Chitosan Nanoparticles as New Ocular Drug Delivery Systems: *in Vitro* Stability, *in Vivo* Fate, and Cellular Toxicity

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Purpose. To assess the potential of chitosan (CS) nanoparticles for ocular drug delivery by investigating their interaction with the ocular mucosa *in vivo* and also their toxicity in conjunctival cell cultures.
Methods. Fluorescent (CS-fl) nanoparticles were prepared by ionotropic gelation. The stability of the particles in the presence of lysozyme was investigated by determining the size and their interaction with mucin, by measuring the viscosity of the mucin dispersion. The *in vivo* interaction of CS-fl nanoparticles with the rabbit cornea and conjunctiva was analyzed by spectrofluorimetry and confocal microscopy. Their potential toxicity was assessed in a human conjunctival cell line by determining cell survival and viability.
Results. CS-fl nanoparticles were stable upon incubation with lysozyme and did not affect the viscosity of a mucin dispersion. *In vivo* studies showed that the amounts of CS-fl in cornea and conjunctiva were significantly higher for CS-fl nanoparticles than for a control CS-fl solution, these amounts being fairly constant for up to 24 h. Confocal studies suggest that nanoparticles penetrate into the corneal and conjunctival epithelia. Cell survival at 24 h after incubation with CS nanoparticles was high and the viability of the recovered cells was near 100%.
Conclusions. CS nanoparticles are promising vehicles for ocular drug delivery.

KEY WORDS: Chitosan; nanoparticles; conjunctival cell toxicity; mucoadhesive polymers; ocular drug delivery.

INTRODUCTION

Topical application of drugs into the eye is severely limited by the protective physiological mechanisms that exist in the precorneal area resulting in considerable drug loss. Because of these biological constraints, the most frequently used dosage forms, solutions and suspensions, are compromised in their effectiveness due to their inability to provide an adequate concentration of the drug at the site of action. Being conscious of these limitations, an important effort in ocular drug delivery has been to improve the bioavailability and to prolong the residence time of drugs instilled topically onto the eye (1). Among the different strategies explored so far, the use of colloidal polymer systems has shown a certain degree of success (2). Previous work by our group and others has indicated that poly(alkylcyanoacrylate) (PACA) nanoparticles (3,4) and poly-ε-caprolactone nanocapsules (5–7) were able to increase the intraocular penetration of drugs, while reducing their systemic absorption. This improved ocular penetration was partially attributed to the interaction and further transport of these colloidal carriers across the corneal epithelium (8). Additionally, it was shown that this transport was determined by the colloidal nature of the particles (9). Nevertheless, despite these interesting data, a limitation of these particulate colloidal carriers is their inability to persist at the eye's surface for extended periods of time and, hence to provide a prolonged drug delivery to the eye.

Another strategy aimed to increase the residence time of drugs in the precorneal area has been the use of mucoadhesive polymers (10,11). Among them, the cationic polysaccharide chitosan (CS) exhibits several favorable biological properties, such as biodegradability (12), nontoxicity (13), biocompatibility (14), and mucoadhesiveness. In fact, an ionic interaction between the CS positively charged amino groups and the negatively charged sialic acid residues in mucus has been proposed as the mucoadhesion mechanism (15). This unique combination of properties makes it a novel versatile biopolymer, which fulfils the requirements for its application in the ophthalmic field.

There are few reports in the literature on the use of CS for ocular drug delivery. Calvo *et al.* (16) observed that CS-coated poly-ε-caprolactone nanocapsules significantly increased the ocular bioavailability of indomethacin compared to uncoated and poly-L-lysine (PLL)-coated poly-ε-caprolactone nanocapsules, even though PLL and CS displayed a similar positive surface charge. The authors concluded that it was not only the positive charge but the specific nature of CS that was responsible for the enhanced bioavailability of the drug incorporated into CS-coated nanocapsules. The effect of the CS coating on the ocular retention of liposomes could not be, however, seen, following their instillation to rats (17). Nevertheless, it should be noted that these authors followed the retention of ^{125}I-labeled bovine serum albumin used as a marker rather than the fate of the CS-coated liposomes. More positive results were obtained for CS microspheres (18), which were shown to increase and prolong the corneal penetration of the encapsulated drug, acyclovir. More recently, Felt *et al.* (19) evidenced that the presence of the polysaccharide significantly prolonged the corneal contact time of tobramicin, following topical instillation to rabbits. In addition, all types of CS investigated exhibited an excellent tolerance.

Based on these considerations, we decided to explore the potential of a different colloidal system consisting of an aqueous suspension of CS nanoparticles. Important advantages of these nanoparticles include their rapid preparation under extremely mild conditions and also their ability to incorporate bioactive compounds (20). It was our hypothesis that CS nanoparticles would increase the residence time of drugs in the precorneal area due to their adhesive properties and, therefore, could prolong the penetration of drugs into the intraocular structures. Thus, the main objective of this work was to quantify and to investigate the mechanism of interaction between CS nanoparticles and the corneal and conjunc-

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tival epithelial surfaces. With this idea in mind, the polymer was previously labeled with sodium fluorescein.

MATERIALS AND METHODS

Chemicals and Animals

The polymer Chitosan SeaCure 123 (CS) (inherent viscosity, 14 mPa·s; deacetylation degree, 85%) was purchased from Pronova Biopolymer A.S. (Drammen, Norway). Sigma Chemical Co. (St. Louis, MO, USA) supplied the sodium tripolyphosphate (TPP) and sodium fluorescein. Other materials were reagent-grade chemicals. Culture plastic material was from Nunc (Roskilde, Denmark), and reagents for cell culture were from Gibco (Life Technologies, Inchinnan, UK). Male albino New Zealand rabbits weighing between 2.0 and 2.5 Kg were used in the *in vivo* study. Animals were allowed free access to food and water during the experiments.

Synthesis of the Chitosan-Fluorescein (CS-fl) Conjugate

The covalent attachment of fluorescein to CS was achieved by the formation of amide bonds between primary amino groups of the polymer and the carboxylic acid groups of fluorescein. A 2.5-g sample of CS was dissolved in 200 ml of (1%, v/v) acetic acid aqueous solution, and the pH value was adjusted to 6.0 with 1 M NaOH. Demineralized water was added to this solution to make the final volume 250 ml. An amount of 100 mg of fluorescein was dissolved in 10 ml of ethanol. Thereafter, both solutions were mixed together, and, to catalyze the formation of amide bonds, EDAC [1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride] was added in a final concentration of 0.05 M. The reaction mixture was incubated under permanent stirring for 12 h in the dark at room temperature. The resulting conjugate was isolated by dialysis (cellulose dialysis tubing, pore size 12,400 Da; Seamless D-0530, Sigma-Aldrich, Madrid, Spain) against demineralized water, 0.05 N NaOH solution, and finally against demineralized water and then lyophilized. The evaluation of the derivatization process was performed by infrared spectroscopy (IR) and also by spectrofluorimetry, using unmodified CS and fluorescein as controls.

Preparation of Chitosan Nanoparticles

Nanoparticles were spontaneously obtained upon addition of a TPP aqueous solution to the CS solution, according to the procedure previously developed by our group (20). CS-fl was dissolved in 0.05% (w/v) acetic acid solution at a concentration of 0.25% and the pH adjusted to 5.5 with a 0.5% (w/v) NaOH solution. TPP was dissolved in purified water at a concentration of 0.2% (w/v). Following this, 0.8 ml of the TPP solution was added to 2.5 ml of the CS-fl solution, thereby leading to the formation of fluorescence-labeled CS nanoparticles. The final pH of the nanoparticles suspension was 6.4.

Physicochemical Characterization of the Nanoparticles

The morphological examination of the nanoparticles was performed by transmission electron microscopy (TEM) (CM12 Philips, Grovewood, UK,). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar films for viewing by TEM.

The mean particle size and size distribution of the nanoparticles were determined by photon correlation spectroscopy (PCS). A suspension sample was diluted to the appropriate concentration with filtered distilled water. Each analysis was performed at 25°C with an angle detection of 90°. The zeta potential was calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). Nanoparticles suspension was diluted with 10^{-3} M KCl and placed in the eletrophoretic cell, where a potential of ±150 mV was established. The PCS and LDA analyses were performed using a Zetasizer 3000 HS (Malvern Instruments, Worcested, UK) (n = 3).

Stability of Chitosan Nanoparticles in the Presence of Mucus Components

Incubation with Lysozyme

The stability of CS nanoparticles was analyzed following their incubation at 37°C in a solution of lysozyme in purified water (1 mg/ml) under moderate stirring. This lysozyme concentration was chosen taking into account the concentration of lysozyme in human tears fluids (3–6 mg/ml) and the dilution of nanoparticles after ocular instillation (10 μl nanoparticles per 7 μl tears). The physicochemical properties of the nanoparticles (mean particle size and zeta potential) were monitored during the incubation process.

Incubation with Mucin

Two *in vitro* methods were used to assess the stability and interaction between nanoparticles and mucin. The first method was based on the measurement of the viscosity of a mucin dispersion in water (0.4 mg/ml) before and after incubation at 35°C in the presence of CS nanoparticles or CS solutions. Dispersion viscosity measurements were carried out by Cannon-Fenske viscometer 5354/2 (Dien, France). The viscometer was submerged in a thermostated bath with a temperature control precision of a 0.1°C. For each time studied, 4–5 measurements were taken, and average values were computed. The second method evaluated the influence of the mucin on the zeta potential of the nanoparticles. CS nanoparticles were incubated at 35°C in aqueous solution of mucin under moderate stirring. At given time intervals (30, 60, 120, 240 min) during incubation, the zeta potential of the nanoparticles was determined as previously described.

In vivo Studies

The nanoparticles were administered to the cul-de-sac of conscious rabbits in order to quantify their *in vivo* interaction with the corneal and conjunctival epithelia. The nanoparticles were not isolated because of the absence of toxic ingredients in the final preparation; consequently, the suspension contained CS in the form of nanoparticles and also CS in solution. The total CS concentration was 1.9 mg/ml. The protocol of administration consisted of 10 instillations of 10 μl of the nanoparticles suspension (5 instillation each eye), given at 10-min intervals. Two control formulations were also administered: one was a solution of fluorescein-labeled CS (CS-fl solution) and the other a fluorescein aqueous solution. After

each instillation, the animals were maintained in an upright position using restraining boxes. The rabbits were sacrificed 1, 2, 4, 8, and 24 h after the last instillation, the eyes enucleated, and the cornea and conjunctiva excised. Each tissue was rinsed with normal saline, blotted dry, and transferred to test tubes where they were digested at 37°C until completely dissolved in 500 μl of a 0.5 M NaOH solution. In order to solubilize the CS retained in the tissues, 1 ml of 1 M HCl was added to the medium. Afterwards, the fluorescein was extracted using buthanol as an extraction solvent. The extraction was performed three times, using 3 ml buthanol volumes, followed by sonication (Branson 250, Sonifier, Barcelona, Spain) for 2 min (40 W) and centrifugation for 30 min, 3000 × *g* at 20°C. Buthanol solution fluorescence was measured by spectrofluorimetry (Luminescence Spectrometer LS50 B, Perkin Elmer, Norwalk, CT, USA), and the fluorescein quantities were calculated (n = 6). A calibration curve was made following extraction of different amounts of CS-fl according to the above-mentioned protocol.

Confocal Laser Scanning Microscopy

Three instillations of 25 μl of the CS-fl nanoparticles suspension containing as well CS in solution were administered to the cul-de-sac of conscious rabbits at 10-min intervals. After each instillation, the animals were maintained in an upright position using restraining boxes. Two hours later, rabbits were killed with an intravenous injection of an overdose of sodium pentobarbital given via a marginal ear vein. Corneal and conjunctival specimens, freshly excised, were directly mounted on a glass slide and examined microscopically without additional tissue processing.

The system (Confocal Bio-Rad MRC 1024E5, Barcelona, Spain) consists of a computer-controlled laser scanner assembly with a Nikon fluorescence microscope (Cambridge, MA, USA). A 100-mW argon lamp ion laser operation at 488 nm wavelength was used as a excitation source. Images were assembled in an integral image processor and displayed on a digital video monitor.

Cell Culture Studies: Conjunctival Cell Line

The commercially available Chang conjunctival cell line (the Wong-Kilbourne derivative of Chang conjunctival cells, ATCC CCL 20.2, clone 1-5c-4) was used for the toxicity experiments (68-72 passages). Chang cells were grown in DMEM/F12 culture medium supplemented with 1 μg/ml bovine pancreas insulin, 2 ng/ml mouse EGF, 0.1 μg/ml cholera toxin, 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The culture conditions were standard (incubation at 37°C in a 5% CO_2/95% O_2 atmosphere), the medium was changed every 2–3 days, and cell growing assessed daily by phase-contrast microscopy.

Cell Culture Studies: Toxicity Measurement

The nanoparticles suspensions were prepared in aseptic conditions, using sterilized solutions (sterile filtration) of CS and TPP. The suspensions were diluted in acetate buffer pH 6.0 and then added to the cells at different concentrations (0.25, 0.5, 1.0, 2.0 mg/ml). The dilution buffer was chosen because of the instability of the particles in a phosphate buffer pH 7.4. After 30 min incubation, nanoparticle suspension was washed out and supplement-free culture medium added. Then cells were incubated for an additional 23.5 h at 37°C. Controls for the toxicity experiments were 0.005% benzalkonium chloride (BAC) (positive control), acetate buffer pH 6.0 (vehicle), and supplement-free culture medium (negative control) and were also added to the cells. After 24 h and several washes in Ca^{2+} and Mg^{2+}-free PBS, cell survival (percentage of cell recovery) and the viability of those surviving cells were assessed. Viability of recovered cells was assessed by Trypan Blue Dye Exclusion Test.

Cell Culture Studies: Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to monitor potential cell alterations or membrane damage in NHC cells after exposure to chitosan nanoparticles. As indicated above, acetate buffer, supplement-free culture medium, and BAC were used as controls. NHC cells were incubated with nanoparticles and controls as above and washed. After that, they were fixed in 1.0% glutaraldehyde in 0.1 M sodium cacodylate-HCl (pH 7.4) for 10 min at 37°C, washed in 0.2 M sucrose solution, dehydrated in a graded ethanol series, critical point dried according to Anderson (21), and gold sputter coated (15–20 nm) in vacuum evaporation under argon gas at a conducting amperage of 20 mA. A JEOL T300 microscope with a Maiya Rolf Holder CS-I photographic system (Tokyo, Japan) was used to examine the cells.

Statistical Analysis

The statistical significance of the differences between nanoparticles and controls at each time was tested by a one-way analysis of variance (ANOVA) with the Pairwise Multiple Comparison Procedures (Student-Newman-Keuls method) for multiple comparison (SigmaStat program; Jandel Scientific, Version 1.0). Differences were considered to be significant at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Development and Characterization of CS-Fluorescein Nanoparticles

In our attempt to evaluate the interaction of CS nanoparticles with biological structures (by spectrofluorimetry and confocal fluorescence microscopy), the first step of the work was the development of a fluorescent derivative of CS that provides a stable fluorescent signal without causing important changes in the characteristics of the nanoparticles. The association of fluorescein to the polymer was first identified by spectrofluorimetry. Indeed, the emission and excitation wavelength values observed for the conjugate were 484 and 514, respectively, whereas those of fluorescein were 188 and 512, respectively. On the other hand, using IR spectroscopy we could see that the amine peak in CS-fl spectrum (at 1562 cm^{-1}) decreased whereas the peak due to amide group (at 1659 cm^{-1}) increased, when compared with the unmodified CS spectrum. This suggests that a reaction takes place between the fluorescein acid group and the CS amine group. Therefore, the result of this reaction is a fluorescent yellow polymer that has a lower number of free CS amino groups. This conclusion led us to accept that the amount of primary amino groups, which are essential for the mucoadhesive properties of chitosan (15), could decrease due to the covalently attached dye. Therefore, once identified the formation of the

conjugate, the next step was to verify if the attachment of fluorescein to some amino groups of CS would affect the zeta characteristics of the resulting nanoparticles. The results showed that the zeta potential of unmodified CS nanoparticles was very similar (+37.5 ± 0.9 mV) to that of CS-fl nanoparticles (+35.6 ± 0.17 mV). On the other hand, the mean nanoparticles size was 384.6 ± 8.5 nm, the polidispersity index 0.34, and transmission electron microscopy evidenced spherical CS-fl nanoparticles with a solid and consistent structure, characteristics that were very similar to those previously reported for unmodified CS nanoparticles (20). Consequently, these results suggest that the fluorescent labeling of CS should not affect significantly the biological behavior of the nanoparticles.

Stability of CS-fl Nanoparticles in the Presence of Mucus Components

The stability of colloidal particles in biological fluids containing important amounts of proteins and enzymes is a crucial issue. At present, it is broadly accepted that the size of the particles plays an important role in their ability to interact with mucosal surfaces and, in particular, with the ocular mucosa (7). Surprisingly, despite the importance of the size, there are very few articles on the stability of colloidal particles in biological fluids. For example, recent work by our group showed that poly(lactic acid) nanoparticles aggregate significantly upon contact with simulated gastric fluids (22). Similarly, we have observed that poly-ε-caprolactone (PECL) nanocapsules suffer an immediate aggregation process upon their incubation with lysome (23). This aggregation was, however, hindered by coating the PECL nanocapsules with CS. This previous information led us to consider the importance of performing a preliminary study of the stability of the particles in the presence of two major components of the precorneal fluid: lysozyme and mucin.

The particle size and zeta potential of CS nanoparticles were determined upon their incubation in the presence and absence of lysozyme. The results presented in Table I indicate that the size was slightly reduced upon incubation with the enzyme. The slight size reduction could be attributed to a partial hydrolysis of some CS molecules externally located, caused by lysozyme. We should, however, be cautious in the interpretation of these data as it has been reported that lysozyme does interact with the acetamide groups but it does not with the free amino groups (24). Hence, given that the deacetylation degree of the CS used in this study is approximately 85%, only a minor degradation of CS nanoparticles must be expected after a 4-h incubation period. This minor degradation might however be enough to generate some polymer fragments, which could eventually detach from the nanoparticles. For example, Sashiwa *et al.* (25) observed that the molecular weight of 77% deacetylated CS went down to half of the original value in 12 h. On the other hand, the results in Table I also show that the incubation of the particles with lysozyme did not lead to a modification in their surface charge. This could be understood by the fact that lysozyme is a cationic protein (pI: 10.5–11.5) and, consequently, its interaction with the nanoparticles might not affect significantly their zeta potential. A similar observation was previously reported for CS-coated PLGA nanoparticles (26). Therefore, the major conclusion from this stability study is that the integrity of CS nanoparticles is not significantly compromised by the presence of lysozyme in the tears fluid.

The stability and interaction of CS in the presence of mucin was also determined by measuring the viscosity of a mucin dispersion before and after incubation with CS nanoparticles. This viscosity was also compared with that of a CS solution incubated in the same conditions. This kind of measurement is important because the blink process requires a low tear viscosity in order to avoid damage to the corneal epithelium (10). Therefore, a significant increase in this parameter should, preferably, be avoided. Results depicted in Table II show that the addition of the CS solution to the mucin dispersion led to a significant increase in the viscosity of the medium. This has been attributed to the mucin carboxylic acid groups, which are ionized at a pH value of 5.5 (pK_a 2.6) and therefore freely accessible for interaction with the positively charged amine groups (24). Conversely, no change in the mucin viscosity was noticeable after a 2-h incubation period with CS-nanoparticles (original viscosity of the mucin dispersion: 0.757 ± 0.004 cSk). This could be explained by the low flexibility of the CS chains forming the solid particles. In fact, it has been reported that chain flexibility is a prerequisite to facilitate interactions with mucin (27). Thus, the lack of viscosimetric changes in the mucin-nanoparticle dispersion suggested that, under the current experimental conditions, no significant interactions of mucin with the nanoparticle ocurred. Additionally, it could be inferred that, upon instillation of the particles, the viscosity of the tear fluid would not be significantly altered. Nevertheless, despite these viscosity data, results of the zeta potential of the nanoparticles upon incubation in a mucin dispersion suggest that a certain interaction may occur between mucin and CS nanoparticles. Indeed, the zeta potential distribution profiles obtained for a

Table I. Physicochemical Properties of CS-fl Nanoparticles Before and After Incubation with Lysozyme (n = 3)

	Particle size (nm)		ζ Potential (mV)	
Time	Control	After incubation with LZM	Control	After incubation with LZM
Initial	394 ± 6	394 ± 6	37.5 ± 0.9	+37.5 ± 0.9
30 min	382 ± 17	367 ± 14	38.4 ± 0.2	+37.6 ± 0.5
1 h	377 ± 13	363 ± 15	38.2 ± 0.3	+37.6 ± 0.4
2 h	377 ± 13	355 ± 8	38.1 ± 0.4	+37.4 ± 0.3
4 h	375 ± 15	348 ± 8	37.6 ± 0.7	+36.3 ± 1.3

LZM, lysozyme.

Table II. Cinematic Viscosity Values of a Mucin Dispersion (0.4 mg/ml) After Incubation with CS Solution and CS Nanoparticles (n = 5)

Time (min)	Viscosity (cSk)	
	Mucin + CS solution	Mucin + CS nanoparticles
5	1.009 ± 0.027	0.767 ± 0.007
15	0.954 ± 0.005	0.764 ± 0.004
30	0.942 ± 0.004	0.761 ± 0.007
60	0.921 ± 0.002	0.758 ± 0.007
120	0.887 ± 0.011	0.759 ± 0.003

The original cinematic viscosity of the mucin dispersion was 0.757 ± 0.004. CS, chitosan.

mucin dispersion and for a suspension of nanoparticles before and after incubation with mucin (Fig. 1) support this observation. In addition, we observed that this slight reduction in the zeta potential values occurs during the first 30 min. This reduction could be attributed to the ionic interaction between the negatively charged particles and chitosan nanoparticles. Therefore, a conclusion from this study is that the nanoparticles are able to slightly interact with mucin; however, this interaction did not lead to a significant modification of the viscosity of the mucin dispersion.

In vivo Study: Quantitative and Qualitative Evaluation of the Interaction of CS Nanoparticles with the Ocular Mucosa

As indicated in the "Materials and Methods" section, isolation of CS-fl nanoparticles is not required for *in vivo* administration because the only major component in the suspending medium is free CS-fl in solution. However, it was important to determine the amount of CS-fl that was in the form of nanoparticles and also that in solution. This parameter (nanoparticles yield) could be determined following centrifugation of the nanoparticles and further quantification of CS-fl by spectrofluorimetry. The result was that 26.8% of CS-fl was in solution and hence 73.2% in the form of nanoparticles.

Figs. 2A and 2B show, respectively, the concentration of CS-fl in cornea and conjunctiva following instillation of the

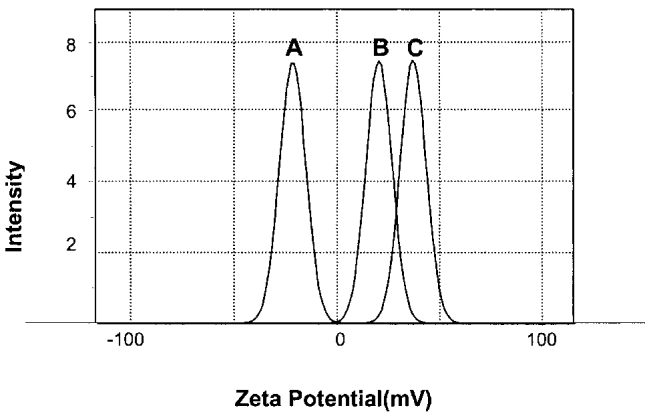

Fig. 1. ζ potential values obtained for (A) a mucin dispersion, (B) CS nanoparticles after incubation with mucin dispersion, and (C) CS nanoparticles.

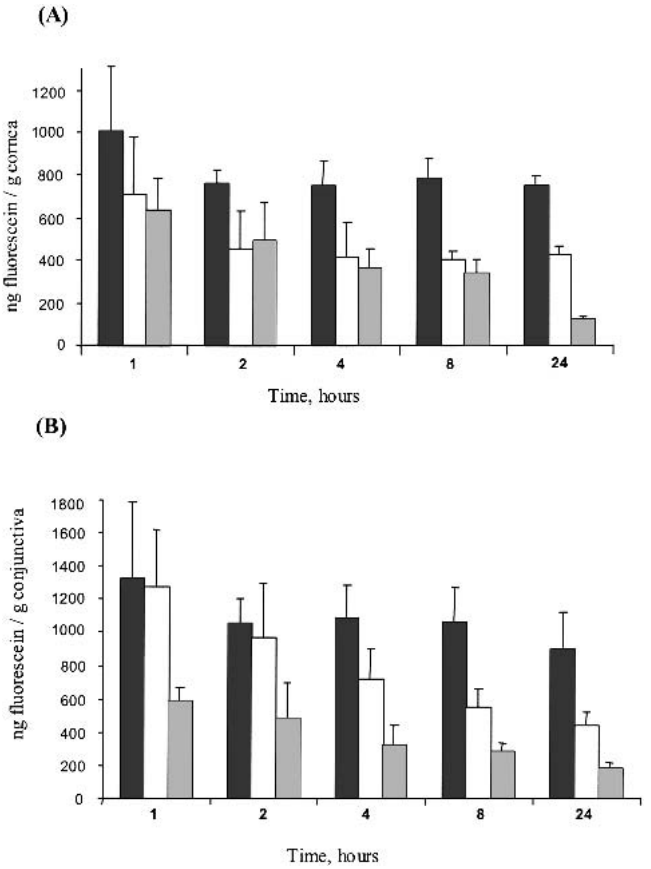

Fig. 2. Evaluation of fluorescein concentration in (A) rabbit cornea and (B) conjunctiva after the instillation of CS-fl nanoparticles (■), CS-fl solution (□), and fluorescein solution (▬) (n = 6).

CS-fl nanoparticles, CS-fl solution, and a control solution of fluorescein. These results show that the behavior of the CS-fl nanoparticles is remarkably different from that of CS-fl solution. The first general observation is that CS-fl nanoparticles provided, to the cornea and conjunctiva, greater concentrations of fluorescein than CS-fl solutions or fluorescein solutions. In fact, the differences in fluorescein concentrations for the nanoparticles and the CS solution were statistically significant at all times assayed with the exception of time 1 h. (cornea) and time 1–2 h. (conjunctiva) ($p < 0.05$). These results indicate that the interaction of CS with the ocular surface (either cornea or conjunctiva) is more persistent when it is in a nanoparticulated form. It also suggests that the mechanism of interaction might be different for both soluble and particulated CS forms. The second important observation from Figs. 2A and 2B is that, following instillation of the nanoparticles, the concentration of fluorescein in both cornea and conjunctiva remains fairly constant for up to 24 h ($p < 0.05$). In contrast, the levels associated with the CS-fl solution and free fluorescein decreased gradually with time. In other words, CS-fl in solution is cleared from the eye faster than CS-fl nanoparticles. Finally, even though CS-fl nanoparticles interact with cornea and conjunctiva, the CS-fl concentration in the conjunctival tissue at early times (2–4 h) is superior to that found in the cornea ($p < 0.05$). This suggests a favorable interaction of the particles with the conjunctiva vs. the cornea.

The more important ocular interaction and retention of the nanoparticles as compared with the solution is in good

agreement with previous work that showed that colloidal particles are taken up and transported across the corneal epithelial cells (8). In order to elucidate whether the particles are simply able to stick to the ocular mucosa or further able to enter the epithelia, we examined cross sections of the corneal and conjunctival epithelia by confocal laser scanning microscopy. The confocal images of a cross section of a rabbit cornea previously treated with CS-fl nanoparticles are shown in Fig. 3. Fluorescent signals can be clearly identified between the corneal epithelial cells and, in a lesser extent, round fluorescent spots appeared to be located inside the cells. These particle-like signals could be associated to the CS-fl nanoparticles as only a light and diffuse fluorescence is visible following application of a simple fluorescein solution. The images suggest that CS nanoparticles are preferably transported by a paracellular mechanism, although the round spots visible inside the cells also support their transcellular transport. This behavior is slighly different from that of other types of nanoparticles, such as poly(alkylcyanoacrylate) (28) and PECL nanoparticles (8), the transport of which was found to occur by a transcellular pathway. Nevertheless, the paracellular transport of CS nanoparticles could be better explained by the intrinsic properties of CS molecules. Previous studies carried out in Caco-2 cell cultures have suggested that CS molecules are able to open the tight junctions between epithelial cells and to allow paracellular transport of large hydrophilic compounds (29). More precisely, this behavior has been attributed to the interaction of the positively charged amino groups of CS with negatively charged sites of the cell surfaces and tight junctions (30). 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. This particular microenvironment in the intercellular spaces and the specific behavior of CS molecules might explain the interaction of CS nanoparticles with the corneal epithelium. In fact, the suspension of CS nanoparticles contains approximately 50% of CS in solution. Therefore, we assume that CS molecules in solution open the tight junction thereby facilitating the transport of CS nanoparticles. This intercellular penetration rather than simple mucoadhesion could explain the high interaction and long residence time of the particles in the cornea. The confocal image of a rabbit cornea treated with CS-fl solution was dramatically different as only a fluorescent background was observed onto the epithelial cells (results not shown). This could also explain the greater persistence of the particles as compared to the CS-fl solution.

The confocal microscopy images of cross sections of the conjunctival epithelium (Fig. 4) were drastically different from those of the cornea. Interestingly, the fluorescence signals were not uniformly distributed on the epithelial cells. Additionally, this image suggests that the particles are located inside the cells rather than in the intercellular spaces. This uneven distribution and intracellular localization could be explained by a greater affinity of the nanoparticles by some specific types of cells. In fact, this epithelium is more heterogeneous than that of the cornea as it contains not only regular epithelial cells, but also goblet cells and antigen presenting cells (APC). Hence, we cannot discard the possibility that CS-fl nanoparticles could be transported to the APC (Langerhans cells and macrophages) located subepithelially (31) or even that a specific cell receptor might be involved in the transport to some epithelial cells. The internalization of CS nanoparticles by conjunctival epithelial cells could also be

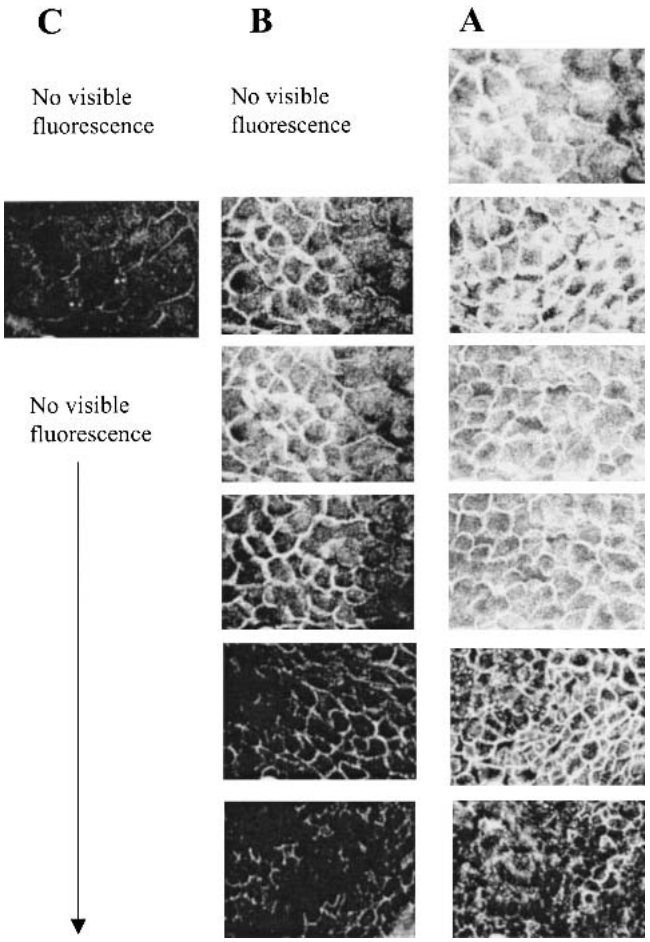

Fig. 3. Confocal fluorescence images at different levels from the rabbit corneal epithelium (5 μm sequential cross sections from the corneal surface) at 1 h postinstillation of (C) CS-fl nanoparticles, (B) CS-fl solution. (A) image of cross section of a nontreated cornea.

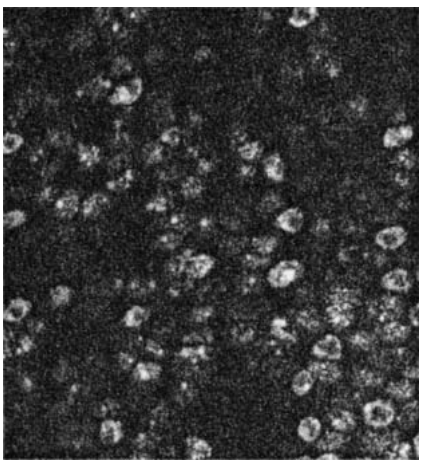

Fig. 4. Confocal fluorescence image at 10 μm from the surface of a conjunctiva excised after 1 h postinstillation of CS-fl nanoparticles in rabbit.

Table III. Percentage of Recovered Cells and Their Percentage of Viability (Assessed by the Trypan Blue Dye Exclusion Test) After 24-h Exposure to the Different Nanoparticles Concentrations in Acetate Buffer (pH 6.0), and Controls

	Medium (C^-)	BAC (C^+)	CS np (0.25 mg/ml)	CS np (0.5 mg/ml)	CS np (1 mg/ml)	CS np (2 mg/ml)	Acetate buffer, pH 6.9
% Recovered cells	100.0 ± 0	2.0 ± 2.0	126.75 ± 10.75	82.3 ± 17.7	56.65 ± 23.35	78.55 ± 1.45	67.42 ± 4.27
% Viability (Trypan Blue)	98.95 ± 1.05	50.0 ± 50.0	95.2 ± 1.3	98.4 ± 1.6	91.25 ± 3.75	96.15 ± 1.15	97.7 ± 1.43

C^-, negative control; C^+, positive control; BAC, 0.005% benzalkonium chloride in culture medium; CS np, chitosan nanoparticles suspended in acetate buffer.

supported by the previous evidence of the transport of other types of nanoparticles using conjunctival primary culture models (32), and also for CS nanoparticles using normal human conjunctival (NHC) cells (33). Therefore, more detailed studies using cell cultures could be useful to further elucidate the mechanism of interaction and internalization of the CS nanoparticles within these cells. Nevertheless, the affinity for some specific conjunctival epithelial cells could explain the more important concentration of CS-fl, following instillation of the nanoparticles, in the conjunctiva as compared to the cornea, as seen in Figs. 3A and 3B. This greater affinity for the conjunctiva as compared to the cornea might also be justified by the more important concentration of mucin in the conjunctiva (34). Consequently, CS-fl nanoparticles may have a greater change to adhere to the conjuntiva, and to subsequently enter some epithelial cells, rather than to the cornea.

Toxicity of Chitosan Nanoparticles in a Conjunctival Cell Line

The results of the toxicity (survival and viability) of CS nanoparticles are shown in Table III. The similar survival values observed for the buffer and for the various concentrations of nanoparticles indicates that no inherent toxicity can be attributed to the nanoparticles at concentrations as high as 2 mg/ml. In addition, the viability of the recovered cells was totally preserved irrespective of the nanoparticles concentration.

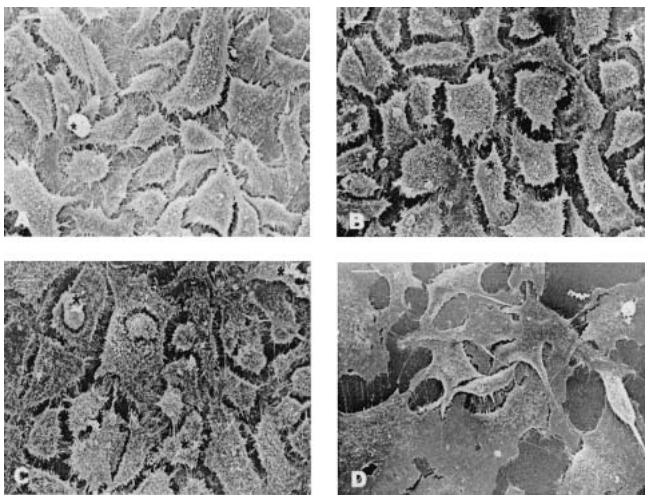

Fig. 5. Scanning electron microphotographs of Chang cells exposed to culture medium, chitosan nanoparticles, and BAC for 24 h. (A) (Negative control, culture medium) Cells showing abundant microvilli and intact membrane details. (B) (CS nanoparticles, 0.25 mg/ml) cells showing well-preserved morphology, an intact cell surface, and abundant microvilli, as expected for an epithelial cell. (C) (CS nanoparticles, 1 mg/ml). (D) (Positive control, BAC) Cells were flat and showed absence of microvilli and broken membrane. Magnification ×750 (bar = 15 μm).

In order to investigate further the tolerance of the particles by the conjunctival cells, we observed the appearance of the cells after exposure to the nanoparticles and corresponding controls, by SEM. The images displayed in Figs. 5A–5C show the general healthy state of the cells, characterized by a well-preserved cell surface. A slight cell loss was observed when cells were exposed to the nanoparticles and also to the acetate buffer, as compared to the control (cells exposed to culture medium). This suggests that the suspensions of nanoparticles cause some deleterious effect that could be simply attributed to the buffer that was used for the resuspension of the nanoparticles. A more detailed observation of the cells indicates that, after treatment with moderate nanoparticles concentrations (0.25–1.0 mg/ml), cells exhibit an intact surface with abundant microvilli and without apparent membrane alterations (Figs. 5B and 5C). The appearance of the cells was very similar to that of the control (Fig. 5A). For the highest nanoparticles concentration (2 mg/ml), a few small membrane holes and some degree of cell flattening and microvilli loss were observed (not shown). Cells exposed to BAC showed enormous alterations and flattering (Fig. 5D). Some membrane alterations and cell loss were observed for those cells exposed to acetate buffer, mostly small holes in the membrane and microvilli loss resulting in a plain surface (not shown). Therefore, these results led us to suggest that increasing concentrations of CS nanoparticles may lead to some cell damage, however, due to the negative effect of the buffer, more experiments need to be performed in order to discriminate the effect of the nanoparticles *per se* with that of the buffer used for their resuspension.

CONCLUSIONS

CS nanoparticles are able to interact and remain associated to the ocular mucosa for extended periods of time, thus being promising carriers for enhancing and controlling the release of drugs to the ocular surface. Current studies are aimed at investigating in further detail how the interaction and internalization of these particles occurs and their toxicity following repeated administration.

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