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Chitosan Based Nanocarriers for Indomethacin Ocular Delivery

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Two different chitosan (CS) nanocarriers namely nanoparticles and nanoemulsion were developed to prolong Indomethacin (IM) precorneal residence time and to improve its ocular bioavailability the main limitations in its management of post-operative inflammation and intraocular irritation after cataract extraction. CS-nanoparticles were developed by modified ionic gelation of CS with tripolyphosphate while nanoemulsion was prepared by spontaneous emulsification technique. Transmission electron microscopy revealed regular well-identified spherical shape. The nanoparticles had a mean size of 280 nm, a zeta potential of + 17 mV and high loading efficiency of 84.8 % while the mean size of nanoemulsion was affected by the nature of the surfactant used and varies between 220-690 nm. In vitro release studies, performed under sink conditions, revealed small initial burst release during the first hour followed by slow gradual drug release of 76 and 86% from nanoparticles and nanoemulsion respectively during a 24 h peroid. In vivo studies and histopathological examination revealed that eyes of rabbits treated with nanoemulsion showed clearer healing of corneal chemical ulcer with moderate effective inhibition of polymorph nuclear leuckocytic infiltration (PMNLs) compared with nanoparticles preparation. Moreover, following topical instillation of CS-nanoemulsion to rabbits, it was possible to achieve therapeutic concentration of IM in the cornea through out the duration of the study and fairly high IM level in inner ocular structure, aqueous humor. These levels were significantly higher than those obtained following instillation of IM solution. Therefore, CS nanocarriers developed in this study were able to contact intimately with the cornea providing slow gradual IM release with long-term drug level thereby increasing delivery to both external and internal ocular tissues.

Key words: Chitosan, Nanoparticles, Nanoemulsion, Indomethacin, Ocular delivery, Ionic gelation

INTRODUCTION

The major problem in ocular therapeutics is to provide and maintain an adequate concentration of the drug at the site of action. Traditional dosage forms for ocular drugs delivery have been and remain, solutions. Conventional ocular solutions have the disadvantage that most of the instilled drug is lost within the first 15-30 seconds after instillation and less than 5% of the applied drug penetrates the cornea and reaches intraocular tissues (Kaur *et al*., 2002 & Vansantvliet *et al.*, 1998).

Limited drug absorption through the lipophilic corneal barriers is mainly due to short precorneal residence time related to tear turn-over, rapid nasolacrimal drainage of instilled drugs from the tear fluid and non-productive absorption through the conjunctiva (Zhang *et al.*, 2004; Indu *et al.*, 2002). This poor ocular bioavailability implies the necessity of frequent instillations to achieve the therapeutic effect, a situation that is frequently associated with undesirable side effects caused by systemic drug absorption.

Many efforts in ophthalmic drug delivery have been devoted not only to prolong the contact time of the vehicle at ocular surface, but at the same time slow down the elimination of the drug and increasing its corneal penetration. These attempts include the use of inserts (Ding, 1998), collagen shields (Hill *et al.*, 1993) and colloidal systems such as liposomes (Pleyer *et al.*, 1993; Bochot *et al.*, 1998) and nanoparticles (Losa *et al.*, 1991; De Campos *et al.*, 2004) and nanocapsules (Losa *et al.*, 1993; De Campos *et al.*, 2003) have received the greatest attention because of their size is less than 1 mm.

Taking into account these limitations and also the fact that the cornea and conjunctiva have a negative charge, it was thought that the use of mucoadhesive cationic chitosan (CS) polymer which might interact intimately with these extraocular structures would increase the concentration and residence time of the associated drug. CS has unique properties such as acceptable biocompatibility and biodegradability with low toxicity and high charge density, (Xu *et al.*, 2003; Dornish *et al.*, 1997). Moreover, CS exhibits interesting physico-chemical characteristic with a good potential for ocular drug delivery such as

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bioadhesion (Illum, 1998; Paul & Sharma, 2000), prolonging the corneal residence time (De Campos *et al.*, 2004; 2001; Felt *et al.*, 1999) and penetration-enhancing properties, which were initially attributed to the modulation of the tight junction barrier between epithelial cells (Koch *et al.*, 1998; Schipper *et al.*, 1997). It was found that, CS increases cell permeability by affecting both paracellular and intracellular pathways of epithelial cells in a reversible manner without affecting cell viability or causing membrane wounds (Dodane *et al.*, 1999; Artursson *et al.*, 1994). In fact, an ionic interaction between CS positively charged amino groups and the negatively charged sialic acid residues in mucus has been proposed as the mucoadhesion mechanism (Lehr *et al.*, 1992). Moreover, chitosan has favorable rheological behavior where, its solutions have shown pseudoplastic and viscoelastic properties. This behavior is particularly important in ophthalmic formulations since it facilitates the retention while it permit the easy spreading of the formulation due to the blinking of the eye (Mucha, 1997).

Indomethacin (IM) a model potent anti-inflammatory drug was used in the present study. Although it has been shown to reduce post-operative inflammation and to decrease the intraocular irritation after cataract extraction (Muchtar *et al.*, 1997) and in cystoid macular oedema (Miyake *et al.*, 1978), the clinical use of its marketed most commonly used eye drops is limited due to its poor bioavailability and topical side-effects, including burning sensation, irritation, and epithelial keratitis (Calvo *et al.*, 1996a). Previous work by Calvo *et al.*, 1996a; 1996b) has indicated that different poly-γ-caprolactone (PECL) colloidal systems namely, nanocapsules, nanoparticles, and sub-micron emulsion were able to increase the ocular penetration of IM. Moreover, Calvo *et al.*, 1997a developed new strategy that combined the features of PECL ocular nanocapsules with the advantages of two different cationic bioadhesive coating polymers, CS and polyaminoacid poly-L-lysine (PLL). The authors observed twice increase in the ocular penetration from CS coated PECL nanocapsules whereas the PLL coating failed to increase IM bioavailability. Therefore, it was not only the positive charge but the specific nature of CS that was responsible for the enhanced bioavailability of IM. Nevertheless, despite the great value of the previous results in improving IM ocular bioavailability, their potential as ocular drug carrier was limited due to their inability to persist at the eye's surface and to provide significant IM level at ocular mucosa for extended periods of time.

Based on these considerations and keeping in mind that enhancement of the precorneal processes such as the slow removal of IM from the absorption site and the improvement of both paracellular and intracellular pathways of epithelial cells would be of great benefit to reduce the dose, its frequent instillation and consequently reduce the local side-effects inherent to this drug. Thus the principle aim of this work was to formulate and evaluate two different CS based mucoadhesive biodegradable nano-systems namely, nanoparticles and nano-emulsions to increase residence time of IM in the precorneal area and to provide these tissues with long-term drug levels. Important advantages of these nanocarriers include their rapid, simple and convenient preparation under extremely mild conditions. A secondary objective was to compare the effects of the developed carriers on the healing rate of rabbits eye induced ulcers (Keratitis) relative to IM solution. The potential of the developed carriers to promote corneal penetration and hence IM therapeutic index was also assessed by measuring IM levels in rabbits ocular corneal tissues and aqueous tissues humor

MATERIALS AND METHODS

Materials and animals

Indomethacin micronized powder (IM), was kindly supplied by EPICO pharmaceutical company (Cairo, Egypt), Chitosan (CS) (low Mw, viscosity, 20 cps, degree of deacetylation 85%) were purchased from Aldrich chemical Co. (Steinheim, Germany), Poloxamer 188 (Pluronic F68), Tripolyphosphate (TPP), was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Poly vinyl alcohol (PVA), medium-viscosity (Mw= 17000) was provided by Fluka company (Buchs, Switzerland). Tween® 80 was supplied by ICI., (Barcelona, Spain). Lecithin Soya, was provided by Lipoid (KG, Ludwishafen, Germany). Migliol 840 oil, medium-chain triglyceride (MCT) was obtained from Hüls AG (Witten/Ruhr, Germany). All other chemicals were of reagent grade and were obtained from EL-Nasr Company, Cairo, Egypt.

Male healthy Albino rabbits weighing between (1.50-2.0 kg) were used in the studies of the indomethacin ocular pharmacokinetics. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed.

Preparation of indomethacin nanoparticles

Chitosan nanoparticles were prepared spontaneously based on ionic gelation by addition of TPP anions aqueous solution to CS solution according to the procedure first reported by Calvo *et al.,* 1997b. Different amounts of CS were dissolved in acetic aqueous solution to give concentrations of (0.1, o.3, 0.5, 0.7, 1.0, and 2.0 mg/mL). The concentration of acetic acid in aqueous solution was 1.5 times that of CS. Under magnetic stirring at room temperature, 4 mL of TPP aqueous solution with various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) was added into 10 mL CS solution respectively. Three kinds of phenomena were observed: solution, aggregates and opalescent suspension. The zone of opalescent suspension was further examined as nanoparticles. For preparation of IM nanoparticles, IM was dissolved in 0.25 mL of methylene chloride and incorporated into the CS solution. The methylene chloride was evaporated during moderate mixing for 30 minutes at 30ºC. IM loaded nanoparticles were formed upon incorporation of 4 mL TPP (0.2 mg/mL) into 10 mL CS solution (0.5 mg/mL) containing IM. The formation of particles was a result of the interaction between the negative groups of the TPP and the positively charged amino groups of CS.

Preparation of nanoemulsions

Nanoemulsion was prepared by spontaneous emulsification method described by (Calvo *et al.*, 1996b) as follows: 100 mg of lecithin was first dissolved in 25 mL of acetone, then 0.5 mL of MCT oil (in which the drug showed higher solubility, data not shown) containing 10 mg of IM was added to acetonic solution. 125 mg surfactant was dissolved in 50 mL of 0.1% w/w aqueous acetic acid CS solution. The acetonic solution was poured slowly under moderate magnetic stirring into 50 mL of surfactant-CS solution. Emulsification of the mixture was carried out by an Ultra-Turrax T25 running at 8000 rpm for 30 min. The acetone was removed under reduced pressure and the colloidal submicron emulsion was concentrated to the desired volume (10 mL, corresponding to 0.1% drug). The composition of different IM nanoemulsions formulae (N1-N5) is summarized in Table I.

Physicochemical characterization of drug carrier systems

The morphological examination of drug-nanoparticles and drug-nanoemulsions was performed using both image analyzer (LEICA Quin Image Analyser, Model Q5501W) equipped with Leica DMLB microscope (Cambridge, England, UK) and connected to computerized digital camera Model TK-C1380 JVC (Victor Company, Japan) and by transmission electron microscope (TEM). In the TEM technique, a sample drop was applied to a collodion-coated 300 mesh copper grid and left for 10 min to allow some of the particles to adhere to collodion. The excess remaining dispersion was drawn off with filter paper. A drop of 2% aqueous solution of uranyl acetate was applied for 1 min. The excess was then removed and the sample was air dried and examined with a transmission electron microscope (Jeol, 1200 EXII, Tokyo, Japan).

The mean particles size and size distribution of the colloidal systems were determined using a Malvern Zetasizer model 1000HSA, Worcester, UK. Light scattering was monitored at a 90° angle and a temperature of 25°C with polystyrene beads used as a standard to check instrument performance. The measurements were performed after diluting samples by 100-fold with water. The viscosity of the different formulae was monitored by Brookfield viscometer model DV-I, Massachusetts, U.S.A. and the refractive index was measured using refractmeter model RIC-I, Index instrument, Kent, U.K. Both measurements were conducted at ambient temperature. The predetermined viscosity and refractive index of the different formulae were incorporated into the computer software of the Zetasizer, which calculated the mean particle size and polydispersity from intensity, mass and number distribution.

The zeta-potential was determined using electrophoretic light scattering technique (Zetasizer 2000, Malvern Instruments Limited, Worcester, UK.) 0.25 mL of the nano carrier system was diluted with 0.001 M KCl and placed in the electrophoretic cell. The zeta-potential values were calculated from the mean electrophoretic mobility values.

The amount of IM encapsulated into nanoparticles and nanoemulsions was calculated by the difference between the total amount used to prepare IM- loaded nano system and the amount of free IM determined in the aqueous phase. The aqueous phase was separated following centrifugation at 14,000 rpm for one hour, (Beckman instruments Inc., Palo Alto, U.S.A.). The amount of free IM was determined spectrophotometrically at 318 nm.

To determine the possibility of chemical interaction between IM and the used polymers, the colloidal suspensions and emulsions were analyzed by Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained with (Genesis II spectrometer TM, Mattson Instruments, Thermo Optak Corp, U.S.A.). Potassium bromide pellets containing the samples were prepared prior to FTIR analysis.

Differential scanning calorimetry (DSC) analysis was performed in order to characterize the physical state of IM in the colloidal systems. Thermograms were obtained using a Shimadzu DSC, TA-50 (Shimadzu Co., Kyoto, Japan).

In vitro drug release

This study was carried out using a USP Dissolution tester (Apparatus I, Hanson SR6, California, U.S.A.). The drug loaded nano carriers, containing the equivalent to 1 mg indomethacin, were placed in glass cylindrical tubes (2.5 cm in diameter and 10 cm in length). Each tube is tightly covered with a Spectra por© (12000 molecular weight cut off, soaked in PBS pH 7.4 overnight) from one end and attached to the shafts of the USP dissolution tester apparatus, instead of the baskets, from the other end. The shafts were then lowered to the vessels of the dissolution apparatus containing 50 mL of PBS pH 7.4. The release study was carried out at 37±0.5°C, and the stirring shafts were rotated at a speed of 25 rpm. At predetermined time intervals, samples were withdrawn and analyzed for IM content spectrophotometrically at λ 318 nm against the samples withdrawn at respective time interval from empty nano carrier dispersions treated in a similar manner. Every withdrawal was followed by replacement with fresh medium to maintain a constant volume. The results were the mean value of 3 runs each representing one batch.

Stability studies

Samples of the nanoparticles and nanoemulsion were sealed in 30 mL clear glass vials and stored for 6 months at 25°C. The stored samples were visually inspected for coalescence, settling, creaming, and changing in color. The entrapment efficiency as well as the mean particle size and pH were determined and compared to the freshly prepared samples.

In vivo studies

Study design

30 Male healthy Albino rabbits weighing between 1.50-2.0 kg were used in this study. The study performed in this section was approved by the university protection for animals care and use committee and the protocol complied with "the Principles of Laboratory Animal Care" [NIH publication # 85-23, revised 1985]. All formulae used in this section were freshly prepared

without any preservative addition and were sterilized using g radiation. The animals were randomly divided into five groups, each of six rabbits. The study was conducted on two phases. In phase I, corneal ulcer inflammation was induced in rabbits using 1N NaOH solution. The animals were anesthetized with intramuscular injections of ketamine hydrochloride 50 mg/kg, Xylazine 10mg/kg and topical benoxinate 0.4%. (Klang *et al.*, 1999). Central corneal chemical injury was made by instillation of 10 μL of 1N NaOH solution into a 7-mm diameter trephine placed on the cornea, After 1 minute, a sponge was applied to adsorb the excess NaOH solution and the trephine was removed. Treatment was initiated one hour post-burn where one drop (20 μL) of nanoparticle formula was instilled in the right eye and one drop (20 μL) of nanoemulsion in the left eye twice daily for three weeks. For corneal ulcer healing, the rabbits were examined every other day during 21 days of the study by the use of hand held slit lamp and photographs of the corneal eye rabbits were taken using digital camera and the rate of healing was evaluated through changes in the transparency and density of corneal opacity, corneal perforation and pthisis. For pathological examination of the inflamed corneal epithelium, the rabbits of the same group were sacrificed with an intravenous injection of an overdose sodium pentobarbital. After sacrificing the animals, the anterior chamber was entered with a scalpel blade and the entire cornea was excised from the eye with corneal scissors. Corneas were placed in 10% formalin. Following fixation they were embedded in paraffin and stained with Hematoxylin and Eosin for routine histological examination. Specimens showed different patterns of cellular activity. Light Microscopy quantification was performed by counting Poly Morph Nuclear Leucocytes (PMNLs) in tissue sections per high power field (×40 objective, ×10 ocular) as: no cells = no infiltration, 1-50 = mild infiltration, 51-100 = moderate infiltration, and 101 or more = marked infiltration.

Phase II was performed on 24 fully-awake rabbits of group II, III, IV, and V to evaluate the distribution of IM loaded nanoemulsion in the cornea and aqueous humor. The rabbits received a volume of 20 μL of the nanoemulsion preparation applied in the cul-de-sac of the right eyes. The left eyes remained untreated and served as a control which received 20 μL of the prepared aqueous 0.1% IM eye drops of pH 7.1 (each 100 mL contain 100 mg drug, 0.28 mL 1N Na OH, 5% sorbitol). After instillation, the rabbits were maintained in an upright position using restraining boxes. At different times post-instillation 1, 2, 4, and 6 h, rabbits were sacrificed and the eyes were enucleated and samples of aqueous humor were needle attached to 1 mL insulin syringe. The samples were collected in glass tubes and stored frozen until the HPLC assay was carried out (De Campos *et al.*, 2001). Cornea was subsequently dissected in situ, rinsed with normal saline, blotted dry and transferred to preweighed tubes. The tubes were reweighed and the weight of the corneas was calculated (Calvo *et al.*, 1997a).

Chromatographic conditions for indomethacin assay

The IM contents in aqueous humor were measured using a modified HPLC assay of Skellern *et al.*, 1975. HPLC system (Hitachi, Tokyo, Japan) consisting of pump (L-6000, Hitachi), Phenomenex C18 column (Luna 5 μm, 250 × 4.6 mm). Mobile phase, a mixture of acetonitrile and 0.1 N acetic acid (50:50) was filtered through 0.45 mm membrane filter and eluted at a flow rate of 2 mL/min. Effluents were monitored using UV detector at 254 nm. The retention time of IM and mefenamic acid (internal standard) was 7.0 and 12.90 min respectively.

Standard solutions

Blank aqueous humor samples were spiked with IM stock solution (50 mg/mL) and 20 mg/mL of internal standard to contain 0.1, 0.5, 1, 2, 4, and 8 mg/mL. A standard curve was constructed by plotting the peak area versus IM concentrations in aqueous humor. All assays were performed in triplicate. During the assay of the samples, the intra -batch precision and accuracy of the analytical procedure were evaluated after replicate analysis (n = 4) of control aqueous humor samples spiked at three concentration levels: 0.5, 2, and 8 mg/mL. The lower limit of quantification was 0.1 mg/mL with a linear response across the full range of concentrations from 0.1 to 8 mg/mL ($R^2 = 0.995$).

Aqueous humor analysis

The samples were thawed at room temperature and aqueous humor (100 mL) was mixed with 100 mL citrate buffer pH 5, 100 mL distilled water and 50 mL mefenamic acid solution (5 mg/mL) as internal standard. The mixture was extracted with 1 mL diethyl ether by mechanical shaking for 15 min. The ether phase was transferred to a tapered tube and evaporated to dryness on a water bath at 30ºC. Residues were reconstituted with 100 mL of the mobile phase and samples of 20 mL were injected into the HPLC system.

Pharmacokinetic analysis

The area under the curve, $AUC_{0\text{-}6}$ (μg·min/mL) of IM concentration in the cornea and aqueous humor were calculated using the trapezoidal rule. The maximum IM concentration C_{max} in the cornea and aqueous humor and the time at which C_{max} is achieved T_{max} were determined from actual data points.

Statistical analysis

The data obtained from different formulations were compared for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey method as a multiple range test using SPSS (version 11, SPSS Inc., U.S.A.). All results were expressed as mean ± standard deviation (S.D). Differences were considered to be significant at a level of p 0.05.

RESULTS AND DISCUSSION

Effect of chitosan concentration

When TPP was 0.2 mg/mL, too high CS concentration (1 mg/mL) made encapsulation extremely difficult, and too low

CS concentration (0.1 mg/mL) made some aggregates with large diameter form. Formation of nanoparticles was only possible within some moderate concentrations of chitosan and TPP. As for gelation between TPP solution of 0.2 mg/mL and CS solution of 0.3-0.7 mg/mL, we usually observed that some opalescent suspension was formed, which was further examined as nanoparticles. Fig. 1 shows that increasing CS concentration led to decrease the encapsulation efficiency of indomethacin. It has been previously reported that the highly viscous nature of the gelation medium hinders the encapsulation (Xu and Du, 2003; Vandenberge *et al.*, 2001; Wu *et al.*, 2005). So it was supposed that relatively lower viscosity of CS with lower concentration such as 0.3-0.7 promote the encapsulation of indomethacin and gelation between CS and TPP.

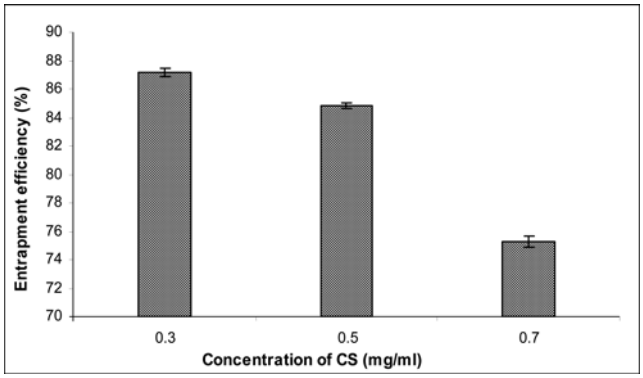

Fig. 1. The influence of chitosan concentration on IM entrapment efficiency (TPP 0.2 mg/mL, mean ± S.D., n = 3)

Formation and characterization of indomethacinloaded chitosan nanoparticles

The preparation of CS nanoparticles based on an ionic gelation process, involves the mixture of two aqueous phases at room temperature. Because of IM hydrophobic nature, the appropriate condition for the incorporation of IM into the CS nanoparticles should be determined. A successful entrapment was achieved by dissolving the hydrophobic IM in 0.25 mL methylene chloride prior to its incorporation into CS solution. This volume was enough to allow precipitation of IM in a very small particles and its entrapment in CS matrix before the addition of TPP solution. In fact, a Tyndall effect with very fine suspension of nanocrystal of IM in CS solution was observed. Ford *et al.* (1999) and De campos *et al.* (2001) had previously shown similar precipitation effect of cyclosporine upon the addition of its organic solution to an aqueous phase in the form of discrete fine spherical particles. Therefore, the nanoparticles formation process, after incorporation of the counter ion TPP involved CS gelling in the form of discrete nanoparticles simultaneously entrapping the IM nanocrystal suspended in the medium.

Physicochemical characterization

Transmission electron microscopy shows the morphological characteristic of the nanoparticles and nanoemulsions. Fig. 2 revealed regular well-identified spherical shape. The mean average diameter of the nanoparticles was 0.28 μm ± 6.4, the polydispersibility index 0.09 with monomodal size distribution and zeta potential of + 17 mV. IM-loaded CS nanoparticles displayed good entrapment efficiency (% of IM entrapped with respect to the total amount of the drug used to prepare the particles) of 84.8 ± 0.81% which considered high if we take into account the hydrophobic character of IM and the hydrophilic nature of CS. Previous results revealed 73% loading of hydrophobic Cyclosporin A and 47% for hydrophilic bovine serum albumin adopting ionic gelation technique (De campos *et al.*, 2001; Xu and Du, 2003). Particle size analysis of the nanoemulsion indicated that, the mean size of the different formulae was affected by the nature of the surfactant used which varies between 220-690 nm (Table I). Statistical analysis of these data revealed significant differences at $p < 0.05$. All formulae revealed a monomodal size distribution with positive zeta potential values. This indicates that CS molecules were localized at the interface and intercalated between the surfactants therefore, a mixed interfacial film was formed at the o/w interface which resulted in an overall positive surface charge (Jumaa *et al.*, 1999). By comparing the zeta potential with the particle size, it was clear that, increasing pluronic F68 in N4 resulted in a decrease in both nanoemulsion droplets size and zeta potential value as well. This decrease in the surface charge suggests the formation of a sterically stabilizing adsorbed

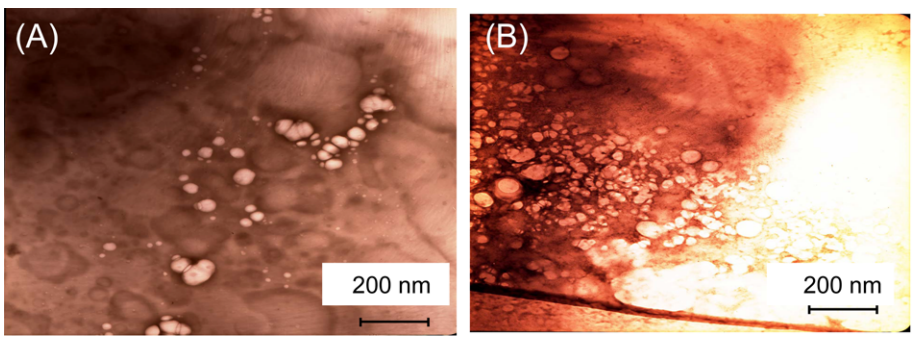

Fig. 2. Electron transmission microphotography of (A) nanoemulsons (N1), (B) Cs-nanoparticles

Table I. Compositions, particle size distribution parameters (mean particle size and polydispersity), zeta potential and entrapment efficiency of indomethacin nanoemulsions (mean ± S.D., n = 3)

Forms no.	Composition*	Particle size (nm) (Polydispersity)	Zeta potential (mV)	Entrapment Efficiency (%)
N1	100 mg Cs and 125 mg Pluronic F68	230 ± 8.35 (0.08)	+ 23	89.0 ± 1.20
N2	100 mg Cs and 125 mg PVA	390 ± 12.26 (0.11)	+ 21	84.5 ± 1.45
N3	100 mg Cs and 125 mg Tween 80	330 ± 16.76 (0.09)	+ 16	83.0 ± 1.60
N4	100 mg Cs and 250 mg Pluronic F68	220 ± 11.98 (0.07)	+ 19	73.0 ± 0.95
N5	200 mg Cs and 125 mg Pluronic F68	690 ± 17.93 (0.14)	+ 17	75.5 ± 2.60

* All formulae contain: 10 mg indomethacin, 100 mg lecithin, 0.5 mL MCT oil, 0.5 mL sorbitol and 0.01% Benzalkonium chloride.

polymer layer. On the other hand, increasing chitosan concentration in formula N5, led to an increase in the mean diameter to largest values (960 nm) with unexpected decrease in the positive zeta value. This can be solely attributed to the increase in the dispersion viscosity. In this sense, it should be mentioned that CS enhances the viscosity of the aqueous medium and hence interferes with the interfacial hydrodynamic phenomena responsible for spontaneous emulsification of the oily solution into the aqueous phase. This led to an increase in the particle size with reduced surface area and hence decreases in surface charge (Davis and Rideal, 1963; Jumaa *et al.*, 1998) .

Fig. 3, displayed the FTIR spectra over the range 4000-500 cm^{-1} of IM, CS, and IM-loaded nanoparticle and nanoemulsion. The IM spectrum shows the characteristic band at 1715 and 1695 cm^{-1} of carbonyl stretches, the aromatic c=c stretch at 1600 cm^{-1} and the O-CH_3 deformation at 1450 cm^{-1}. There are three characteristic peaks of CS at 3428 cm^{-1} of (OH), 1092 cm^{-1} of (C-O-C), and 1627 cm^{-1} of (NH_2). In IM-loaded nanoparticles and nanoemulsion, the peak of 3428 cm^{-1} becomes wider and shifted to 3456 and 3435 cm^{-1} respectively indicating that the hydrogen bonding is enhanced. New sharp peak at 1635 or 1636 cm^{-1} appeared in both nanocarriers with the disappearance of carboxyl group absorption peak of IM at 1715 cm^{-1} and NH2 bending of CS at 1627 cm^{-1}. According to this result, we suppose that the tripolyphosphoric groups of TPP or carboxyl group of IM were linked by an electrostatic interaction with ammonium group of CS (Wu, *et al.*, 2005). Therefore, inter and intramolecular action are expected in IM-loaded nanopaticles.

Fig. 4, displayed DSC thermograms obtained for TPP, CS, IM, and IM loaded nanoparticles and nanoemulsion. The IM, CS, TPP showed an endotherm at 160, 108, and 40&113ºC respectively corresponding to their melting temperatures. However, the endothermic peak characteristic of IM was not detected in the thermograms obtained for IM-loaded nanoparticles and nanoemulsion. These results suggest that IM was dispersed molecularly in the CS polymer network in both carrier systems.

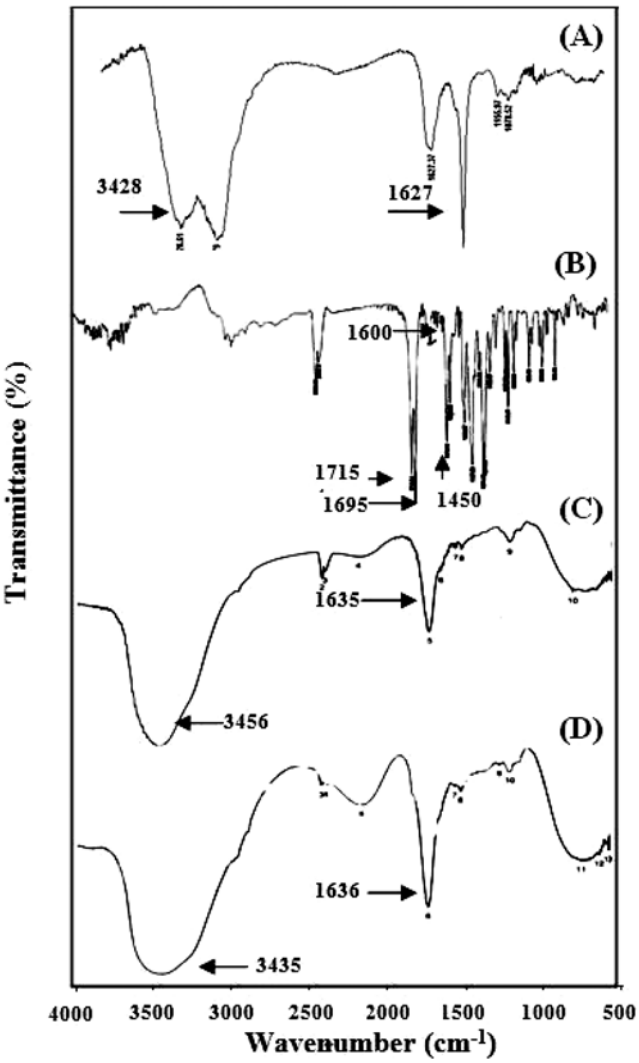

Fig. 3. FT-IR spectra of (A) chitosan, (B) indomethacin, (C) IM-loaded nanoparticles, and (D) IM-nanoemulsion

Long term stability study

A long term physicochemical stability study was performed. The nanoparticles and nanoemulsions formulae (N1-N3) remained visually almost unchanged when stored at room temperature for six months. No IM crystals were detected and no coalescence, settling or creaming was observed except nanoemulsion of formula N4 and N5 had reversible creaming that were easily redispersed by gentle shaking and the samples

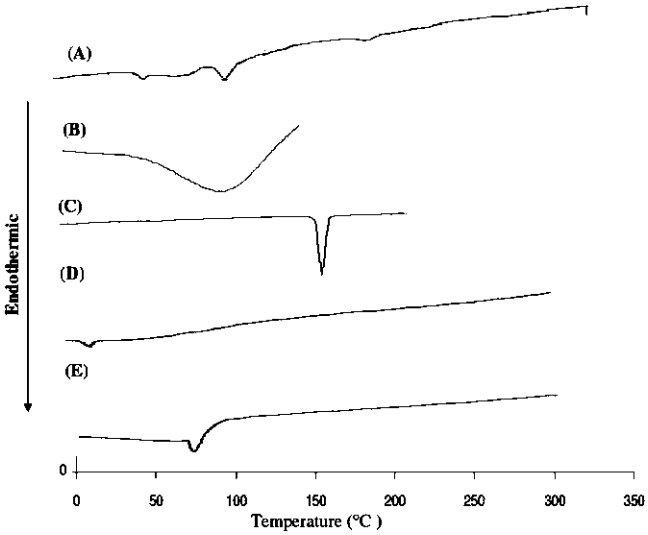

Fig. 4. DSC of (A) TPP, (B) chitosan, (C) indomethacin, (D) IM-loaded nanoparticles, and (E) IM nanoemulsion

acquired their original appearance. As shown in Table II, nanoparticles did not suffer an increase in their mean size or decline in the pH during storage, however the size of the different nanoemulsion formulae increased with evident decrease in their pH. This could be understood by the possibility of triglyceride and phospholipids hydrolysis leading to the release of free fatty acids (Grit *et al.*, 1989). Therefore, IM nanoparticles (formula G) and IM nanoemulsion (N1) with higher drug entrapment efficiency and little observed increase in their particle size after 6 months were chosen for further studies.

Table II. Evaluation of particle size, pH, and entrapment efficiency of indomethacin nanoparticles and nanoemulsion during storage for 6 months at 25°C (mean ± S.D., n = 3)

Formulae No.	Time (months)	Particle size (nm)	pH	Entrapment efficiency (%E)
G	0	280 ± 6.4	5.2	84.8 ± 0.81
	1	283 ± 7.9	5.1	
	3	291 ± 10.6	5.0	
	6	301 ± 8.9	4.8	82.3 ± 1.9
N1	0	230 ± 8.4	5.0	89.0 ± 1.20
	1	240 ± 10.2	4.8	
	3	249 ± 12.6	4.4	
	6	256 ± 9.7	4.1	86.5 ± 2.72
N2	0	390 ± 12.3	5.0	84.5 ± 1.45
	1	410 ± 16.3	4.7	
	3	423 ± 20.7	4.2	
	6	433 ± 18.4	3.8	80.0 ± 1.11
N3	0	330 ± 16.8	5.1	83.0 ± 1.60
	1	339 ± 17.6	4.8	
	3	358 ± 21.3	4.0	
	6	380 ± 16.9	3.5	77.0 ± 2.32
N4	0	220 ± 11.9	5.1	73.0 ± 0.95
	1	300 ± 9.5	4.6	
	3	340 ± 10.1	4.2	
	6	460 ± 12.8	3.8	68.5 ± 1.05
N5	0	690 ± 17.93	5.0	75.5 ± 2.60
	1	890 ± 12.6	4.6	
	3	1005 ± 21.2	4.0	
	6	1800 ± 20.8	3.6	65.0 ± 3.12

In vitro release

Results of IM release from chitosan nanoparticles (G) and nanoemulsion (N1) are depicted in Fig. 5. It was apparent that the release profiles obtained were almost similar and exhibited a small initial burst release of about 9.5 and 12.0% respectively in the first hour followed by slow steady state drug release with an average constant of 4.47 and 5.44 percent per hour. It should be emphasized that practically incomplete drug release was observed from both nanoparticle and nanoemulsion systems with only 76 and 86% of the drug loaded were released within 24 h respectively. The obtained results were contraverse with the previous work done by Calvo *et al.*, 1996b; 1997a) where 85% of indomethacin diffused out from the colloidal systems in the second hour. This could reflect the ability of the prepared nanocarriers to modulate the release rate of indomethacin.

The burst release of IM was associated with those IM molecules dispersing close to the nanocarriers surface, which easily diffuse out in the initial incubation time in addition, huge specific surface area that characterized nanocarriers can adsorb the drug so the first burst release is possibly due the part of IM desorbed from nanocarriers surface (Xu and Du, 2003; Agnihotri *et al.*, 2004). On the other hand, the slow pattern dipicted could be attribulted to the retention capacity of the oily nanodropltes, lipophilic bilayers in which IM is expected to be incorporated owing to its low aqueous solubility or probably to the Pluronic surfactant micelles present in the aqueous phase (Ammoury *et al.*, 1990). The successful entrapment of IM due to chitosan interaction was another reason.

Two kinetic models could govern the overall release profile of IM from the prepared nanocarriers in phosphate buffer sink solution: the partition rate of the drug from the oily phase to the sink solution or the diffusion of the drug through the thin polymeric membrane. The release data was kinetically analyzed using the empirical equation: $\text{Log } Q = \text{Log } k + n \text{ Log } t$ (Ritger and Peppas 1987) where, Q is the fraction of drug released in time *t*, *k* is a constant characteristic of the drug polymer interaction and *n* is an empirical parameter characterizing the release mechanism. Based on the diffusional exponent, the nanoparticle and nanoemulsion formulae revealed n-values of ($1>n>0.5$), meaning non-Fickian diffusion behavior that was obtained as a result of contributions from diffusion and polymer erosion (Agnihotri *et al.*, 2004).

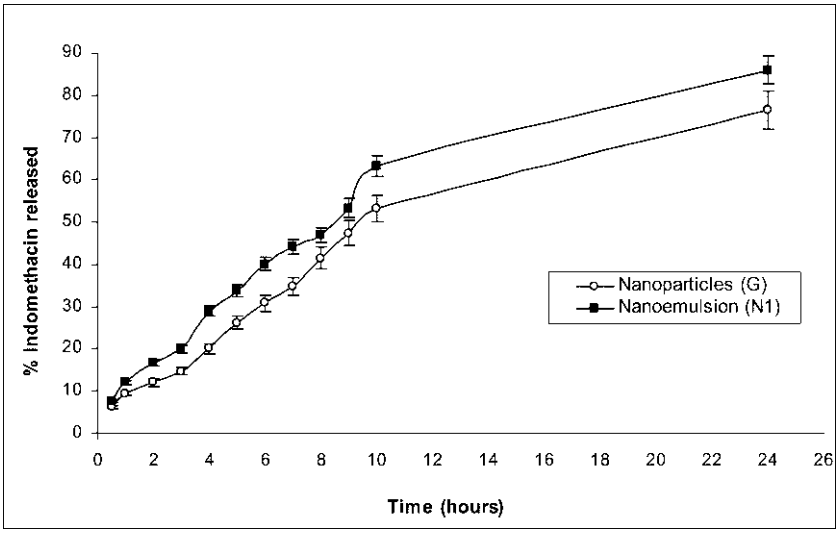

Fig. 5. Indomethacin release profiles from Cs-nanoparticles and nanoemulsion (N1) in phosphate buffer pH 7.4 at 37±0.5°C (mean ± S.D., n = 3)

***In vivo* experements**

After 21 days post induced chemical injury the eyes of rabbits treated with nanoemulsion (N1) showed clear healing of corneal chemical ulcer compared with nanoparticles (G). Fig. 6 showed different patterns of cellular activity. These include no corneal cellular infiltration, mild, moderate and marked polymorph nuclear leukocytic (PMNLs) infiltration. The Reached results showed that IM nanoemulsion was more effective and better in reducing the corneal ulceration comparable to moderate infiltration when nanoparticles were used.

Srinivasan and Kulkarni (Srinivasan and Kulkarni, 1981) studied polymorphonuclear leukocyte response inhibition by nonsteroidal anti-inflammatory agents. They described that only IM was able to inhibit the PMNL response following complete corneal de-epithelialization. Our results showed that IM in the nanoemulsion formula was more effective in PMNLs inhibition and better in reducing the corneal ulceration comparable to moderate infiltration when nanoparticles were used. This trend is in agreement with drug released from nanoemulsion and might be attributed to dose uniformity and the homogeneity precise of emulsion compared to suspension. In addition, to the synergistic effect between the drug and the components of nanoemulsion (Klang, 1999). Therefore based on healing rate of corneal ulceration, nanoemulsion formula was further selected for phase II of in vivo corneal penetration.

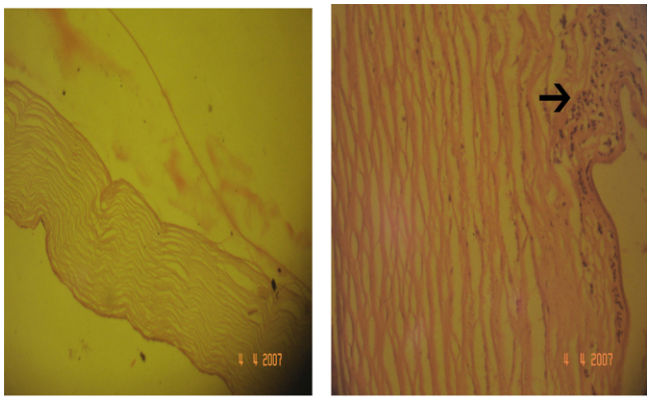

Fig. 6. Histopathologic of rabbits corneal tissue 21 days post-administration of (A) the IM-nanoemulsion; and (B) IM-nanoparticles

As shown in Fig. 7, after post instillation of nanoemulsion, the IM exhibited an early increased transport across the cornea. Indeed, after 1 h the concentration of IM in cornea was 30 times higher than the one observed after instillation of IM solution. Similar results were obtained in the aqueous humor. These differences were statistically significant ($p<0.05$)

It was also interesting to note that, the corneal level showed a maximum at 1 h post administration of nanoemulsion which decreased gradually afterwards however, during at least 6 h, IM concentration in the cornea was sufficiently high to adequately suppress inflammatory process. Therefore, using IM-CS nanoemulsion developed in this study, therapeutic concentration of IM was maintained in the cornea through out the duration of the study. This suggests that this clinically relevant ocular tissue may act as a reservoir for IM-loaded nanoemulsion. The second important observation to note that intraocular IM level attained in the aqueous humor following administration of nanoemulsion was fairly high for up to 6 h in contrast to its intraocular level from IM solution where it was cleared from the eye faster and its level went down below the limit of detection at 4 h post-administration.

The ocular bioavailability of IM was illustrated by the area-under the curve (AUC), maximum IM concentration (C_{max}) and the time at which the C_{max} is achieved (T_{max}). The AUC was 17

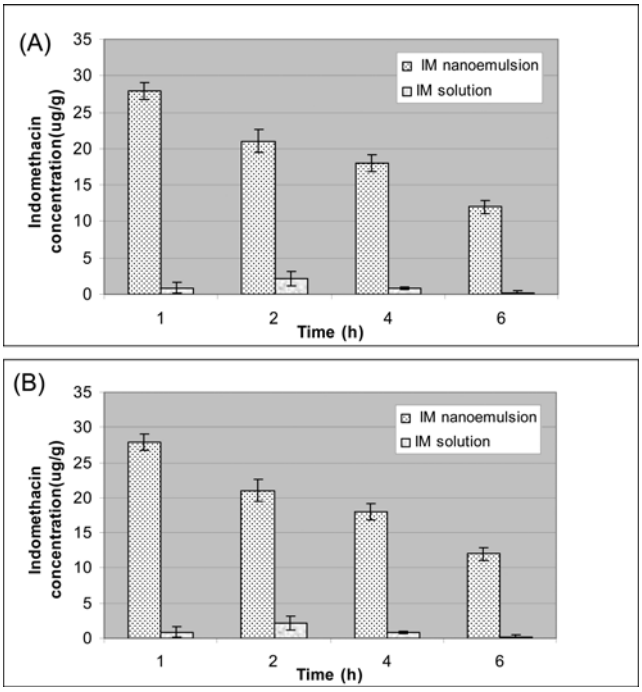

Fig. 7. Indomethacin concentration attained in (A) cornea and (B) aqueous humor after application of nanoemulsion and solution (mean ± S.D., n = 6).

times greater for nanoemulsion and C_{max} was 13 fold those of IM solution. The reason for this improved ocular bioavailability could be found in (i) the mucoadhesive properties of CS, (ii) the electrostatic interaction between the positively charged CS nanoemulsion and the negatively charged corneal tissues which plays a key role in prolonging the residence of IM in these epithelia, (iii) the ability of CS to open the tight junctions between epithelial cells which mainly attributed to the interaction of the positively charged amino groups of CS with negatively charged sites of the cell surfaces and tight junctions (Schipper *et al.*, 1997). The interior of tight junctions is highly hydrated and contains fixed negative charges. An alteration in the relative concentration of specific ion species in the pore volume would result in changes in tight junction resistance, which might lead to loosening or opening of the pore (De Campos *et al.*, 2004). (iv) Moreover, based on our results it should be noted that it was not only the combined CS mechanisms of mucoadhesion and the effect on the tight junctions but the presence of non-ionic surfactant poloxamer F 68 (HLB = 17) could be responsible for the increased penetration and the observed improved healing rate.

In conclusion, this paper described the development of two different mucoadhesive chitosan based nanocarriers, nanoparticles and nanoemulsion adopting simple and convenient way for IM ocular delivery. Unlike previously investigated colloidal systems, the interest of these formulations was their ability to interact and remain associated to the ocular mucosa thus prolonging the residence time in the cornea and slow gradual IM release during 24 h was achieved. Furthermore, CS nanoemulsion developed in this study can be proposed as promising carrier to enhance the therapeutic index of clinically challenging IM with potential application at both extra and intra ocular levels.

Indeed, it is of concern to check that the increased residence dose not enhance the systemic concentration of the drug and hence its side effects.

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