Abstract

The aim of this work was to formulate chitosan (CS) based nanoparticles (NPs) loaded with ketorolac tromethamine (KT) intended for topical ocular delivery. NPs were prepared using ionic gelation method incorporating tri-polyphosphate (TPP) as cross-linker. Following the preparation, the composition of the system was optimised in terms of their particle size, zeta potential, entrapment efficiency (EE) and morphology, as well as performing structural characterisation studies using FT-IR and DSC. The data suggested that the size of the NPs was affected by CS/TPP ratio where the diameter of the NPs ranged from 108.0 ± 2.4 nm to 257.2 ± 18.6 nm. A correlation between drug EE and the corresponding drug concentration added to the formulation was observed, where the EE of the NPs increased with increasing drug concentration, for up to 10 mg/mL. FT-IR and DSC revealed that KT was dispersed within the NPs where the phosphate groups of TPP were associated with the ammonium groups of CS. The *in vitro* release profile of KT from CS NPs showed significant differences (P<0.05) compared to KT solution. Furthermore, mucoadhesion studies revealed adhesive properties of the formulated NPs. The KT loaded NPs were found to be stable when stored at different storage conditions for a period of 3 months. The *ex vivo* corneal permeation studies performed on excised porcine eye balls confirmed the ability of NPs in retaining the drug on the eye surface for a relatively longer time. These results demonstrate the potential of CS based NPs for the ocular delivery of KT.

Introduction

Ketorolac tromethamine (KT) is a non-steroidal anti-inflammatory drug (NSAID) from the family of heterocyclic acetic acid derivatives (Li et al., 2015). It is effective in inhibiting post-operative eye inflammation, reducing conjunctivitis with no alteration of corneal opacity, yet has been shown to increase intraocular pressure (Sunil et al., 2013). KT is a non-selective cyclooxygenase (COX) inhibitor, being marketed as a racemate mixture (Savaroglu & Genc, 2013). It is administered as its tromethamine salt orally, intramuscularly, intravenously and as a topical ophthalmic solution (Sinha et al., 2009). KT is available as a topical ophthalmic solution (0.4% - 0.5% w/v), tablets (10 mg) and solution for injection (15-30 mg/ml). Topical KT is a more effective inhibitor of miosis than topical diclofenac sodium during cataract surgery (Unlu et al., 2010).

Only small amounts (1% - 3% w/v) of ophthalmic solutions such as those of KT eye drops penetrate the cornea and reaches intraocular tissues. Also, side effects of KT including gastrointestinal bleeding, perforation and peptic ulceration along with the short mean plasma half-life ($t_{1/2}$ ~5.5 h) has prompted research for the development of improved formulations of KT (Sinha, et al., 2009).

Most efforts in ophthalmic drug delivery have been devoted to increasing the corneal penetration of drugs with the final goal of improving the efficacy of treatments of different ocular diseases (Nagai et al., 2015). These attempts included the use of colloidal drug delivery systems, such as liposomes and biodegradable nanoparticles (NPs) and nanocapsules. However, the short precorneal residence time of these colloidal systems represents a limitation for treatment of ocular surface diseases, such as conjunctivitis and dry eye (Achouri et al., 2013). Substantial efforts have been directed towards the development of

ocular drug delivery systems that would prolong the drug precorneal residence, thus increasing ocular bioavailability (Abdelkader et al., 2014; Rupenthal, 2011).

NPs for ophthalmic drug delivery have been shown to enhance the ocular bioavailability while providing sustained release action (Hafner et al., 2015). There have been studies over the years examining the mechanism of drug release and reporting on ocular therapeutic efficacy of drugs delivered using nanoparticles (Zhu et al., 2012). Furthermore, such drug delivery systems are advocated as an ophthalmic drug delivery systems that may enhance dosage form acceptability while providing sustained release action (Kaur & Smitha, 2002).

Polymeric nanoparticles (PNPs) enable manipulation of surface properties for site-targeting of drugs; improve overall stability, enhance safety and efficiency for local and systemic drug delivery (Abdelkader et al., 2012). Their nanometre–size promotes effective permeation through membrane barriers enabling enhanced bioavailability. They have been shown to cross mucosal barriers, i.e. the intestinal, nasal and ocular membranes (Zhang & Zhao, 2015).

Chitosan (CS), obtained (from marine crustaceans) via the deacetylation of chitin is a naturally occurring and abundantly available polysaccharide (Ngo et al., 2015). Commercially available CS has an average molecular weight ranging between 3800 and 20,000 Daltons and is 66% to 95% deacetylated (Agnihotri et al., 2004). CS exhibits many advantages when used to formulate NPs for drug delivery applications, including biocompatibility, biodegradability, and low immunogenicity. The high positive charge density also confers its mucoadhesive properties and renders it attractive for mucosal drug delivery. CS possesses amino group on the D-glucosamine residue which upon protonation forms an electrostatic bond with negatively charged sialic acid on mucin. Thus, the interaction between mucin and CS, which is related to the electrostatic charge of both the molecules usually affect mucoadhesive characteristics of CS, depending on the pH of the

media (Silva et al., 2012). According to the Henderson–Hasselbach equation, the degree of ionisation of CS and mucin at pH 5 was 96.93% and 99.75% respectively (Qaqish & Amiji, 1999). Felt et al. (1999) identified CS as a well-tolerated mucoadhesive cationic polymer that affects epithelial pathways of the ocular tissues and thus, increasing the permeability of drug, making it suitable for the development of ocular dosage forms (Felt et al., 1999). Also, CS has a relatively low toxicity in ocular delivery; which has been confirmed by Di Colo et al. (2004) who described the absence of apparent irritation signs following topical instillation of CS solution (Di Colo et al., 2004). Its LD50 in laboratory mice is 16 g/kg body weight, which is close to sugar and salt (Karthikeyan et al., 2013). It has been reported that CS has wound healing properties, permeability enhancing characteristics as it enhances drug permeation through the cornea by opening the tight junctions between epithelial cells (Almeida et al., 2014). Furthermore, it has antimicrobial properties and is used in the production of wound healing dressings (Almeida et al., 2013). Thus, CS is considered as the most suitable polymer for the formulation of ophthalmic dosage forms (Fabiano et al., 2015). Therefore, CS-NPs are considered to be promising carrier systems for the drug and gene delivery. In addition, CS has special properties such as protonation of its amine groups under acidic condition; it can be cross-linked with other polymers such as tripolyphosphate (TPP) (Hassani et al., 2015) and sodium alginate to form NPs. The preparation of CS NPs via the cross-linking method was found to be simple and mild as reported previously by (Ji et al., 2011).

This paper discusses the design and characterisation of CS based NPs for the ocular delivery of KT. The specific objectives include:

- Investigating effect of formulation parameters on the size, size distribution and morphology along with physicochemical and spectroscopic characterisation of the formed CS-TPP NPs.

- Establishing the effect of drug concentration on the percentage entrapment efficiency (EE%); and conducting the *in vitro* release study of KT from prepared CS-TPP NPs.
- Studying the mucoadhesive properties and *ex vivo* permeation of KT across excised porcine corneas as well as testing the physical stability of prepared NPs at different storage conditions.

Experimental

Materials

Ketorolac tromethamine (KT), low molecular weight chitosan (deacetylation degree 85%, viscosity 20,000 cP), sodium tripolyphosphate (TPP) and porcine mucin were purchased from Sigma Aldrich chemicals Co., UK. Glacial acetic acid was purchased from Fisher Scientific, UK. All other solvents and buffer salts were obtained from Sigma Aldrich chemical Co., UK; were of analytical grade and used as received.

Preparation of chitosan-sodium tripolyphosphate nanoparticles (CS-TPP NPs)

CS-NPs were prepared using the ionic gelation method, previously reported by Calvo. et al.(Calvo et al., 1997b). In brief, CS solutions were prepared by sonication at various concentrations (1, 1.25, 1.75, and 2 mg/mL) in dilute acetic acid (1% v/v) at room temperature; the pH of the formed solution was 3.2. The pH of the CS solution was adjusted to pH 5.5 with 1M NaOH as at this pH, CS is ionised. Then, the solution was filtered using 0.45 µm filter membrane. An aqueous solution of 4 mL of TPP at various concentrations i.e. 0.5, 0.75, and 1 mg/mL was added drop wise to 10 mL CS solution with magnetic stirring (900 rpm for 30 min) at room temperature to yield a final volume ratio of (2.5:1 v/v) of CS:TPP.

Particle size and zeta potential measurement

The particle size and Poly Dispersity Index (PDI) of the prepared NPs were determined by dynamic light scattering (DLS) using Malvern Zetasizer 3000HSA at 25^{o}C. Zeta potential was measured by Laser Doppler Electrophoresis (LDE) using the same instrument under the same conditions. Size values were reported as mean hydrodynamic diameter (MHD). All measurements were undertaken on at least three independent preparations.

Physicochemical characterisation of prepared nanoparticles

- **Scanning electron microscopy (SEM)**

Morphological examination of KT-loaded CS-NPs was performed using Zeiss EVO 50, tungsten source SEM. A drop of NPs suspension was spread on a 10 mm glass cover slip which was fixed onto an aluminium support stub, air-dried and sputter coated with gold/palladium alloy. NPs morphology was determined at 20.00 kV.

- **Differential scanning calorimetry (DSC)**

The thermal analysis of CS, KT, the physical mixture (PM) of both CS and KT and also the freeze-dried powder of the KT-loaded NPs was carried out on Mettler Toledo DSC 822e0, Switzerland. The calorimeter was calibrated with indium; samples were weighed separately in aluminium pans; covered with aluminium lids and hermitically sealed using a pan press (Thermal Science, USA). Once in the calorimeter, the temperature of the pan was gradually increased from 25^{o}C to 300^{o}C at a rate of 10^{o}C/min. Nitrogen was purged at a flow rate of 45 mL/min. The data generated was consolidated using Mettler STARe software version 8.10.

Freeze drying of selected CS-NPs was carried out using TELSTAR – CRYODOS-I-rev0105MA freeze dryer (Telstar Industrial, S. L., Spain). Freeze dried samples were generated by subjecting 15 mL of NPs formulation to two drying steps following freezing.

- **Fourier Transform Infrared Spectroscopy (FT-IR)**

FT-IR spectrometer (Thermo Scientific Nicolet iS5, Thermo fisher, USA) was used to record the FT-IR spectra of CS, KT, their PM and freeze-dried powder of KT-loaded NPs. A clean diamond window was used to measure the background spectra. Sufficient amount (approx. 2-4 mg) of the sample was placed to form a thin film covering the diamond window. The FT-IR spectra were recorded at a resolution of 2 cm^{-1} with an average of 120 scans. The data was acquired and analysed using Omnic software (Omnic version 8.2, USA).

HPLC analysis of KT

Isocratic HPLC separation was achieved using reversed phase Phenominex C18 column (150mm×4.6mm×5µm).The mobile phase consisted of methanol and phosphate buffer (pH 3) with ratio of 55:45 v/v respectively and was selected to achieve good separation and resolution. A flow rate of 1 mL/min and injection volume of 10 µL were used to give an optimal signal-to-noise ratio with a reasonable separation time and run time was 20 min. The detection was performed at 314 nm.

Determination of drug entrapment efficiency

The NPs were separated from the aqueous medium that contained free drug by centrifuging the samples at 12,000 × g for a period of 30 min at 4°C. This was followed by the redispersion of NPs in PBS (pH 7.4). Varying amounts of KT were used (5, 10, and 15 mg/mL) and its effect on selected NPs ratio was determined. The content of KT in the supernatant was measured using HPLC method. The entrapment efficiency (EE%) of the NPs was determined using equation 1. All experiments were performed in triplicates.

$$\text{Entrapment Efficiency (EE \%)} = \frac{(Tp - Tf)}{Tp} \times 100 \qquad \text{Equation 1}$$

Where, Tp is the total amount of KT used in the preparation of NPs and T_f is the free KT present in the supernatant (Soppimath K.S et al., 2001).

Stability and mucoadhesion studies

The stability and mucoadhesive properties of KT loaded CS-NPs were investigated through incubation of these NPs with porcine mucin. Two *in vitro* methods were used in this study. The first method was based on the measurement of the viscosity of mucin dispersion (0.4mg/mL) in water before and after incubation at 35^{o}C in the presence of CS-NPs or CS solutions. Dispersion viscosity measurements were carried out by Brookfield viscometer (DV-II +Pro, USA). For each time interval, three measurements were taken, and average values were computed. The second method was employed to evaluate the influence of the mucin on the zeta potential of NPs. CS-NPs were incubated at 35^{o}C in an aqueous solution of mucin under stirring. At predetermined time intervals (0, 30, 60, and 120 min) during incubation, the zeta potential of the NPs was determined. Equal volumes of mucin solution (0.4 mg/mL) and CS-NPs as well as CS solution were vortexed for 1 min and the zeta potential of the mixtures were measured by Zetasizer (De Campos et al., 2004).

***In vitro* release study**

In vitro release studies were performed using standard Franz diffusion cells. The Franz–diffusion cells had a 15 mm diameter orifice (providing a diffusion area of 1.7 cm^2) and were thermostated by means of a water jacket connected to a VTC-220 heat circulator (Logan Instrument Corporation, Somerset, NJ, USA). Receptor chambers (12 mL volume) were filled with phosphate buffer (PBS, pH 7.4; osmolality 297 mOSm/kg) and stirred constantly using small magnetic bars. The donor and the receptor compartments were separated by a dialysis (cellophane) membrane with a molecular weight cut-off of 12,000-14,000 Da. The

membrane was pre-soaked in the receptor medium overnight prior to the experiment. The temperature was set at 35°C ± 0.5°C. The centrifuged samples i.e. each formulation consisting of 1 mL of the NP dispersion in PBS (pH 7.4) loaded with 5 mg of KT was placed in the donor compartment before occluding the chamber with Parafilm. 1 mL samples were withdrawn at predetermined time intervals for up to 6 h, and replaced with an equal volume of fresh receptor medium. The amount of KT released at each time point was determined by HPLC method.

***Ex vivo* permeation study**

Corneas used in the *ex vivo* experiments were obtained from porcine eye balls, which were freshly collected from a local abattoir. The eyes were examined for any visual defects. They were kept in a normal saline solution, directly transported to the laboratory and used within few hours of enucleation. Corneo-scleral buttons were dissected using standard eye bank techniques, and care was taken to minimise tissue distortion. Franz diffusion cell was used and the tissue was placed between the donor and the receiver compartments with the endothelial side facing the receptor compartment; temperature was maintained at 35°C. The receiver compartment and the donor compartment were filled with 12 mL of PBS solution (pH 7.4), and 1 mL of the formulation respectively. For each formulation, three corneas were used (n = 3). Samples (1 mL) were withdrawn from the receiver compartment for analysis of drug permeation at predetermined time points, and were replaced with fresh PBS solution to compensate the withdrawn sample volume. The amount of KT that permeated across the cornea was quantified by HPLC method (Gratieri et al., 2011).

***Ex vivo* data analysis**

The apparent permeability coefficient (P_{app}, cm/s) was calculated according to the following equation (Abdelkader et al., 2011)**.**

$$P_{app} = \frac{\Delta Q}{\Delta t(3600)ACo}$$ Equation 2

Where ΔQ/Δt is the permeability rate constant of KT across the excised porcine corneas. It was calculated from the gradient of the plot of the amount of KT permeated (Q) versus time (t); C_o is the initial drug concentration (µg/mL), A is the corneal surface area (cm^2) in contact with the formulation from the epithelial side and the release media from the endothelial side and 3600 is a factor used for the conversion of hours to seconds. The lag time (t_L) was determined by extrapolating the linear plot to the x-axis.

Stability of the prepared nanoparticles

Samples of the drug loaded NPs were stored in clean, capped 20 mL glass vials at three different temperatures (4°C, 22°C and 40°C) for three months. At specified time intervals, the stored samples were visually inspected for appearance, change in the colour and were also evaluated for change in the pH and particle size. The EE% of the stored samples was also determined after the 3 month time point.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) by GraphPad Software Version 3.05, USA. A *p* value of < 0.05 was considered to be statistically significant.

Results and discussion

Two different pH conditions were used in this study. The NPs were prepared at pH 5.5 initially as at this pH CS is highly ionised enabling dissolution. Once the NPs are formed, they were dispersed in PBS, pH7.4, which is the final formulation pH as the normal pH of the

tear fluid is between 6.5 and 7.4. The NPs were mixed with PBS to yield a final KT concentration of 5 mg/mL. As reported by Ludwig et al. (2005), it is necessary that the pH and tonicity of the solution instilled onto the ocular surface is physiologically compatible to minimise tissue irritation, reflex tearing and blinking, as this may lead to a loss of the dose. Also, any disturbance in the physiological pH of tear fluid results in increased blinking rate and lacrimation. Therefore, the pH of the ophthalmic preparations is adjusted close to the natural pH of the tear fluid to minimise irritation of the eye surface (Ludwig, 2005).

Particle size and zeta potential measurements

Results (Table 1) showed that the particle size is dependent on CS concentration, with size reduction being proportional to the CS concentration. At CS concentration of 1 mg/mL, the mean particle size was 108.0 ± 2.4 nm (F1), upon increasing CS concentration to 2 mg/mL, the average particle size increased to 257.2 ± 18.6 nm (F7). Thus, showing a significant difference ($P < 0.001$) between F7 and other formulations. The same applied when the CS concentration was increased from 1.25 mg/mL to 1.75 mg/mL, without altering the concentration of TPP (0.5 mg/mL); the size of the F4 and F6 formulations increased significantly ($P < 0.01$; Table1), which is an indication of dependence of the size of the NPs on the CS/TPP ratio (Aydın & Pulat, 2012). According to Zhao et al., the success of CS-NPs with nano-sized scale, the concentration of CS and TPP should be controlled at a suitable range (Zhao & Wu, 2006). Also, it is well accepted that the particle size of ocular formulations should not exceed 10 µm in order to avoid eye irritation as well as foreign body sensation. In addition, Zhou et al. (2013) reported that NPs with size range between 1 nm and 1000 nm improved passage of poorly water soluble drugs through the ocular tissues (Zhou et al., 2013). Also, ibuprofen loaded Eudragit RS100 nanosuspension with a mean particle size

of 100 nm and with a positive zeta potential has been successfully tested for ophthalmic applications previously (Pignatello et al., 2002).

The poly dispersity index (PDI) for all the prepared formulations ranged between 0.111 ± 0.041 and 0.274 ± 0.157, thus complying with the accepted limits (less than 0.4) of PDI. This indicates a relatively narrow particle size distribution and uniformity of the prepared NPs. The difference in the PDI values was found to be insignificant ($P > 0.05$) among the NP formulations (Table 1).

The increase in the mass ratio of CS led to an increase of the particle zeta potential. This was presumed to be due to the availability of free (unbound) amino groups on the CS molecule, contributing to an increased positive charge (Ji, et al., 2011). However, an increase in the TPP concentration led to a decrease in zeta potential and an increase in particle size as shown in (Table 1). For formulation F1, the zeta potential was found to be 22.9 ± 0.95 mV, however, when the TPP concentration was increased, as seen in F3, the zeta potential decreased significantly ($P < 0.05$) to 16.6 ± 0.26 mV. This decrease in the zeta potential was thought to be due to the neutralisation of positive charge contributed by the amino groups of CS molecule upon interaction with the negatively charged phosphate groups on TPP. However, upon comparing F1 and F7 formulations, there was no significant difference ($P > 0.05$) in their zeta potential values as the mass ratios for these two formulations were kept constant. On the other hand, when the CS concentration was doubled (formulation F7) in relation to formulation F3, a significant increase ($P < 0.001$) in the zeta potential was noted (Table 1). From the literature (Calvo, et al., 1997b), it is known that increasing CS concentrations as well as TPP concentrations will lead to increasing particle diameters and agglomeration of the produced NPs and similar observations were made in the present study.

The effect of changing the pH of CS solution from around (3.0 to 5.5) on the average particle size as well as zeta potential of prepared NPs was investigated. The size of the NPs increased, presumably due to the aggregation of particles as CS is highly protonated at pH 5.5. On the other hand, the zeta potential of the NPs decreased with increasing the pH of CS solution which may be due to the decreased positive charge density of the polymer with the rise in the pH, Figure 1. Our results are in agreement with the previous reports (Ji, et al., 2011); it suggests that the most suitable pH for NPs formation in this study would be pH 5.5.

Entrapment of KT in CS-TPP nanoparticles

The effect of different concentration of KT on the average particle size, surface charge and EE% of the prepared NPs has been investigated. It is clear from Table 2 that the average particle size of the NPs has been increased upon loading the drug compared to drug free NPs. Moreover, the particle size appeared to increase significantly ($P < 0.05$) with increasing concentration of KT i.e. from 5mg/mL to 10 mg/mL. Incorporation of KT into the NPs had an influence on the zeta potential as with formulations F4KT and F7KT the zeta potential decreased significantly ($P < 0.01$) upon incorporation of KT compared with drug free NPs i.e. F4 and F7, respectively (Table 1). This indicates an electrostatic interaction between the drug and CS. This is in agreement with what has been reported by Boonsongrit et al. (2006). The EE% of KT has increased upon increasing drug concentration (Table 2). When KT was loaded into the formulations at a concentration of 5 mg/mL, a significantly lower ($P < 0.001$) EE% was noted for formulation F1KT (5.23 ± 2.14) compared to the remaining NP formulations tested at this KT concentration. The EE% among the remaining formulations ranged between 18.60 ± 0.32 and 35.03 ± 4.40 with F2KT yielding a significantly ($P < 0.05$) higher entrapment than others barring F6KT. On the other hand, a significant increase ($P < 0.001$) in the EE%, i.e. from 22.0 ± 3.80 to 71.70 ± 1.55 was noted for F3KT formulation,

when the drug concentration was increased from 5mg/mL to 10 mg/mL respectively. The entrapment of KT within the NPs is presumed to be primarily that of matrix type. In addition, drug adsorption on the surface of NPs may also take place. DSC results (Figure 4) showed disappearance of the endothermic peak of KT indicating dispersion of KT in a molecular form due to electrostatic interaction and H-bonding between polymers used in the formulation process and the drug. Also, this has been confirmed by the decrease in the zeta potential of the NP formulations upon incorporation of different concentrations of KT (Table 2).

Effect of drug concentration on the Entrapment Efficiency

Drug loading into NPs has been optimised by varying drug concentrations i.e. 5, 10, and 15 mg/mL of KT. Data in Figure 2 demonstrate that increasing the concentration of KT within F2KT formulation from 5mg/mL to 10 mg/mL, there was a pronounced enhancement in the entrapment efficiency (EE%) ($P < 0.05$). However, when drug concentration was further increased to 15 mg/mL for the same formulation, the NPs did not show a significant variation in EE% ($P > 0.05$). Moreover, the drug precipitated within the NP dispersion presumably due to the drug concentration reaching the saturation capacity within NPs at a concentration of 10 mg/mL. Thus, increasing KT concentration during NP preparation from 5 mg/mL to 10 mg/mL showed a significant increase in the EE% ($P < 0.05$) due to increasing drug/polymer ratio and increasing the drug concentration available for encapsulation, while there is no change in EE% with higher concentration i.e. over 10 mg/mL. NPs with high EE% (loaded with 10 mg/mL KT) have been chosen for further characterisation studies.

Morphological characterisation of the nanoparticles

The nanoparticles appeared to be spherical and regular in shape with an average size of 200 nm (Figure 3). The diameter of the particles is in agreement with the particle size data obtained using dynamic light scattering technique. A low polydispersity index (PDI = 0.272 ± 0.010) further confirmed the homogeneity of the particle size distribution observed in the SEM images (Figure 3).

Differential scanning calorimetry (DSC) and FT-IR study

Figure 4 shows the DSC thermograms of KT, CS, their physical mixture (PM) and drug loaded NP formulation (F2KT), loaded with 10 mg/mL KT. The DSC trace of KT shows two endothermic peaks at 160^{o}C and 168.9^{o}C. The first peak is associated with the melting of KT while the second peak is related to the decomposition of the drug. These results are in good agreement with the data reported previously (Kavithab et al., 2010); (Chopra et al., 2008). On the other hand, the DSC thermogram of CS showed a broad endothermic peak over the temperature range 70-100^{o}C which is associated with water loss. The physical mixture of KT with CS, showed the same melting behaviour (168.2^{o}C) indicating no chemical interaction between the drug and the polymer. However, the endothermic peak characteristic of KT melting was not detected in the thermogram obtained for the KT-loaded NPs which indicates chemical interaction and suggests that KT was presumably entrapped within the NPs in the amorphous form.

The FT-IR spectra for KT, CS, their physical mixture (PM) and drug loaded NP formulation (F2KT) loaded with 10 mg/mL KT have been obtained over a range of 4000 - 500 cm^{-1}. Figure 5 shows major peaks (3,341 cm^{-1}; NH stretch, 1,145 cm^{-1}; C = O stretch

(diarylketone), and 1611 cm^{-1} within the spectrum of KT, this is most likely due to C-C aromatic stretching). There are three characteristic peaks of CS at 3355 cm^{-1} relating to -OH stretching, 2873 cm^{-1} corresponding to -CH stretching vibration, 1061 cm^{-1} of C-O-C, and 1601 cm^{-1} of NH_2. The FT-IR spectrum of loaded NPs confirms that the amino groups of CS are capable of interacting with anionic groups of TPP by ionic bonding as a primary binding force. Also, absence of the characteristic peak of the drug at 3,341 cm^{-1} demonstrates the fact that drug-polymer interactions may exist within the NPs.

Mucoadhesion study

The stability and interaction of CS-NPs with mucin has been investigated by measuring the viscosity of mucin dispersion when incubated at 35^{o}C for up to 2 h with KT loaded CS-NPs. The viscosity values were also compared with that of CS solution incubated under similar conditions. The viscosities of mucin dispersion were not significantly affected or altered ($P > 0.05$) by the addition of NP formulation F6KT (loaded with 10 mg/mL KT; Table 3). The viscosities of mucin dispersion were 4.73 ± 0.01 cP and 4.64 ± 0.100 cP at 0 and 120 min of incubation respectively, compared with viscosity of mucin alone (4.5 ± 0.003 cP). However, the viscosity of mucin dispersion showed a significant decrease ($P < 0.05$) upon incubation with CS solution (1.75 mg/mL). Comparing the viscosity values before and after incubation i.e. 5.93 ± 0.12 cP and 4.67 ± 0.12 cP at 0 and 120 min respectively. This may be attributed to the interaction of negatively charged carboxylic group of mucin with the ionised amino group of CS as has been reported previously (De Campos, et al., 2004). According to De Campos et al. (2004), this measurement is important as the viscosity is relevant to the blinking process which requires a low tear viscosity in order to avoid damage to the corneal epithelium. The current data indicates that no change in the viscosity of the tear film may be occurring upon instillation of the formulation. It has been reported that increasing viscosity

between 12 - 20 cP has doubled the ocular bioavailability, however, excessive increase of viscosity did not make any further difference but resisted free movement of eye lids during blinking and caused blurred vision. A highly viscous formulation may compromise proper instillation of the dosage form and may cause inconvenience during application (Lang et al., 2002).

Comparing the zeta potential values of mucin dispersion before and after incubation, there was a small decrease in the zeta potential for mucin + CS:TPP NP formulations (previously used in viscosity measurement) compared with mucin + CS solution. Figure 6 indicates that the zeta potential of CS solution mixed with mucin before incubation was 66.16 ± 0.21 mV and after incubation 61.5 ± 0.11 mV. This significant reduction ($P < 0.05$) could be related to interaction between the negative groups within mucin with the positive groups of CS. On the other hand, zeta potential of NPs before and after 2 h incubation was 23.2 ± 0.40 mV and 22.8 ± 0.41 mV, respectively which appears to be an insignificant change ($P > 0.05$). Thus, it could be concluded from the above data that the prepared NPs have a tendency to interact with mucin without altering the corneal viscosity.

***In vitro* release study**

The results of KT release from CS-NPs are depicted in Figure 7. The formulation which gave the slowest release rate was F3KT, as a complete release of the drug occurred within a period of 6 h compared to the release profile of pure KT solution. This formulation had a highest EE% of 71.70 ± 1.55 (Table 2). Also, there was no significant difference ($P > 0.05$) among the release patterns of other selected formulations and nearly more than 10% of KT was released in the first hour and the total amount of drug was released within 5-6 h compared to 3h release period for the KT solution. Thus, it was apparent that the NPs showed a more retarded drug release. Interestingly, when the drug release was modelled using the zero

order, first order, Hixson-Crowell and Higuchi's square root of time and Korsmeyer-Peppas equations (data not shown) (Shankar & Mishra, 2003), the drug release from the NPs resembled a zero order profile.

As it can be seen from the data (Figure 7), initial burst release of drug from the NPs occurred and this rapid release may primarily be due to the surface adsorbed drug that would easily diffuse into the release medium (Ji, et al., 2011). According to Agnihotri et al. (2004)**,** drug release via diffusion involves three steps. First, water penetrates into particulate system, which causes swelling of the matrix; secondly, the conversion of glassy polymer into rubbery matrix takes place, while the third step involves the diffusion of the drug from the swollen rubbery matrix (Agnihotri, et al., 2004). However, this was not the case for the release pattern of these formulations as nearly a steady amount of the drug was released from NPs independent of drug concentration entrapped inside the particles.

Physical stability of the prepared nanoparticles

A long term stability study was carried out by storing the prepared NPs at various environmental conditions. Visual inspection of the prepared NPs revealed that, loaded NPs stored for 3 months at $4^{o}C$ remained unchanged in terms of colour, absence of particle aggregation or sedimentation. Also, there was no change in the pH (pH 5.5). However, NPs stored at room temperature ($22^{o}C$), showed some characteristics of aggregation; however, upon gentle shaking the formulation reverted to a uniform dispersion, with its pH remaining unaltered. From the data in Figure 8, it is clear that the average particle size of the formulations showed an insignificant increase ($P > 0.05$) presumably due to the swelling of the NPs at pH 5.5 owing to the presence of the cationic polymer (Lin et al., 2010). On the other hand, the samples that were stored at $40^{o}C$ did not show any pH change. However, a

colour change i.e. to pale yellow was observed. This might be attributed to high temperature storage conditions that affected the drug adsorbed on the surface of NPs.

On the other hand, upon measuring the drug entrapment efficiency (EE%) of the stored NPs (containing 10 mg/mL KT), it was noted that the EE% of loaded CS-NPs stored at 4^{o}C, were the most stable formulations in terms of drug retention as the data indicated an insignificant change ($P > 0.05$) in the EE% of all the formulations (F2KT, F3KT, and F6KT) compared to their counterparts stored at 22^{o}C and 40^{o}C where the drug retention was significantly lower ($P < 0.01$) (Table 4). Moreover, formulations showed a remarkable decrease in the EE% at 40^{o}C. This stability data indicates that the storage condition of 4^{o}C was most suitable for storing NPs as they are able to retain their structural and morphological characteristics i.e. colour, consistency, pH and also EE%.

***Ex vivo* permeation study**

The *ex vivo* permeation profiles of KT from selected NPs were obtained using excised porcine corneas (Figure 9). The NPs have been chosen for corneal permeation study based on the size, zeta potential, EE% and physical stability. The following permeation parameters were calculated: steady-state flux, apparent permeability coefficient (P_{app}) and t_L (Table 5). The steady-state flux calculated for KT solution was 1.9-fold and 2.7-fold higher than those from F6KT and F3KT formulations, respectively. It is clear that steady-state flux from KT solution was significantly higher ($P < 0.001$) than that of NP formulations. However, the apparent permeability coefficient (P_{app}) of KT calculated for NPs was significantly ($P < 0.05$) lower than that of free drug solution. In addition, all the three permeation parameters for F3KT formulation were significantly ($P < 0.05$) lower compared to F6KT formulation. The permeation parameters obtained from NPs i.e. (F3KT) and (F6KT) was 3.77-fold and 1.89-fold lower than that for drug solution. These results correlated well with the *in vitro* release

data suggesting that the corneal permeation process of KT was dependent on its release characteristics and the permeation across the cornea is rate limiting.

With regard to the t_L data (Table 5), KT solution produced the shortest t_L and produced the highest steady-state flux. On the other hand, F3KT and F6KT produced longer t_L compared to KT solution. These results could be attributed to the drug that would take a longer time to release from the cross-linked polymer chains within NPs as opposed to free drug solution which permeated faster through the cornea.

Conclusion

The preparation of chitosan based nanoparticles loaded with ketorolac tromethamine in the present study had been successful. The DSC chromatograms and FT-IR spectra revealed that there was no chemical interaction between drug and CS suggesting that the drug is dispersed in a soluble form inside the NPs. The *in vitro* release study indicated that the prepared formulation has the ability to sustain drug release over a period of 6 h as opposed to yielding a rapid release profile, noted for KT solution (3 h). Also, the formulation exhibited stability and mucoadhesive characteristics when tested with mucin. The stability of NPs was found to be temperature dependent, with a lower temperature favouring the formulations. Corneal permeation studies confirmed that the prepared NPs have ability to retain the drug on the eye surface for longer time compared to the drug solution. Thus, these results demonstrate that the chitosan based nanoparticle systems are a promising candidate for the ocular delivery of ketorolac tromethamine in terms of improving its efficacy and safety.

Declaration of interest and acknowledgments

The authors would like to report that they have no conflicts of interest relevant to the contents of this article. This work is undertaken with the financial support from the Culture Affairs and Mission Department, Ministry of Higher Education, Cairo, Egypt.

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Table 1: Average particle size, zeta potential and poly dispersity index (PDI) of CS-TPP nanoparticles prepared at different concentrations of CS and TPP. Results are expressed as mean values ± SD, (n=3).

Formulation code	CS (mg/mL)	TPP (mg/mL)	Average particle size (nm)	Zeta potential (mV)	PDI***
F1	1	0.5	108.0 ± 2.4	22.9 ± 0.95	0.274 ± 0.157
F2	1	0.75	110.3 ± 1.8	20.1 ± 0.25	0.165 ± 0.019
F3	1	1	141.6 ± 6.8	16.6 ± 0.26**	0.157 ± 0.006
F4	1.25	0.5	151.3 ± 2.5	24.4 ± 0.40	0.205 ± 0.006
F5	1.25	0.75	175.2 ± 2.0	22.3 ± 0.23	0.111 ± 0.041
F6	1.75	0.5	181.7 ± 5.6*	27.8 ± 1.13	0.115 ± 0.076
F7	2	1	257.2 ± 18.6*	24.8 ± 1.69**	0.152 ± 0.008

*denotes significant difference ($P < 0.001$) between F6 as well as F7 and other formulations.

** Significant difference between F3 and F7 ($P < 0.001$).

*** PDI variation not significant among different NP formulations ($P > 0.05$).

Table 2: Effect of ketorolac tromethamine (KT) concentration on the average particle size, zeta potential and entrapment efficiency (EE%) of KT loaded CS-TPP nanoparticles. Results are expressed as mean values ± SD, (n = 3).

	KT concentration					
	5 mg/mL			**10 mg/mL**		
Formulation code	**Average particle size (nm)**	**Zeta potential (mV)**	**(EE%)**	**Average particle size (nm)**	**Zeta potential (mV)**	**(EE%)**
F1KT	227.4 ± 4.40	21.4 ± 0.21	5.23 ± 2.14	352.9 ± 16.10	20.0 ± 0.31	34.90 ± 0.52
F2KT	144.5 ± 6.90	19.4 ± 0.87	35.03 ± 4.40	202.2 ± 9.90	16.7 ± 0.42	73.48 ± 7.51
F3KT	154.9 ± 13.0	13.1 ± 0.40	22.0 ± 3.80	208.8 ± 2.40	16.5 ± 0.15	71.70 ± 1.55
F4KT	201.8 ± 6.40	15.3 ± 0.12	20.30 ± 1.10	281.5 ± 2.50	21.2 ± 0.52	53.55 ± 8.13
F5KT	220.0 ± 10.8	16.0 ± 0.21	18.60 ± 0.32	265.2 ± 1.30	21.1 ± 2.10	47.70 ± 4.38
F6KT	335.5 ± 6.40	24.1 ± 0.95	33.85 ± 7.42	299.5 ± 37.10	23.2 ± 0.65	50.10 ± 0.42
F7KT	291.9 ± 22.7	18.5 ± 0.98	23.10 ± 3.30	312.1 ± 4.20	17.4 ± 0.68	43.30 ± 0.99

Table 3: Viscosity values (cP) of mucin (0.4 mg/mL) before and after incubation with CS solution and CS-TPP nanoparticles. Results are expressed as mean values ± SD, (n = 3).

	Viscosity (cP)	
Time (min)	**Mucin + CS solution**	**Mucin + CS-TPP NPs**
0	5.93 ± 0.12	4.73 ± 0.01
5	5.92 ± 0.06*	4.73 ± 0.06
15	5.77 ± 0.06	4.69 ± 0.10
30	5.35 ± 0.14	4.67 ± 0.03
60	5.00 ± 0.10	4.66 ± 0.12
120	4.97 ± 0.12*	4.64 ± 0.10

*Significant difference ($P < 0.001$) between viscosities of (mucin + CS solution) at 5 min and at 120 min.

N.B. Significant differences ($P < 0.001$), ($P < 0.01$) upon comparing viscosities of (mucin + CS solution) at 5 min and at 120 min, respectively with mucin on its own as the viscosity of mucin alone without any solutions added was found to be (4.5 ± 0.003 cP).

Table 4: Entrapment efficiency (EE%) of different KT (10 mg/mL) loaded nanoparticle formulations following their storage at different temperature conditions for three months. Results are expressed as mean values ± SD, (n = 3).

		EE%		
Formulation code	**Time period (month)**	**4°C**	**22°C**	**40°C**
F2KT	0	73.48 ± 7.51*	73.48 ± 7.51	73.48 ± 7.51
	3	70.11 ± 0.97	62.32 ± 3.54	60.11 ± 7.33
F3KT	0	71.70 ± 1.55	71.70 ± 1.55	71.70 ± 1.55
	3	69.22 ± 0.94	67.23 ± 2.33	57.23 ± 0.82
F6KT	0	50.10 ± 0.42	50.10 ± 0.42	50.10 ± 0.42
	3	46.12 ± 1.12	41.33 ± 2.76	28.12 ± 9.88

* The reduction in the EE% was not significant ($P > 0.05$) for formulations stored at 4°C as opposed to their counterparts stored at 22°C and 40°C over a 3 month period.

Table 5: Steady-State Flux, Apparent Permeability Coefficient (P_{app}), and Lag Time (t_L) of KT. Results are expressed as mean values ± SD, (n = 3).

Formulation code	Steady-State Flux (µg/h)	$P_{app} \times 10^{-6}$ (cm/sec)	t_L (h)
Drug solution	117.65 ± 0.96*	5.31 ± 0.13	0.198 ± 0.34
F3KT	43.70 ± 0.88	1.41 ± 0.24	0.450 ± 0.41
F6KT	62.34 ± 1.25	2.81 ± 0.85	0.483 ± 0.22

* A significant difference was noted ($P < 0.001$) when the data from the three permeation parameters between the drug solution and the KT loaded formulations was compared.

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Figure 5: FT-IR spectra for KT, CS, their PM and loaded CS-NPs (F2KT).

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Figure 6: Zeta potential of mucin solution (0.4 mg/mL), CS solution (1.75 mg/mL), CS solution + mucin (1.75 mg/mL) and F6KT NPs + mucin before and after incubation (35^{o}C) at different time intervals. Results are expressed as mean values ± SD (n=3).

Figure 7: In vitro release profiles of KT from KT-loaded CS-NPs, i.e. F2KT, F3KT and F6KT in relation to KT solution. Results are expressed as mean values ± SD (n=3).

Figure 8: Average particle size of different KT-loaded CS-NPs following storage for 3 months (A) at 22^{o}C, (B) at 4^{o}C and (C) at 40^{o}C. Results are expressed as mean values ± SD (n=3).

Figure 9: Transcorneal permeation profiles of KT-loaded CS-NPs, i.e. F3KT and F6KT in relation to KT solution on excised porcine corneas. Results are expressed as mean values ± SD (n=3).