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Nanodispersion-loaded mucoadhesive polymeric inserts for prolonged treatment of post-operative ocular inflammation

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ABSTRACT

Mucoadhesive polymeric films incorporated with ketorolac tromethamine-loaded nanodispersion aiming the sustained delivery of the drug to the cornea have been developed and characterised for the treatment of post-operative ocular inflammation. Nanodispersions were prepared by ionic gelation method with various concentrations of chitosan and sodium tripolyphosphate. The developed nanodispersions were analysed for morphology, particle size, dispersion homogeneity, zeta potential, entrapment efficiency and drug release. The nanodispersion that showed the smallest particle size and the highest entrapment efficiency was incorporated in optimised HPMC E15 and Eudragit RL100/HPMC K4m films. The formulation with optimum physico-mechanical properties was selected to study its in vivo transcorneal permeation through freshly excised bovine cornea in comparison with the nanodispersion and the marketed eye drops (Acular®). The polymeric ocular film showed greater permeation than aqueous eye drops. Moreover, the ocular film revealed a prolonged anti-inflammatory effect compared to eye drops when applied to inflamed rabbit’s eyes.

Introduction

Ketorolac tromethamine (KT), an aryl-acetic acid non-steroidal anti-inflammatory drug (NSAID), is a non-selective cyclooxygenase (COX) inhibitor effective in inhibiting post-operative inflammation of the eyes following cataract surgery and intraocular lens implantation (Flach et al., 1988; Heier et al., 1999). Also, it is effective in reducing conjunctivitis with no alteration of corneal opacity (Fraser-Smith and Matthews, 1988) and does not increase intraocular pressure (Fu and Lidgate, 1986). It can be considered as a viable alternative to corticosteroids in treating ocular inflammation in the presence of pathogens (Fu and Lidgate, 1986). Ketorolac tromethamine is available in the market as aqueous eye drops. However, this conventional system cannot be considered optimal in the treatment of vision-threatening ocular diseases, as most of the drug is washed from the eye and only less than 5% of the administered drugs could penetrate the cornea to reach the desired intraocular tissue due to various mechanisms as lacrimation, tear dilution and tear turnover (Diebold et al., 2007; Baba et al., 2011). Considerable efforts have been directed towards the development of ocular drug delivery systems that would enhance the ocular bioavailability while providing sustained release. These approaches included the use of colloidal drug delivery systems, such as liposomes and polymeric nanoparticles (NPs). Polymeric nanoparticles offer an important advantage over liposomes being more stable (Calvo et al., 1997a). Polymeric nanoparticles enable manipulation of surface properties for site targeting of drugs, promote effective permeation through membrane barriers and slow down the rate of ocular elimination (Das et al., 2010).

The cationic polymer chitosan (CS), a deacetylated chitin, has attracted a great deal of attention because of its unique properties, such as acceptable biocompatibility, biodegradability and potential ability to enhance transmucosal drug delivery (Alonso and Sánchez, 2003). CS, as a cationic polysaccharide, is able to form nanoparticle dispersion when it comes in contact with specific multivalent polyions, such as sodium tripolyphosphate (TPP).

In order to take advantage of the bioavailability enhancement of nanoparticle dispersions and to overcome their disadvantage in being thermodynamically unstable as a result of their large surface area, mucoadhesive polymeric films loaded with nanoparticles were developed as a novel ocular drug delivery system (Kapanigowda et al., 2015).

An ideal polymer used for ocular inserts should be biocompatible, non-toxic, non-irritant and non-reactive with drug. It also should facilitate a stable and uniform rate and extent of drug release, have good adherence property at site of application and have no interference with the normal vital ocular functions such as vision and oxygen permeability (Kesarwani et al., 2011).

It was reported that the formulations, which were prepared with hydroxypropyl methylcellulose (HPMC) have great potential for the delivery of drugs to moist surfaces owing to their bioadhesive nature, ease of hydration and subsequent swelling and controlled drug release (Boateng and Popescu, 2016).

The purpose of this work was to develop and characterise HPMC-based polymeric ocular inserts containing KT-loaded CS/TPP nanodispersions, aiming to increase stability, prolong corneal contact time and control drug release and transcorneal permeation.
Materials and methods

Materials

Ketorolac tromethamine was kindly supplied by European Egyptian Pharmaceutical Industrial Company (Alexandria, Egypt). Chitosan (low Mw, viscosity, 20 cps, degree of deacetylation 85%) was purchased from Aldrich Chemical Co. (Stingham, Germany). Sodium tripolyphosphate was purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxypropyl methylcellulose (HPMC K4m and HPMC E15) were kindly supplied by Colorcon (Kent, England). Glycerin and polyethylene glycol 400 (PEG 400) were obtained from El Nasr Pharmaceutical Chemical Company (Cairo, Egypt). Eudragit RL100 was purchased from Rhom Pharma (Hamburg, Germany). All other reagents were of analytical grade. Cellophane membrane (Spectra/Por Membrane, molecular porous MWCO: 6–8000) was obtained from Spectrum Laboratories (Los Angeles, CA).

Methods

Preparation of chitosan/tripolyphosphate (CS/TPP) nanoparticles

CS nanoparticles were prepared according to ionic gelation method (Calvo et al., 1997b). Different concentrations of CS solution in 1% v/v acetic acid (0.3, 0.45, 0.6, 0.75, 1 and 1.2 mg/ml) were prepared and adjusted to pH 5.5 with 1 N NaOH solution. Aqueous solutions of TPP of various concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2 mg/ml) were prepared. Four millilitres of TPP aqueous solution were added dropwise to 10 ml of CS solution under magnetic stirring at room temperature. The formulations, which showed opalescence, were considered as suitable plain CS/TPP nanodispersions for incorporation of KT. For the preparation of KT-loaded nanoparticles (NPs), 1 ml of 0.5% w/v KT solution was added first to the CS solution. The TPP solution was then added to the medicated CS solution as explained above for plain nanodispersions. The composition of plain nanodispersions is represented in Table 1.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>CS (mg/ml)</th>
<th>TPP (mg/ml)</th>
<th>Physical appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.30</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>C2</td>
<td>0.30</td>
<td>0.40</td>
<td>v</td>
</tr>
<tr>
<td>C3</td>
<td>0.30</td>
<td>0.60</td>
<td>v</td>
</tr>
<tr>
<td>C4</td>
<td>0.30</td>
<td>0.80</td>
<td>v</td>
</tr>
<tr>
<td>C5</td>
<td>0.30</td>
<td>1.00</td>
<td>v</td>
</tr>
<tr>
<td>C6</td>
<td>0.30</td>
<td>1.20</td>
<td>v</td>
</tr>
<tr>
<td>C7</td>
<td>0.45</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>C8</td>
<td>0.45</td>
<td>0.40</td>
<td>x</td>
</tr>
<tr>
<td>C9</td>
<td>0.45</td>
<td>0.60</td>
<td>x</td>
</tr>
<tr>
<td>C10</td>
<td>0.45</td>
<td>0.80</td>
<td>v</td>
</tr>
<tr>
<td>C11</td>
<td>0.45</td>
<td>1.00</td>
<td>x</td>
</tr>
<tr>
<td>C12</td>
<td>0.45</td>
<td>1.20</td>
<td>x</td>
</tr>
<tr>
<td>C13</td>
<td>0.60</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>C14</td>
<td>0.60</td>
<td>0.40</td>
<td>x</td>
</tr>
<tr>
<td>C15</td>
<td>0.60</td>
<td>0.60</td>
<td>x</td>
</tr>
<tr>
<td>C16</td>
<td>0.60</td>
<td>0.80</td>
<td>x</td>
</tr>
<tr>
<td>C17</td>
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<td>1.00</td>
<td>x</td>
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<tr>
<td>C18</td>
<td>0.60</td>
<td>1.20</td>
<td>x</td>
</tr>
<tr>
<td>C19</td>
<td>0.75</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>C20</td>
<td>0.75</td>
<td>0.40</td>
<td>x</td>
</tr>
<tr>
<td>C21</td>
<td>0.75</td>
<td>0.60</td>
<td>x</td>
</tr>
<tr>
<td>C22</td>
<td>0.75</td>
<td>0.80</td>
<td>v</td>
</tr>
<tr>
<td>C23</td>
<td>0.75</td>
<td>1.00</td>
<td>x</td>
</tr>
<tr>
<td>C24</td>
<td>0.75</td>
<td>1.20</td>
<td>x</td>
</tr>
</tbody>
</table>

x: clear solution; v: opalescent dispersion; /: aggregates.

Evaluation of KT-loaded nanoparticles

Particle size, polydispersity index and zeta potential. The mean particle size and size distribution of freshly prepared nanoparticle dispersions (plain and KT-loaded NPs) were determined using Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). The measurements were performed after diluting the samples by 100-fold with water at ambient temperature.

Entrapment efficiency (EE %). The amount of drug entrapped in the nanoparticles was determined by calculating the difference between the total amount of KT used to prepare the NPs and the amount of free, non-entrapped, drug remaining dissolved in the aqueous suspending medium. The drug-loaded nanoparticles were separated from the supernatant containing unloaded KT by centrifugation at 13,500 rpm and 4°C for 60 min (cooling ultracentrifuge 30–30 K, Sigma, Germany). The amount of free drug in the supernatant was determined by spectrophotometric analysis (UV spectrophotometer; Shimadzu, Columbia, MD) at 321 nm. The calibration curve was done in a concentration range of 2–16 μg/ml and the R2 value of the regression line was 0.999. The drug entrapment efficiency (EE %) of NPs was calculated as indicated below:

$$EE \% = \frac{A - B}{A} \times 100$$

where A is the total amount of drug in the nanodispersion and B is the free amount of drug in the supernatant.

In vitro release of KT from CS/TPP nanodispersions. The in vitro release testing was carried out in a USP-rotating basket dissolution apparatus (Schleuniger pharmacon, Switzerland). The baskets were replaced by glass cylindrical tubes (2.5 cm in diameter and 10 cm in length) containing one millilitre of KT-loaded NP. One end of the glass tube was attached to the shaft of the USP dissolution tester apparatus instead of the baskets and the other end was tightly covered with a cellophane membrane soaked overnight in phosphate buffer saline (PBS, pH 7.4) and immersed in 50 ml of PBS (pH 7.4). The release study was performed at 35 ± 0.5°C and a rotation speed of 50 rpm. Aliquots of 2 ml of the release medium were withdrawn at specific time intervals and replaced with equal volume of PBS. The samples were analysed spectrophotometrically at 321 nm against samples withdrawn at respective time intervals from plain nanodispersions treated similarly. The experiment was performed in triplicate, and the percentage released of KT was calculated.

Release kinetics of KT from nanodispersions. In order to understand the kinetics of drug release, the results of the in vitro drug release study of different NP dispersions were fitted to various kinetic equations like zero (cumulative % drug released vs. time), first order (log cumulative % drug remained vs. time), Higuchi’s model (cumulative % drug release vs. square root of time) and Korsemeyer–Peppas (log % released vs. log time). The release rate constant (K) and correlation coefficient (R2) were calculated. The best fitting model, the one with the highest correlation coefficients between the observed and the fitted data, was selected.

Transmission electron microscopy (TEM). The morphological examination of the selected optimum nanoparticle dispersion was performed by high-resolution transmission electron microscope (HR-TEM, Tecnai G20, FEI, the Netherlands). A drop of the NP dispersion was placed on a carbon-coated grid and left for two minutes to allow the adsorption of the nanoparticles to the carbon film. Then, a drop of 2% w/v aqueous solution of uranyl acetate was applied for one minute, and the excess was removed with distilled water, and the sample was examined by TEM.
Freeze drying. Aliquots of three different batches of the selected optimum nanoparticle dispersion were freeze-dried to study the Fourier transform infra-red (FTIR) spectroscopy of the nanoparticles. The aliquots were frozen in liquid nitrogen and lyophilised for 48 h at a pressure of 0.05 mmHg and a primary drying temperature of −40 °C (Freezone lyophilizer; Labconco Corporation, Kansas City, MO).

Fourier transform infra-red (FTIR) spectroscopy. FTIR spectra of KT, CS, CS/TPP physical mixture and freeze-dried KT-loaded CS/TPP nanoparticles were recorded on a Genesis II Mattson FT/IR spectrometer (Madison, WI). The scanning was done in the range of 400–4000 cm⁻¹ at a speed of 2 mm/s and a resolution of 4 cm⁻¹ at room temperature. The band width was measured at 50% of height of the peaks.

Stability studies on nanodispersions. Samples of the selected optimum nanoparticle dispersion were sealed in 30 ml clear glass vials and stored away from light for 6 months at different temperatures (4 °C and 25 °C). The stored samples were visually inspected for coalescence, settling and changing in colour. The entrapment efficiency as well as the mean particle size and pH were determined and compared to the freshly prepared samples.

Preparation of polymeric ocular inserts containing KT-loaded nanoparticles

Two different polymeric films (NF1 and NF2) containing KT-loaded nanodispersions were prepared by solvent-cast technique (evaporation method) (Giovino et al., 2012). For the formulation of NF1 an aqueous solution of HPMC E15 (10% w/v) and glycerine (10% w/v) was prepared, whereas for the formulation of NF2 a methanolic solution of HPMC K4M (1.25% w/v), Eudragit RL 100 (2.5% w/v) and PEG 400 (30% w/v) was prepared. The nanodispersion that showed optimum physicochemical properties was incorporated, by stirring for 3 h, into the polymeric solutions in a ratio of 1:1. The mixture was placed into a Petri dish (diameter = 5 cm) and dried in an oven at 40 °C for 24 h. The prepared film was cut by a punch into small round pieces (diameter = 0.5 cm).

Evaluation of the prepared polymeric films (inserts)

Determination of thickness. A screw gauge was used to measure the thickness of films, which were cut from different places of the same formulation. The thickness was determined at three different points of the film (Abhilash et al., 2005) and the test was done on ten films.

Determination of folding endurance. The films (n = 10) were folded in the centre, between the fingers and the thumb, and then opened. The process was repeated until the films showed breakage or cracks in its centre. The total folding operation is termed as folding endurance value (Khana et al., 1997).

Evaluation of weight uniformity. The weight variation test was carried out by weighing 10 films that were cut from different places of the same formulation and their individual weights were determined by using the digital balance.

Determination of surface pH. Surface pH was determined by allowing the films to swell in a closed Petri dish at room temperature for 30 min in 0.1 ml distilled water. The swollen films were removed and placed on a pH paper. After 60 s, the developed colour was compared with the standard colour scale. A mean of three readings was recorded.

Determination of percentage of moisture loss. Three films from each formulation were weighed individually (initial wt) and placed in a desiccator containing anhydrous calcium chloride. After three days, the films were taken out and reweighed (final wt) (Gorle and Gattani, 2009). The percentage moisture loss was calculated using the following equation:

\[
\text{Moisture loss } \% = \frac{\text{initial wt} - \text{final wt}}{\text{initial wt}} \times 100
\]

Determination of percentage of moisture absorption. The percentage of moisture absorption test was carried out to ensure physical stability and integrity of the inserts. The films were weighed (initial wt) and placed in a desiccator containing 100 ml of saturated solution of sodium chloride, where a relative humidity of 75±5% was maintained. After three days, the films were taken out and reweighed (final wt). The percentage of moisture absorption was calculated using the following equation:

\[
\text{Moisture absorption } \% = \frac{\text{final wt} - \text{initial wt}}{\text{initial wt}} \times 100
\]

Determination of swelling index. The swelling index of the prepared mucoadhesive ocular inserts was determined gravimetrically. A small filter paper (diameter = 55 mm) was placed on 1% w/v agar gel plate. This experimental set-up was left to equilibrate for 60 min (constant weight of filter paper). The accurately weighed films (n = 3) were then placed individually on the upper side of the filter paper and the weight of the swollen films (the weight of equilibrated filter paper subtracted from the weight of filter paper together with the swollen film) was determined at different time intervals. The swelling index was calculated using the following equation:

\[
\text{Swelling index } (S_w) = \frac{\text{wt} - \text{w}_0}{\text{w}_0} \times 100
\]

where, \(S_w\) = equilibrium percent swelling. \(w_0\) is the weight of swollen insert after time \(t\) and \(w_0\) is the original weight of insert at zero time.

Determination of bioadhesive strength. Freshly excised conjunctiva membrane of an adult goat, obtained from a local slaughter house, was used as a model membrane for the measurement of bioadhesive strength. The membrane was washed before use with distilled water and isotonic phosphate buffer (pH 7.4). Bioadhesive strength of the film (n = 3) was determined using a modified physical balance (Sultana et al., 2006). Sections of membrane were fixed with mucosal side out onto the opening of two glass vials using rubber bands. One pan of the balance was removed and a vial was connected to the balance instead in an inverted position (first vial). The other vial (second vial) was placed on a height adjustable plate below the first vial. The sample was attached to the mucosa of the first vial. On the other pan of the balance, an empty beaker was placed and the balance was tared. Then, the height of the second vial was adjusted so that the mucosal surfaces of both vials came in intimate contact. A time of contact of 2 min was given, and then, water was dropped into the beaker until the film and the mucosal tissue were detached. The weight of water required to detach the film from the conjunctival surface was recorded. The force required for detachment of the film from the mucosal surface (adhesion force of the film) was calculated in Newton (N), using the following equation (Mishra and Gilhotra, 2008):

\[
\text{Adhesion force } (\text{N}) = \frac{\text{bioadhesive strength } (\text{g}) \times 9.81}{1000}
\]

Determination of mechanical properties of the films. Mechanical parameters, tensile strength and elongation at break, were calculated from the load time profiles of the films using INSTRON® tensile tester (Norwood, MA) equipped with a 5000 N load cell. Upper and lower grips of the sample with a gauge length of 5×1 cm, were attached to the crosshead and the base plate, respectively,
in such a way that the former was located exactly 2 cm above the latter. The crosshead was moved upwards at a speed of 30 mm/s until the film broke. At the time of film break, the magnitude of applied force and the increase in film length were recorded. The tensile strength and elongation % were calculated according to the following equations (Khan et al., 2000):

\[
\text{Tensile strength} \left( \frac{g}{mm^2} \right) = \frac{\text{breaking force} (g)}{\text{cross sectional area of sample} (mm^2)}
\]

\[
\text{Elongation at break} \% = \frac{\text{difference in length at breaking point} (mm)}{\text{original length} (mm)} \times 100
\]

The results were reported as the mean (±SD) of three replicates.

Sterilisation and test for sterility for ocular inserts. In the present study, the films \((n = 10)\) were sterilised by exposing them to ethylene oxide (EtO) gas. The characteristics of the gas steriliser are: EtO pressure 650 bar, EtO/CO2 mixing ratio of 30%/70%, sterilisation temperature 55 °C, relative humidity 32%, exposure time 8h. After the sterilisation cycle, air-flushing cycles for 48 h were applied to remove the EtO retained gas. After sterilisation, the inserts were evaluated for sterility according to the USP guidelines for sterility testing. For microbial contamination, test fluid thioglycolate medium was used, while soybean-casein digest medium was used to test the presence of fungi with incubation under aerobic conditions (El-Bagory et al., 2010; Singh et al., 2010). The tests were carried out in triplicate under aseptic conditions to avoid accidental contamination of the product during the test.

In vitro drug release study of KT from inserts. The in vitro drug release from the ocular inserts was studied using a USP dissolution testing apparatus through cellophane membrane, as described above under in vitro release study of nanodispersions.

Ex vivo transcorneal permeation. Transcorneal permeation of the drug from the optimum formulations (nanodispersion, C9b and ocular insert, NF2) in comparison to Acular® eye drops was performed through freshly excised bovine corneas. The whole bovine eye ball was transported from a local butcher shop to the laboratory in cold normal saline within 1 h of slaughtering the animal. The corneas were carefully excised along with 2–4 mm of the surrounding scleral tissue and washed with cold normal saline until the washing was free from proteins. The experiment was performed in a USP paddle dissolution apparatus (Schleuniger Pharmatron, Switzerland). For the experiment, glass cylindrical tubes (diameter = 0.5 cm) open from both ends were used. The corneas were attached to one end of the glass cylinders by means of gauze. The formulations were placed onto the corneas inside the cylinders. The glass tubes were then tied to the paddle shafts and the position of the cylinders was adjusted so that, the corneas and the above formulations were immersed in the receptor medium. The study was done in 50 ml PBS (pH 7.4) at 35 °C ± 0.5 with a constant stirring rate of 50 rpm. Aliquots of the withdrawn samples were analysed for KT by measuring the absorbance at 321 nm (UV spectrophotometer; Shimadzu) and the percent of KT permeated per unit area was calculated.

In vivo ocular anti-inflammatory study. The experiment was conducted in New Zealand rabbits having an average weight of 1.5 kg. All experiments were approved by the Institutional Animal Ethics Committee, Misr University for Science and Technology, Egypt and they comply with the ARRIVE guidelines. Ocular inflammation was induced by installing Turpentine liniment BP in the rabbits’ eyes (Mitra et al., 2000). The inflammation inhibitory effect of the formulated ocular insert loaded with KT nanoparticles (NF2) and the marketed eye drops (Acular®) was compared. Rabbits were divided randomly into two groups of three each. Animals were housed in an institutional animal room under standard conditions with free access to food and tap water. Ocular insert was inserted into the left eye of rabbits of Group I and Acular® eye drop was instilled into the left eye of rabbits of Group II. The right eye of all the rabbits was treated with normal saline and served as the control. After 15 min of administration of formulations in respective eyes, 100 μl of turpentine liniment was instilled in both eyes of all the rabbits. All eyes were then evaluated for polymorphonuclear leukocyte (PMN) migration by counting PMN in tear fluid. Three drops of normal saline were instilled into the inferior cul-de-sac of the rabbit eye. After gentle mixing, 50 μl of the tear fluid was withdrawn with the help of micropipette (Eppendorf® Research® pipette, adjustable volume, 2979698, Sigma Aldrich, St. Louis, MO) at 1, 2, 3, 4, 5 and 6 h following instillation of turpentine liniment. The withdrawn tear fluid was diluted with Turke’s fluid in WBC pipette and number of PMN was counted in Neubauer’s haemocytometer. This part of anti-inflammatory testing completed after 6 h then began the second part of the study, where the inflamed left eyes of both groups received a treatment. Inserts and Acular eye drops (3 drops) were applied to the left eyes of Group I and Group II, respectively. The right eyes of both groups (control) were left without treatment. The ocular inflammation was evaluated at 0, 6 and 12 h after applying the medication. Severity of discharge, chemosis and conjunctival congestion were scored from 0 to 3, while the clarity of iris structure and degree of flare were scored from 0 to 2 (Homburger, 1983).

Stability study on ocular inserts. The stability study was carried out on the selected optimum formulation (NF2), according to International Conference on Harmonisation (ICH) guidelines, by storing replicates of ocular inserts (packaged in aluminium foil) in a humidity chamber, with a relative humidity of 75% and a temperature of 40 °C (Gorle and Gattani, 2009). Samples were withdrawn at 0, 1, 2, 3 and 6 months. For stability testing, they were examined for break down or degradation as well as physical characteristics (viz. thickness, weight, and folding endurance). Samples were also analysed for in vitro drug release at 6th hour after 6 months.

Statistical analysis. Student’s t-test was used to analyse data of two groups obtained in different experiments at the 0.05 level of significance using GraphPad InStat 3 software. One-way analysis of variance (ANOVA) analysed all other data obtained for more than two groups by the same software.

Results and discussion

Preparation of chitosan/tripolyphosphate (CS/TPP) nanodispersions

The ability of CS to form nanoparticles, when it comes in contact with TPP, relies on the formation of inter- and intramolecular cross-linking between the amino groups of CS and the phosphate groups of TPP (Hu et al., 2008). However, upon addition of TPP to CS, three kinds of formulations were observed: clear solution, colloidal dispersion and aggregated suspension (Table 1). A clear solution was observed when concentration of TPP was lower or equal to that of CS, which indicates that the concentration of TPP was below the limits needed for the formation of crosslinks. When concentration of TPP exceeded that of CS to a certain limit a colloidal dispersion was formed, but a further increase in TPP concentration resulted in the formation of a precipitating suspension.
It is believed that, at appropriate quantities, TPP forms dominant inter- and intramolecular crosslinks with CS to form small nanoparticles. However, when the available quantity of TPP increases the superfluous TPP would probably link the nanoparticles to form larger particles (Stoica et al., 2013). This finding indicates that the formation of the nanoparticles is dependent on concentrations of both chitosan and TPP. This result is in accordance with that reported by Liu and Gao (2009) in preparation of ionically cross-linked chitosan nanoparticles of ciprofloxacin hydrochloride.

The formulations, which showed an opalescent dispersion (C2, C3, C9, C16, C22 and C23) were assumed therefore to be nanodispersions and were loaded with KT. Interestingly, a turbidity was noticed upon the addition of KT solution to CS solution before the addition of the crosslinking agent (TPP), indicating the formation of nanoparticles at that stage. This finding could be attributed to the fact that KT is a weak acid (pKa = 3.54) and the major fraction of which is anionic at physiological pH (Tiwari and Udupa, 2003). Therefore, the opposite charges of CS and KT resulted in a spontaneous formation of nanoparticles, which was further cross-linked by adding TPP. De La Fuente et al. (2008) reported that, the establishment of electrostatic interactions of the active compounds, either with the positively charged polymer CS or with the negative polyanion TPP, is the main mechanism that governs their entrapment.

**Evaluation of the prepared nanoparticle formulations**

The KT-loaded CS/TPP nanodispersions (C2, C3, C9, C16, C22 and C23) were characterised for particle size, zeta potential, dispersion homogeneity, entrapment efficiency and *in vitro* drug release.

**Particle size, polydispersity index and zeta potential**

From Table 2, it is clear that, the size of all KT-loaded formulations were in nanorange, except C16, which was in the micron size, and was therefore excluded from further investigations. In addition to that, all formulations showed a positively charged zeta potential.

The entrapment efficiency (EE %) of KT-loaded CS/TPP nanoparticles ranged from 40.11% to 57.99%. The excellent solubility of KT (pKa = 3.5) at pH 5.5 (pH of the CS/TPP nanodispersion), may have hindered the high entrapment (Boonsongrit et al., 2006).

The polydispersity index (PDI) is a factor that represents the dispersion homogeneity. The PDI of the formulations ranged from 0.23 to one. The formulations having a PDI below 0.5 (C9, C22) were of higher homogeneity.

**Effect of concentration of TPP.** As noticed from Table 2, when the concentration of CS was kept constant and TPP concentration was increased (C2/C3 and C22/C23), a significant increase (p < 0.05) in particle size was observed which was associated with a significant decrease (p < 0.05) in zeta potential. As CS/TPP NPs are formed by the interaction between the protonated amino group in CS and the polyanion phosphate groups in TPP, it is to be expected that, with a higher TPP concentration, neutralisation of $\text{NH}_3^+$ increases with a subsequent decrease in zeta potential. In addition, it is believed that, upon increasing the quantity of anionic TPP, the superfluous TPP would link more nanoparticles to form larger particles and decrease the homogeneity of the nanodispersion (Xu and Du, 2003). However, the TPP did not significantly affect the entrapment efficiency of KT which may be due to the fact that the addition of TPP comes after the interaction between CS and KT (Boonsongrit et al., 2006).

**Effect of CS concentration.** Raising the initial concentration of CS during the encapsulation process while keeping the concentration of TPP constant (C3/C9 and C16/C22) increased the number of protonated amino groups in the system, which was evidenced by a significant increase in zeta potential (p < 0.05). This resulted in stronger electrostatic attractions between CS and TPP with the subsequent formation of more compact particles of smaller size. Moreover, with increased CS concentration, more drug could be incorporated in the nanoparticles due to increased interaction between the oppositely charged CS and KT (Latha et al., 2012) (Table 2).

**Effect of KT concentration.** Based on the previous results the nanodispersion C9 was selected as the optimum formulation because it possessed the smallest particle size, the highest entrapment efficiency, the smallest PDI and a relatively high zeta potential. This formula was therefore selected to study the effect of increasing the concentration of KT, from 0.3 mg/ml (C9) to 5 mg/ml (C9b), which is the concentration of the market product Acular® on the physicochemical properties of the prepared nanodispersions.

The particle size significantly increased (p < 0.05) by increasing the initial concentration of KT (Table 2). However, the entrapment efficiency of KT decreased despite the particle size enlargement, because the entrapment was calculated as percentage in relation to the total amount of drug present in the dispersion, which is in the case of C9b much greater. This finding is in accordance with results observed by Wu et al. (2005), which may indicate that the nanoparticles have certain drug-loading capacity governed by the amount of CS present. Thus, any excess drug would be just electrostatically adsorbed on the surface of particles and is probably easily separated by centrifugation leading to an increased amount of unentrapped drug and a decreased EE% (Hou et al., 2015). Also, the zeta potential significantly (p < 0.05) decreased almost to the half, which is probably due to the partial deposition of the negatively charged KT on the particle surface reducing thereby the total net charge (Rampino et al., 2013).

**In vitro release of KT from nanodispersions**

As illustrated in Figure 1(a) all the nanodispersions showed a biphasic release pattern with an initial burst release followed by a more gradual sustained release. The initial burst of KT was probably due to the free KT present in the nanodispersion, which was

<table>
<thead>
<tr>
<th>Formula</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Poly dispersity index (PDI)</th>
<th>Entrapment efficiency (EE) (%)</th>
<th>Drug released after 7 h (%)</th>
<th>Diffusion exponent (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>615.1 ± 21.5</td>
<td>+19.3 ± 1.5</td>
<td>0.84 ± 0.06</td>
<td>40.1 ± 1.3</td>
<td>51.6 ± 1.54</td>
<td>0.38</td>
</tr>
<tr>
<td>C3</td>
<td>729.2 ± 18.6</td>
<td>+15.1 ± 1.3</td>
<td>1.01 ± 0.02</td>
<td>44.4 ± 1.4</td>
<td>46.4 ± 1.76</td>
<td>0.32</td>
</tr>
<tr>
<td>C9</td>
<td>147.2 ± 15.3</td>
<td>+19.4 ± 0.8</td>
<td>0.22 ± 0.04</td>
<td>57.9 ± 0.9</td>
<td>43.6 ± 1.66</td>
<td>0.25</td>
</tr>
<tr>
<td>C16</td>
<td>2040 ± 20.6</td>
<td>+16.7 ± 0.6</td>
<td>0.31 ± 0.03</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C22</td>
<td>3888 ± 24.1</td>
<td>+24.8 ± 1.9</td>
<td>0.39 ± 0.07</td>
<td>42.3 ± 1.6</td>
<td>53.7 ± 1.05</td>
<td>0.38</td>
</tr>
<tr>
<td>C23</td>
<td>878.3 ± 19.9</td>
<td>+17.3 ± 2.0</td>
<td>0.62 ± 0.08</td>
<td>40.1 ± 1.1</td>
<td>47.3 ± 1.63</td>
<td>0.31</td>
</tr>
<tr>
<td>C9b</td>
<td>565.1 ± 26.3</td>
<td>+10.5 ± 0.3</td>
<td>0.58 ± 0.04</td>
<td>41.8 ± 2.14</td>
<td>65.9 ± 1.55</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*Not determined.*
not entrapped in the NPs, in addition to the amount of drug adsorbed onto the surface of the NPs (Shafie and Fayek, 2013). During this phase, the structure integrity of the particles is believed to be maintained, while the second sustained phase is probably characterised by pore formation and particle deformation (Panyam et al., 2003), which allowed slow diffusion of encapsulated drug.

It is clear from Figure 1(a) that, increasing the concentration of CS from 0.3 to 0.45 mg/ml (C3/C9) was associated with a decrease in the percentage of KT released. This may be attributed to increased viscosity of the formulations by increasing the concentration of the polymer.

Also, the concentration of crosslinking agent (TPP) affected the release of KT. Increasing the concentration of TPP, from 0.4 to 0.6 mg/ml (C2/C3) and from 0.8 to 1 mg/ml (C22/C23), showed a significant decrease (p < 0.05) in the percentage of drug released as shown in Figure 1(a) and Table 2. This finding is probably because increasing the concentration of crosslinking agent may result in increased density of the polymer and reduction in its chain mobility. This could cause the formation of more stable and rigid spheres which would probably delay the drug release (El-Nahas and Hosny, 2011).

Figure 1b illustrates the release of KT from C9b and the commercial eye drops Acular®. It can be seen that about 100% of the KT was released after 5 h from the eye drops (Acular®), while in case of the CS/TPP nanodispersion (C9b) only about 65% of KT was released within 7 h. This could reflect the ability of the prepared CS/TPP nanocarrier to sustain the release of KT.

**Release kinetics of KT from CS/TPP nanodispersions**

In order to determine the release model that describes the pattern of drug release, the in vitro release data were analysed according to different models. According to the highest linearity, all CS/TPP nanodispersion formulations (C2, C3, C9, C22, C23 and C9b) were best fitted to Korsmeyer–Peppas release kinetics. It is obvious that the values of n for all the tested formulations were less than 0.45 indicating that the release from nanoparticles followed Fickian diffusion through polymeric matrix, but it was greater than 0.45 in case of C9b (0.534) indicating a non-Fickian diffusion which probably means that the polymer erosion plays also a role in the drug release pattern (Table 2).

**Transmission electron microscopy (TEM)**

Morphological examination was carried out on the selected optimum nanoparticle dispersion (C9b). As shown in Figure 2 the CS/TPP nanoparticles were mostly destinct, almost spherical with smooth surface. Furthermore, the nanoparticles appeared to be considerably smaller (20–30 nm) than the average particle size that was determined by the zetasizer. This result was consistent with that obtained by Wu et al. (2005), who reported that the CS/TPP NPs loaded with ammonium glycerrhizinate showed particle size more than 120 nm by dynamic light scattering but was in the range of 20–80 nm when measured with TEM (Wu et al., 2005). This may be attributed to that the zetasizer measures the apparent size of particles, including hydrodynamic layers that form around hydrophilic particles leading to an overestimation of NPs’ size (Prabha et al., 2002).

**Fourier transforms infra-red spectroscopy (FTIR) of KT-loaded nanoparticles**

The spectrum of pure KT in Figure 3(a) shows peaks at 3346.5 cm⁻¹ [NH stretch], 3354.21 cm⁻¹ [OH (acid)], 1759.08 cm⁻¹ [C=O stretch (acid)], 1170.79 cm⁻¹ [C=O stretch (diaryl ketone)]. The spectrum also demonstrates peaks at 1471.68 cm⁻¹ and 1413.18 cm⁻¹ due to C=C aromatic and aliphatic stretching and peaks at 704.02 cm⁻¹, 731.02 cm⁻¹, 781.17 cm⁻¹, 798.52 cm⁻¹ for C-H bending (aromatic). In Figure 3(b), the spectrum of chitosan shows a broad band at 3421 cm⁻¹, which corresponds to the amine and hydroxyl groups, a peak at 2879.72 cm⁻¹ caused by –OH stretching and an absorption band of the carbonyl (C=O) stretching of the secondary amide (amide I band) at 1656.85 cm⁻¹. The bending vibrations of the N–H (N-acetylated residues, amide II band) appear at 1597.06 cm⁻¹ (Sankalia et al., 2007). The peaks at 1423.47 and 1379.1 cm⁻¹ belong to the N–H stretching of the amide and ether bonds and N–H stretching.

**Figure 1.** Percent of KT released versus time in PBS (pH 7.4) at 35°C from (a) nanodispersions of different CS/TPP compositions containing 0.3 mg/ml KT (n = 3), (b) nanodispersion of 0.45 mg/ml CS and 0.6 mg/ml TPP containing 5 mg/ml KT (C9b) compared to the commercial eye drops Acular® (n = 3).

**Figure 2.** TEM photomicrograph of KT-loaded CS/TPP nanoparticles of the formula with optimum characteristics (C9b).
(amide III band), respectively. The peaks observed at 1076.28 and 1029.99 cm\(^{-1}\) correspond to the secondary hydroxyl group (characteristic peak of \(-\text{CH-OH}\) in cyclic alcohols, C-O stretch) and the primary hydroxyl group (characteristic peak of \(-\text{CH}_2\text{-OH}\) in primary alcohols, C-O stretch) (Chen et al., 2004). The FTIR spectrum of the physical mixture of KT and chitosan (Figure 3(c)) exhibited the characteristic bands of the drug, which indicates the absence of chemical interaction between them. However, observing the FTIR analysis of KT-loaded nanoparticles (C9b) (Figure 3(d)), the characteristic peak of CS at 3421 cm\(^{-1}\) (OH) became wider and was shifted to 3456 cm\(^{-1}\) indicating the enhancement of hydrogen bonding. Moreover, many of the characteristic peaks of the drug disappeared or became overlapped by those of CS, which confirms the interaction between KT molecules which are anionic at physiologic pH and the cationic CS during the formation of nanoparticles. This supports the observation stated above that, when KT solution was added to CS solution nanoparticles began to form even before the addition of TPP.

Figure 3. FTIR spectra of (a) KT, (b) CS, (c) physical mixture of KT and CS (d) lyophilised KT-loaded nanoparticles of the formula with optimum characteristics (C9b).
**Stability studies on nanodispersion**

Samples of the NP formulation with optimum characteristics (C9b) were stored at different temperatures (25°C and 4°C) for six months and were evaluated at specified time intervals for visual examination, particle size, pH and entrapment efficiency. The results are demonstrated in Table 3. After 6 months, an increase in particle size and a decrease in EE% were detected. It was also noticeable that, the systems were more stable at lower temperature. This result indicates that, during a six months storage a slight aggregation occurred; however, the particles were still in the nanometre range. The decrease in EE% indicates some leakage of the entrapped drug from the nanoparticles during storage, which is probably due to the high hydrophilicity of the drug. Formulating the nanodispersion in form of ocular film thus seemed not only efficient to prolong corneal contact time and control the drug release but also to enhance the physical stability of the nanodispersion.

**Evaluation of nanodispersion-loaded polymeric inserts (films)**

On visual examination, all prepared films were found to be translucent, uniform, and homogenous with no visible cracks or imperfections and easily to be peeled from the casting surface proving the efficiency of solvent casting method for preparation. The composition of the films with regard to type and content of polymers and nature of plasticiser affected the characteristics of the films considerably.

**Thickness and weight uniformity**

Thickness and weight of the films were almost uniform (Table 4), but NF1 formula was significantly thicker and heavier than NF2. The increased thickness and weight observed for NF1 films is probably due to greater total polymer concentration (10%) compared to NF2 films (3.75%) (Gorle and Gattani, 2009).

**Folding endurance**

The folding endurance is frequently used to estimate the ability of the film to withstand repeated bending and folding. As shown in Table 4, NF1 films (HPMC E15/glycerine) started to crack but not break after 285 folding, while NF2 (Eudragit RL100/HPMC K4m/PEG 400) did not show any cracks up to 300 folding. This result indicates the good film-forming property of the polymer mix as well as the good plasticising effect of PEG 400 which was previously reported to impart flexibility and softness to polymeric films (Rajput et al., 2011).

**Mechanical properties**

The results listed in Table 4 show that NF1 films had higher tensile strength but lower elongation percentage than NF2 films. The higher tensile strength revealed by NF1 may be due to its consistence of higher polymer percentage. It is reported by Rajput et al. (2011) that the increase in polymer concentration results in high tensile strength, as the chain pack becomes denser with greater inter-chain interactions, which would make it harder for chains to entangle and lowers their flexibility. In general, polymer films that have a greater tensile stress have less ductility. In contrast, the settlement of plasticiser between the polymer molecules increases the distance between the monomers and this could reduce the linkage between monomers and loosen the chain pack, leading to a reduction in tensile strength. The increased free volume between the polymer chains results at the same time in a greater chain mobility and film flexibility (Abha et al., 2011). Therefore, the higher ductility and lower tensile strength of NF2 films indicate that the plasticiser (PEG 400) was more efficient in making bonds with the polymer molecules, which subsequently weakened the bonds between polymer chains. It has been reported that the plasticising effect of PEG 400 on Eudragit RL films is mainly due to the interaction between carbonyl groups in trimethyl-ammonioethyl methacrylate chloride segments of Eudragit RL and hydroxyl groups of PEG 400 (Fujimori et al., 2005). Also, hydrogen bonds between PEG 400 and the polymer molecules could impart flexibility to the film (Tandale and Wagh, 2011). In addition, the presence of HPMC K4m combined with Eudragit improves the flexibility of the polymeric films as the HPMC forms hydrogen bonds with the Eudragit RL100 molecules (Mohamed et al., 2013). Furthermore, based on the definition of plasticisation, the elongation should increase with increase in the plasticiser concentration (Wypych, 2004), thus NF2 films containing 30% PEG 400 as plasticiser would have greater flexibility than NF1 films having only 10% glycerine.

**Surface pH**

The pH of ophthalmic formulations may affect their comfort in the eyes. Ophthalmic products that produce irritation increase tear fluid secretion, and hence, a rapid loss of the drug may occur with a probable reduction in the therapeutic response (Mohamed et al., 2013). The ideal pH for maximum comfort should be in the range of 7.2 ± 0.2, but the eye can tolerate a pH range between 4.5 and 11.5 due to the buffering action of the tears (Shanmugam et al., 2011). The NP-loaded ocular films NF1 and NF2 had a pH within the acceptable range (Table 4), which would eliminate any difficulty or irritation when placed in the cul-de-sac of the eye.

**Percentage moisture loss and moisture absorption**

It is clear from the data recorded in Table 4 that, NF1 film that was prepared with the hydrophilic polymer, HPMC E15 alone, showed a greater percentage in both moisture loss and moisture absorption than NF2 film containing in addition to HPMC K4m the

---

**Table 3.** Evaluation of particle size, pH and entrapment efficiency of KT-loaded nanodispersion that showed optimum physicochemical characterisation (C9b) during storage for 6 months at 25°C and 4°C (mean ± SD, n = 3).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (months)</th>
<th>25°C</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>0</td>
<td>565.1 ± 26.3</td>
<td>565.1 ± 26.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>705.7 ± 17.8</td>
<td>589.4 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>934.5 ± 21.6</td>
<td>764.1 ± 17.1</td>
</tr>
<tr>
<td>pH</td>
<td>0</td>
<td>6.5 ± 0.0</td>
<td>6.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.3 ± 0.0</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.0 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Entrapment efficiency (EE %)</td>
<td>0</td>
<td>418.2 ± 2.1</td>
<td>418.2 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>39.9 ± 1.2</td>
<td>40.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30.4 ± 2.9</td>
<td>35.9 ± 2.2</td>
</tr>
</tbody>
</table>

**Table 4.** Physicochemical and mechanical evaluation of ocular polymeric films (mean ± SD, n = 3) incorporated with the nanodispersion that showed optimum physicochemical characterisation (C9b).

<table>
<thead>
<tr>
<th>Film code</th>
<th>Film thickness (mm)</th>
<th>Weight uniformity (mg)</th>
<th>Folding endurance</th>
<th>Tensile strength (g/mm²)</th>
<th>Elongation at break (%)</th>
<th>pH</th>
<th>Moisture loss (%)</th>
<th>Moisture absorption (%)</th>
<th>Swelling index (24h) (%)</th>
<th>Force of adhesion (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF1</td>
<td>0.46 ± 0.01</td>
<td>23.0 ± 0.16</td>
<td>285</td>
<td>6.7 ± 0.2</td>
<td>26.3 ± 0.8</td>
<td>7.0 ± 0.0</td>
<td>6.8 ± 0.05</td>
<td>12.66 ± 0.21</td>
<td>36.21 ± 0.43</td>
<td>0.095 ± 0.05</td>
</tr>
<tr>
<td>NF2</td>
<td>0.36 ± 0.004</td>
<td>14.7 ± 0.12</td>
<td>&gt;300</td>
<td>2.6 ± 0.4</td>
<td>65.3 ± 1.7</td>
<td>6.5 ± 0.1</td>
<td>3.6 ± 0.06</td>
<td>6.42 ± 0.22</td>
<td>29.13 ± 0.62</td>
<td>0.240 ± 0.08</td>
</tr>
</tbody>
</table>
hydrophobic polymer Eudragit RL 100. The hydrophobicity of Eudragit in NF2 thus offered a considerable hindrance in moisture transfer (Tandale and Wagh, 2011). The increased moisture absorption shown by NF1 may be also attributed to higher hygroscopicity of the plasticiser (Shivalingam et al., 2014). It was previously reported that PEG 400 (NF2) possesses about half the hygroscopicity of glycerine (NF1) (Uglea, 1998).

**Swelling index**

Hydration of mucoadhesive polymers permits a mechanical entanglement of polymer chains, which exposes the bioadhesive sites and initiates intimate contact of the film with the mucous network (Eouani et al., 2001). The swelling index of the developed NP-loaded polymeric films was evaluated and illustrated in Figure 4(a). The results reveal that, the swelling index of NF1 was greater than NF2. This is probably due to the presence of higher percentage of HPMC in the former. The ability of HPMC to absorb water is due to the presence of hydrophilic groups, which permit water entry into the polymer network leading to swelling and, as the chains uncoil and extend, more locations become available for hydrogen bonding and further molecular entanglements (Ju et al., 1995). Furthermore, the hydrophobicity and poor water uptake of Eudragit RL100 in NF2 might have led to resistance of the matrix network structure to the movement of water (Deore et al., 2013). The reduction in water uptake of HPMC films by the addition of Eudragit-RL100 was in agreement with the results reported by Semalty et al. (2008) in the evaluation of buccal films of glibenclamide. However, the difference in percentage swelling of the formulae was not in accordance with the difference in HPMC concentration and the hydroporphicity of Eudragit, as the swelling index at 120 min of NF1 was only greater by 20% than that of NF2 (Table 4). This finding may be attributed on one hand due to the high proportion of quaternary ammonium groups in Eudragit RL100, which could promote water uptake (Shivhare et al., 2010) and on the other hand due to the difference between HPMC K4m and HPMC E15 in side chain substitution and viscosity. HPMC K (HPMC 2208) has lower average percent methoxyl as well as hydroxypropoxyl substitution than HPMC E (HPMC 2910) (Ford, 2014), which would indicate greater proportion of OH groups in the polymer. The viscosity of HPMC K4m is also greater than HPMC E15. It is reported that about 6.2 mol of water were associated with each polymer repeating unit of low viscosity HPMC E (Joshi and Wilson, 1993) in contrast to 8.5 mol of water being associated with each polymer repeating of high viscosity HPMC K (Ford and Mitchell, 1995).

The swelling index profiles of the formulae seemed to be biphasic with an initial fast rate of swelling due to rapid formation of a surface ‘gel’ layer on exposure to aqueous media. This was followed by a reduced water uptake rate, as when the thickness of the gel layer surrounding the matrix increases, further rapid water penetration into the matrix is retarded (Adler et al., 1999).

The NF1 film reached a swelling equilibrium (maximum water uptake) faster than NF2, as illustrated in Figure 4(a). This finding indicates that the water influx in low viscosity HPMC E probably weakened the network integrity of the polymer, thus influenced structural resistance of the swollen matrix, which in turn resulted in pronounced erosion of the lose gel layer (El-Khodairy, 2001).

**Bioadhesive strength**

Bioadhesion is a very important aspect for maintaining high drug levels at the site of administration and preventing drainage of ophthalmic formulation. The rate and the extent of patch hydration and swelling affect considerably the patch adhesion, thus any polymer with good swelling property is expected to be a good candidate for bioadhesive application (Alanazi et al., 2007). In contrast to this belief, NF1 exhibited lower force of adhesion than NF2 despite possessing higher swelling index (Table 4). NF1 may have exceeded the critical degree of hydration, where optimum swelling and bioadhesion occurs, as over-hydration leads to an abrupt drop in adhesive strength due to disentanglement at the polymer tissue interface (Semalty et al., 2008; Deore et al., 2013). The high content of plasticiser (30% PEG 400) in NF2 film could be another reason for the greater bioadhesion. It was previously reported that, the adhesion of Eudragit films is markedly increased when the concentration of the plasticiser is greater than 25% (Snejdrova and Dittrich, 2012). Also, it was established that polyethylene glycols (PEGs) have a mucoadhesion promoting effect (Giovino et al., 2012). Furthermore, the presence of positively charged functional groups in the polymer chain of Eudragit RL100 (NF2) enhances the strength of bioadhesion when compared with the neutral non-ionic polymer HPMC, which is unable to interact electrostatically with mucin (Werner, 2004; Sankalia et al., 2008).

**In vitro drug release study of KT from ocular inserts**

The release of KT from nanodispersion-loaded ocular inserts (NF1 and NF2) in comparison to nanodispersion (C9b) was investigated. It is clear from Figure 4(b) that, the incorporation of KT-loaded CS nanodispersion into polymeric films significantly (p < 0.05) decreased the rate of KT release. This is because the polymeric systems containing the nanodispersions have to dissolve first in order for the NPs to escape into the buffer solution (Giovino et al., 2012). From Figure 4(b), it is also noticeable that, the release of the drug from NF1 was significantly greater than from NF2.
Furthermore, the films showed different release patterns. The release profile of NF1 was biphasic with an initial burst release phase followed by a sustained release phase in contrast to NF2, which showed a slow monophasic gradual release.

It was previously reported that, the rate and extent of drug release, as well as the mechanism of release from polymeric films are affected by the type and amount of polymer as well as the polymer hydration characteristics (Williams et al., 2002; Salsa et al., 2003). As expected, the higher swelling characteristics of NF1 was accompanied by an increase in drug release. The hydrophilic polymer, HPMC E15 in NF1, absorbs water quickly which results in rapid dissolution of the polymer molecules imparting therefore high porosity of the film, which in turn permits rapid diffusion of external solvent. This enhances the erosion rate of the film (Semalty et al., 2008) and allows easy release of free KT. This fast initial release of the drug was followed by a sustained release phase due to slow release of the drug from the chitosan nanoparticles. On the other hand, the presence of Eudragit RL100 in NF2 film caused a significant retardation of KT release from the polymeric matrix. Tiwari et al. (2003) reported that, the incorporation of Eudragit RL100 into polymeric films increases the hydrophobicity, which accordingly decreases the porosity and wetting of the matrix and delays its dissolution, which results subsequently in sustained diffusion of the drug from the matrix (Tiwari et al., 2003). This result was in agreement with that obtained by Ali and Shafie (2012), who found that, the incorporation of hydrophobic polymer (Eudragit RL100) into hydrophilic polymer (methyl cellulose) retarded the release of timolol maleate.

**Ex vivo permeation study**

Based on all previous investigations, nanodispersion-loaded ocular insert NF2 was selected for ex vivo permeation study, as it showed better mechanical characteristics, greater mucoadhesive strength and slower drug release in comparison to NF1. The permeation of the drug through excised bovine cornea from the selected formula (NF2) and the marketed eye drops (Acular®) was investigated. The results illustrated in Figure 5(a) reveal that, the amount of KT permeated from nanoparticle-based formula was significantly ($p < 0.05$) greater than from Acular® eye drops despite the fact that, swelling of the polymer network to uncoil the polymeric chains is crucial for the escape of the NPs out of the polymer matrix. According to Handerson Hasselbalch equation, KT with a $pK_a$ value of 3.5 will be mainly in the ionised form at pH 7.4, the matrix. According to Handerson Hasselbalch equation, KT with a $pK_a$ value of 3.5 will be mainly in the ionised form at pH 7.4, the pH of Acular® eye drops and tear fluid, which would reduce the permeability of the drug through the cornea. A similar result was obtained by Dave and Paliwal, who studied the effect of pH on permeation of aceclofenac through goat cornea (Dave and Paliwal, 2014). On the other hand, when drug is formulated as nanoparticle, its penetration across the cornea can be significantly improved due to possible uptake of the NPs by corneal epithelial cells (Nagai and Ito, 2014). In addition, this uptake could be enhanced by using positively charged NPs due to electrostatic interaction with negatively charged mucin of cornea. This finding was consistent with Vega et al. (2008), who found that ex vivo corneal permeation of flurbiprofen-loaded nanoparticles exhibited fourfold increase in the corneal penetration than the aqueous drops. Furthermore, chitosan is known to possess permeation enhancing properties for hydrophilic drug molecules due to its ability to open epithelial tight junctions by structural reorganisation of the proteins. This allows a greater paracellular transport of drug molecules (Bravo-Osuna et al., 2007). It was also reported that chitosan improves intracellular transport of drug molecules possibly by enhancing endocytic activities.

**In vivo ocular anti-inflammatory study**

The in vivo anti-inflammatory study consisted of two parts. The first part involved the application of ocular insert loaded with KT nanoparticles (NF2) and marketed KT eye drops (Acular®) in rabbits’ eyes followed by the induction of inflammation by turpentine liniment. The PMN count in tears was used for the evaluation of the inflammation inhibitory effect of ocular insert compared to eye drops. The results illustrated in Figure 5(b) show that after the first hour, the PMN count was clearly less ($p < 0.05$) for the eyes treated with eye drops compared to the eyes treated with the ocular insert. This indicates that the inhibitory effect of eye drops was greater than the insert during the first hour, which may be due to the larger area of absorption available for the eye drops compared to the insert. However, because of longer time of contact, the ocular inserts produced a more effective and longer lasting effect ($p < 0.05$) till the end of the first part of the study (6 h). Afterwards began the second part of the study, which involved the application of insert and eye drops in the inflamed eyes for treatment. As shown in Table 5 and Figure 6, the ocular insert loaded with KT nanoparticles could suppress the inflammatory signs including discharge and lid closure, chemosis, conjunctival congestion, undefined iris structure and flare (foggy appearance) much better than the eye drops. The results of anti-inflammatory studies thus indicate a better potential of ocular inserts over the eye drops in controlling ocular inflammation. The lower effect of eye drops may be attributed to the fast drainage of the majority of drug through the nasolacrimal duct, in addition to the short residence time of the hydrophilic eye drops on the surface of cornea (Li et al., 2013). In contrast, the ocular film possesses a longer ocular residence time, due to the mucoadhesive properties of
both, the film and the loaded chitosan nanoparticles, which would thus prolong ocular absorption. Furthermore, the ocular availability of the drug was found to be greater when formulated as nanoparticles (Morsi et al., 2016) due to endocytic uptake mechanism (Qaddoumi et al., 2003).

**Physical stability study**

The stability study was carried out on the selected ocusert (NF2). It was found that, during the period of the study (6 months), there was no significant change \( (p < 0.05) \) in the physicochemical properties of the film (thickness, weight and folding endurance) and the total % drug released at 6th h (data not shown). This result shows that the film was of good stability.

**Sterility testing**

After incubation of all the prepared broths, (negative control, positive control and test for the selected formula (NF2) in each type of media) growth of bacteria and fungi was checked. The overall results of the sterility test showed that, the prepared ophthalmic formulation passed the sterility test, as there was no evidence of the growth found in the negative control or the test tubes. These results proved the efficiency of ethylene oxide gas as a method of sterilisation at the mentioned operation conditions.

**Conclusions**

In the present study, CS/TPP nanodispersions were successfully prepared by ionic gelation method. In order to improve the stability of nanodispersion and prolong the contact time with corneal surface the formulation with optimum characteristics was incorporated into polymeric ocular inserts. \textit{Ex vivo} permeation study and \textit{in vivo} ocular anti-inflammatory study has shown the superiority of nanodispersion-loaded ocusert over the commercial eye drops (Acular\textsuperscript{R}). This formulation could be thus considered as a potential ophthalmic drug delivery system to enhance and prolong the ocular availability of anti-inflammatory drugs in postoperative surgery.

| Table 5. Anti-Inflammatory effect of ocular insert (NF2) and Acular\textsuperscript{R} eye drops in turpentine liniment-induced ocular lesions in rabbits (n = 3). |
|-----------------|--------|--------|--------|
| Eye treatment   | 0 h    | 6 h    | 12 h   |
| Control         | 13.00 ± 0.00 | 13.00 ± 0.00 | 10.33 ± 0.57 |
| Eye drops       | 11.00 ± 1.00 | 9.33 ± 0.57  | 7.66 ± 0.57  |
| Ocular insert   | 7.33 ± 0.57  | 5.00 ± 1.00  | 2.00 ± 0.00  |

**Figure 6.** \textit{In vivo} anti-inflammatory study in albino rabbits. The signs of ocular inflammation induced in rabbits’ eyes at 0, 6 and 12 h after applying (A) ocular insert loaded with C9b (NF2) to the left eyes of group I and (B) Acular\textsuperscript{R} eye drops to the left eyes of Group II in comparison to (C) untreated eyes (right eyes of Groups I and II).
inflammation, which would improve patient compliance due to less frequent administration.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**


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