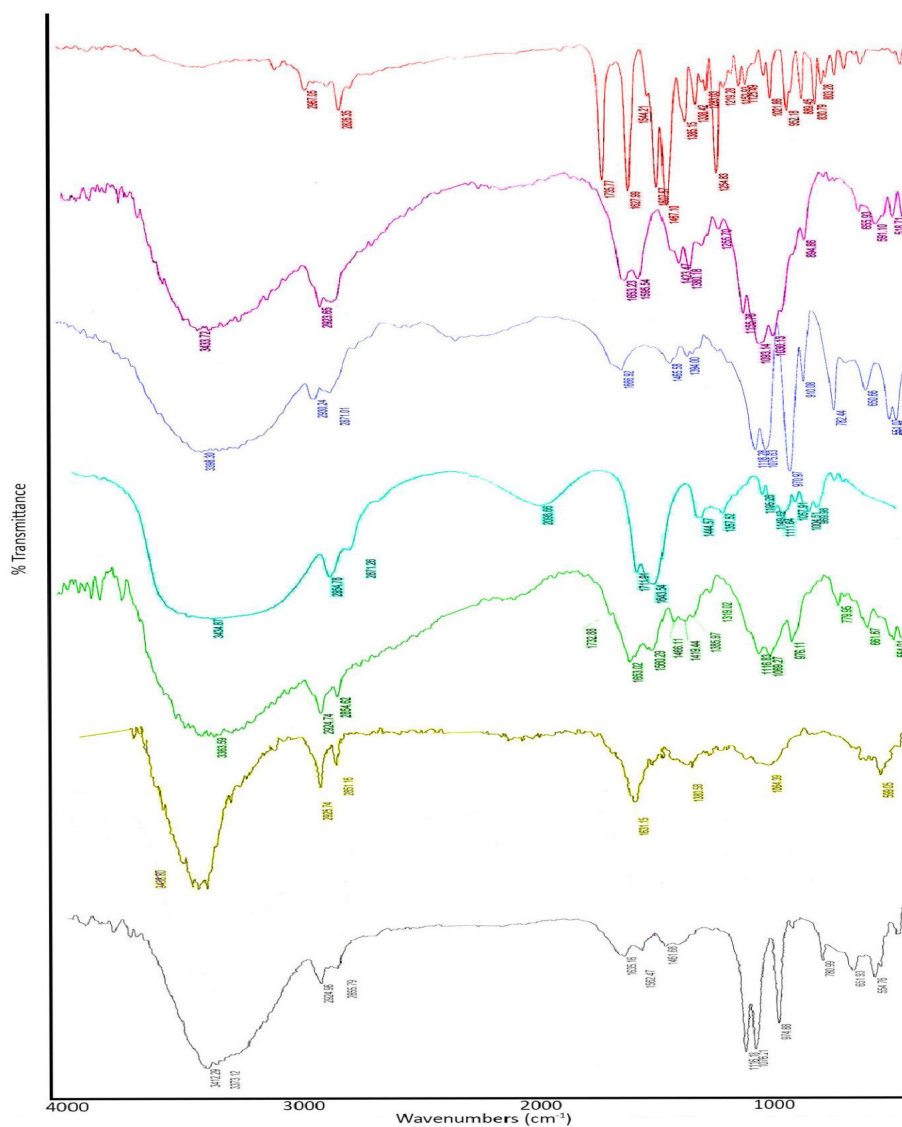


Figure 3.
The FTIR spectra of A: ENR, B: Chitosan, C: β -GP, D: Glutaraldehyde E: Chitosan/ β -GP, F: Chitosan/Glutaraldehyde, G: single-layer formulation.

from the ring of ENR. For the ENR film, the band 1628 cm^{-1} was shifted to 1635 cm^{-1} , respectively.

In the wave number range $800\text{--}1200\text{ cm}^{-1}$, the FTIR spectrum of chitosan shows three bands at 1155 , 1030 and 894 cm^{-1} . The wide band at $1030\text{--}1155\text{ cm}^{-1}$ represents the bridge -O- stretch of the glucosamine residues in chitosan. The spectrum of chitosan shows a band at 1595 cm^{-1} that is assigned to the NH_2 group of chitosan. These bands indicate that chitosan is a partially deacetylated product of chitin. The chitosan molecule shows four peaks at 1423 , 1380 , 1315 and 1255 cm^{-1} . The bands at 1423 and 1315 cm^{-1} are associated with oscillations characteristic for OH and C-H bending of CH_2 groups. The band at 1380 cm^{-1}

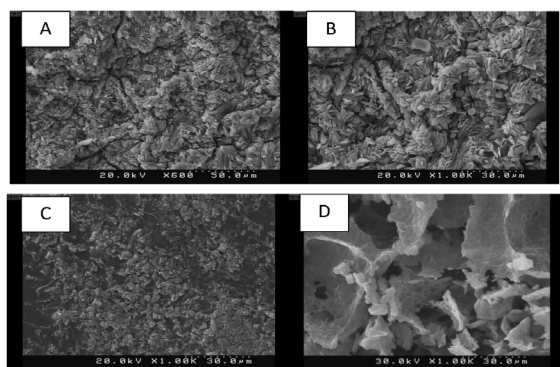


Figure 4. Scanning electron microscopy of freeze-dried chitosan based films. A and B: single layer film, C: triple-layer film, and D: Swelled film (F4) in phosphate buffer for 3 days.

represents the C-O stretching of the primary alcoholic group $-\text{CH}_2-\text{OH}$. A weaker amino characteristic peak at 1255 cm^{-1} is associated with O-H bending vibration. Chitosan exhibited a broad peak at 3434 cm^{-1} , which was assigned to the stretching vibration of N-H and O-H bond. Peaks at 2924 cm^{-1} were due to the

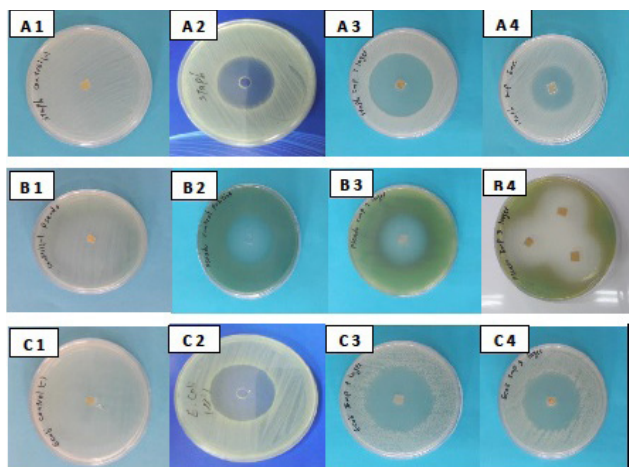


Figure 5. Antibacterial activity of ENR films against some bacterial strains. Letter A: *S. aureus*, B: *P. aeruginosa*, C: *E. coli*. Number 1: negative control, 2: positive control (ENR suspension), 3: single layer film, 4: triple-layer film.

Table 2.

The zones of inhibition of microbial growth around the enrofloxacin films and the cylinder containing enrofloxacin suspension (positive control).

Bacteria	Zone of inhibition (mm)		
	Enrofloxacin Film		Positive control
	Single layer	Triple-layer	
<i>S. aureus</i>	45.3 ± 1.53^a	30.7 ± 0.58^b	43.2 ± 2.02^a
<i>E. coli</i>	45.7 ± 4.04^a	34.2 ± 0.29^b	44.3 ± 1.75^a
<i>P. aeruginosa</i>	35.7 ± 1.53^a	28.0 ± 0^b	34.7 ± 1.53^a

Note: Data represent mean \pm SD (n=3). Different letters denote significant differences ($p < 0.05$).

C-H stretch vibrations. A peak at 1653 cm^{-1} was due to the C=O stretch of amide bond.

Two bands characteristic of GP appear at 971 cm^{-1} and 1076 cm^{-1} , with a minor shoulder at 910 cm^{-1} . The band at 1076 cm^{-1} is characteristic for GP and indicates the $-\text{PO}_4^{2-}$ group. The band at 971 cm^{-1} is characteristic for the aliphatic P-O-C stretching and the band at 910 cm^{-1} may indicate the presence of the $-\text{HPO}_4^{2-}$ group.

Morphological findings

The appearance of films was creamy, smooth, non-sticky, homogenous and flexible. The SEM photographs of the samples are shown in Figure 4 (A-D). It showed that the surface of single layer film was rough, obviously dense, porous, homogenous and integrated. The triple-layer film also had a continuous and porous structure, but its texture was softer than single layer films, while the swelled film (F4) in phosphate buffer (pH=7.4) had a more porous structure than non-swelled films as shown in Figure 4 (D).

Antimicrobial activities

The zones of inhibition of microbial growth provided by positive control and ENR films were presented in Figure 5 and Table 2. It showed that the inhibition zones of positive control and single layer films were significantly greater than those of triple-layer films ($p < 0.05$). However, no statistical differences in inhibitory zones were observed between the positive control and single layer films against *S. aureus*, *P. aeruginosa* and *E. coli*. In addition, no inhibition zone was observed for the films without ENR (the blank formulations).

Discussion

Infection control in animals is a primary clinical objective in veterinary field and is usually achieved by drug therapy, once or twice a day for at least 3-5 days. The objective of the present study was to prepare and

evaluate ENR films as sustained release formulations. The polymeric matrix used in this study consisted of chitosan polymer and β -Glycerophosphate (β -GP). Glycerol was included in the films in order to achieve the desired plasticizing action.

Our results showed that formulation F2 had the highest burst release (65%) within 1 hour. Formulation F1 also exhibited a high burst effect (38%) within 1 hour, but there were no burst effects in formulations F3 and F4 within the first hours. In triple-layer films, following an initial burst release within 24 hours, drug was released gradually from chitosan films. The formulations F3 and F4 could sustain ENR delivery for 96 h and 168 h, respectively, which is a reasonable time span for antimicrobial therapy.

The rate of drug release was higher in F2 formulation in comparison to that of F1 formulation containing the same amount of chitosan but with different amounts of β -GP (750 mg in F2 and 500 mg in F1). This can be explained by the fact that the pore size increases with an increase in β -GP content [11] and the porous structure of the films are made by interactions between chitosan and β -GP. Moreover, the ENR molecules (with molecular weight of 359.4 Da) are small enough to move out through the pores of polymeric network. This might be the cause of faster drug release from F2 films as compared to F1 films [9, 12].

The strength and water preservation efficiency of the hydrogel depends on the amount of crosslinking agent used because the chitosan molecules can be transformed into polymeric network structure by the addition of crosslinking agent and consequently, water molecules can be retained in the network [13]. This issue correlates well with the results of the present study where the *in vitro* releases of drug were prolonged from the formulations F3 and F4 that contained glutaraldehyde in lower and top layer. These formulations could prolong the release of ENR for 4 and 7 days, respectively. Based on the *in vitro* dissolution profiles, Iqbal et al. (2012) also reported that the implants containing tramadol which were not exposed to crosslinking agent showed rapid release and completed the release within 8 days, while the implants which were exposed to glutaraldehyde could sustain the release up to 17 days [2].

In triple-layer implants, the drug release was effectively modified by changing the preparation method. At the beginning, the release of drug from formulation F3 was carried out only through the perimeter of the second layer because the lower and top layers were drug-free. This property controlled the burst effect and sustained the drug release. Regarding the formulation F4, a novel preparation method was used,

where the second layer which contained the drug was entirely covered with polymer. Thus, the drug could not release fast but released gradually by diffusion through the polymer matrix and erosion of the polymer.

With regard to release kinetics, it has been proposed that drug release from matrices usually implies water penetration into the matrix, hydration, swelling, diffusion of the dissolved drug, and/or the erosion of the gelatinous layer. The release mechanism of a drug would depend on the selected dosage form, pH, and the nature of the drug and polymer [2]. In the present study, formulations F3 and F4 fitted best with Higuchi release model, in which the release rate decreased with time. The Higuchi model indicates a diffusion-controlled mechanism of release. The erosion of the polymer in formulation F3 and F4 could gradually reduce the diffusion path length, which in turn attenuates the reduction of the release rate in Higuchi model [14].

The FTIR spectrum of the chitosan/ β -GP system after gelation indicates the characteristic bands for chitosan and glycerol phosphate disodium salt. There was no additional band observed. For the chitosan/ β -GP system, the bands of chitosan at 1380 and 1315 cm^{-1} were shifted to 1385 and 1319 cm^{-1} , respectively, but the bands of GP at 1076 and 971 cm^{-1} were shifted to 1069 and 976 cm^{-1} .

The cross-linked chitosan films showed peaks at 1572 cm^{-1} , indicating the formation of imine bonds due to cross-linking reaction of free amino groups of chitosan with the aldehyde groups of glutaraldehyde (GA). The spectra of chitosan cross-linked with GA showed typical aliphatic groups of GA at 1631 cm^{-1} due to N=C bond and 1558 cm^{-1} attributed to C=C bond. For the film formulation, the bands at 1558 and 1631 cm^{-1} were shifted to 1562 and 1635 cm^{-1} , respectively. The absence of peak in the 1720–1740 cm^{-1} region indicated the absence of an aldehyde group in the cross-linked product and, hence, any residual unreacted GA.

The various components of the formulations, especially the glycerol, resulted in loss of infrared features owing to severe background absorbance, and complicated the interpretation of spectra. Three basic infrared features of chitosan powder, namely the amide C=O stretch, amide N-H bend, and CH_2 scissoring modes, were compared with that of film. The C=O stretching frequency of amide was found to be lower in the film. This shift is attributed to hydrogen bonding with hydroxyl groups of glycerol, whereas shifts in N-H bending and CH_2 scissoring frequencies indicate altered interaction/mobility of polymer chains [15].

The morphological tests of the film formulations

showed similar properties as shown by other researchers such as Jiang et al. (2011) who fabricated bioabsorbable chitosan/ β -GP composite membranes. They also reported rough surface and porous structure of the membranes caused by interaction between chitosan and β -GP [12].

Regarding microbiological studies, the findings indicated that the inhibition zones were significantly ($p < 0.05$) smaller for the triple-layer films as compared to the single layer films and the free drug (positive control). It seems that it is mainly due to slow drug release from triple-layer films in comparison to

Table 3.
The compositions of the film formulations of Enrofloxacin.

Ingredient	Formulation Code			
	F1	F2	F3	F4
Chitosan (mg)	200	200	400	400
β -GP (mg)	500	750	1000	1000
Glutaraldehyde (μ l)	30	30	50	50
Glycerol (μ l)	100	100	200	200
ENR (mg)	100	100	100	100

Note: Composition of triple-layer films (F3 and F4) are the same, but their preparation methods are different with regard to the method of the addition of second layer (see text).

single-layer formulations during 24 hours. Aviv et al. (2007) also prepared polymer films consisting of poly (L-lactic acid) or poly (D, L-lactide-co-glycolide) and gentamicin for prevention of bacterial infections as-

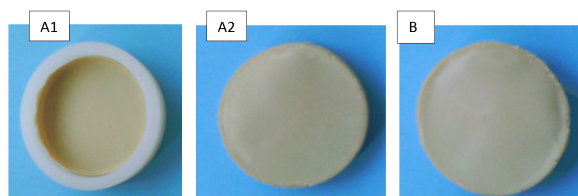


Figure 6.
Single layer films. A1 and A2: F1 formulation, B: F2 formulation

sociated with orthopedic implants. All films exhibited marked gentamicin release, which was responsible for the dramatic decrease in bacterial survival (10^3 /mL CFU after 24 h). Practically, no bacteria survived after 1–3 days, so the film preparation process did not affect



Figure 7.
Triple-layer films. Left: F3 formulation, Right: F4 formulation

the potency of gentamicin as an antibacterial agent [16]. The findings of the present study also showed that ENR maintained its antibacterial properties in the films and demonstrated the antimicrobial activity against all the bacterial strains tested.

In conclusion, the present study showed that the triple-layer chitosan/GP films were more effective than single layer formulations to deliver ENR for a long period and this approach represents an attractive technology platform for the delivery of other clinically important hydrophobic drugs. Both F3 and F4 formulations can be considered for further research and use in antimicrobial therapy to release ENR for 4 and 7 days, respectively. The major advantages of these systems include targeted local delivery of drugs at a constant rate such as postsurgical use to prevent infection.

Materials and methods

Materials

Medium molecular weight chitosan with degree of deacetylation (DDA) of 75-85% and β -glycerophosphate disodium salt pentahydrate were purchased from Sigma-Aldrich (St. Louis, MO). ENR (99.57%, assay by HPLC) was obtained from TEMAD Pharmaceutical Co. (Tehran, Iran). Acetic acid, glutaraldehyde and glycerol were purchased from Merck (Darmstadt, Germany). Other chemicals were reagent grade.

Preparation of the films

The solvent evaporation technique was used for preparation of the films. The compositions of the film formulations of ENR have been presented in Table 3. To make single layer films (F1 and F2 formulations), a chitosan solution was prepared by stirring 200 mg powdered chitosan in 6 ml aqueous acetic acid (1%, v/v) and maintaining at 4°C overnight. The insoluble particles were removed by filtration. The chitosan solution was mixed with 100 mg ENR by stirring at room temperature for 2 h and 100 μ l glycerol was added. Then, 500/750 mg β -GP (for preparation of F1/F2 formulation, respectively) along with 30 μ l glutaraldehyde was dissolved in deionized water. The prepared chitosan and β -GP solutions were placed in an ice-water bath for 15 min and then the β -GP solution was added to chitosan solution drop-wisely. Chitosan films were prepared by pouring the final 8 ml solution onto the Teflon molds.

The contents in the molds were left to dry at ambient temperature for 72 h to form the circular films. The dried transparent film was carefully peeled off from the plates, washed with distilled water, and then air dried (Figure 6).

The triple-layer films were prepared by pouring successive layers onto a mold in two ways. In the first way (F3 formulation), chitosan solution was prepared by stirring 150 mg powdered chitosan in 4 ml aqueous acetic acid (1%, v/v). This solution was maintained at 4°C overnight. Then, 500 mg β -GP along with 50 μ l glutaraldehyde was dissolved in deionized water. The chitosan and β -GP solutions were placed in an ice-water bath for 15 min. The β -GP solution was added to chitosan solution drop-wisely. Then, 100 μ l glycerol was added to solution. The final 5 ml solution was cast as the first layer. The third layer was prepared similar

to the first layer. The second layer was contained 100 mg chitosan dissolved in 4 ml aqueous acetic acid. The chitosan solution was mixed with 100 mg ENR by stirring at room temperature for 2 h. The only difference between two ways was how to pour the second layer in the mold. In the first way (F3), the second layer was poured on the whole surface of the first layer whereas in the second way (F4), the second layer was poured on the centre of the first layer. The first and the third layer left to dry for 48 h but the second layer left to dry for 24 h (Figure 7).

In vitro drug release studies

In vitro drug release tests were performed under sink conditions using a piece of film immersed at 37°C in 500ml of phosphate buffer, pH = 7.4, containing 0.5% Tween 80. The dissolution system was shaken at 100 rpm. Samples from dissolution medium were removed periodically and the medium was replenished. The absorbance of the samples was measured at 273 nm by using a UV-Vis spectrophotometer. All measurements were performed in triplicate.

In vitro drug release kinetic study

The drug release kinetic is directed by one or more mechanisms that depends on the composition of the matrix, geometry, preparation method and dissolution media. This can be explained by mathematical models in accordance with the desired or required predictive ability and accuracy of the model [17]. To assess the drug release kinetic, data were analysed per the zero order, first order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas models. The coefficient of correlation (r^2) values were calculated for a linear curve obtained using regression analysis of the plots from these models [18].

Morphological Studies

For sample preparation, all formulations were initially placed in a freezer at -20°C for a short-term and then freeze-dried overnight. To study the morphology of formulations, dried films were cut with a sharp blade to expose internal microstructure and coated with platinum-gold for SEM imaging at 30 kV using scanning electron microscope (FESEM, Hitachi. S4160, Japan).

FTIR spectra

FTIR spectroscopy was used to assess the polymer chemical groups and investigate the formation of cross-linked networks through the reaction of chitosan with glutaraldehyde. FTIR spectra of ENR, chitosan, β -GP, dried chitosan/ β -GP film, glutaraldehyde and formulation F1 were recorded in KBr pellets. To prepare 0.50-mm-thick KBr pellets, 3–5 mg of powder films (extra fine) mixed with 200 mg dried KBr. The FTIR spectra between 400–4000 cm^{-1} were recorded using a FTIR spectrophotometer (Nicolet, Model Impact 410; Madison, WI) at room temperature.

Microbiological studies

To determine the antibacterial activity of ENR films, the “diffusion test” was carried out by using *Escherichia coli*, ATCC35218, and *Pseudomonas aeruginosa* ATCC10145 as gram-negative pathogenic strains and *Staphylococcus aureus* ATCC29213 as gram-positive pathogenic strain. The antimicrobial efficacies of all films were evaluated by placing films on the solid agar medium. Pieces of 0.5×0.5 cm of each ENR film (containing 20 μg of ENR) were used as well as the blank preparation (formulated exactly the same without adding ENR). The blank formulation was used as a

negative control to investigate the antimicrobial properties of chitosan. ENR suspension was also used as a positive control. Wells with 8mm diameters were prepared for delivery of aliquots of 20 μl of positive control (containing 1 $\mu\text{g}/\mu\text{l}$ of ENR) into medium. After incubation for 24h at 37°C, the zones of inhibition around the wells were measured using a calliper [19]. All experiments carried out in triplicate.

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Author contributions

Conceived and designed the experiments: SKF, AR, HAJ. Performed the experiments: SKF, KK. Analyzed the data: YHA, SKF. Provide research space and equipment: HAJ, TZS. Contributed reagents/materials/analysis tools: AR, YHA, TZS. Wrote the paper: AR, SKF.

Conflict of interest

The authors declare that they have no competing interests.

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