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Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A

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Abstract

Present limitations in the management of extraocular diseases include the inability to provide long-term extraocular drug delivery without compromising intraocular structures and/or systemic drug exposure. In the present study, the potential of chitosan (CS) nanoparticles as a new vehicle for the improvement of the delivery of drugs to the ocular mucosa was investigated. Cyclosporin A (CyA) was chosen as a model compound because of its potential usefulness for the treatment of these local diseases. An ionic gelation technique was conveniently modified in order to produce CyA-loaded CS nanoparticles. These nanoparticles had a mean size of 293 nm, a zeta potential of + 37 mV and high CyA association efficiency and loading (73 and 9%, respectively). In vitro release studies, performed under sink conditions, revealed a fast release during the first hour followed by a more gradual drug release during a 24-h period. In vivo experiments showed that, following topical instillation of CyA-loaded CS nanoparticles to rabbits, it was possible to achieve therapeutic concentrations in external ocular tissues (i.e., cornea and conjunctiva) during at least 48 h while maintaining negligible or undetectable CyA levels in inner ocular structures (i.e., iris/ciliary body and aqueous humour), blood and plasma. These levels were significantly higher than those obtained following instillation of a CS solution containing CyA and an aqueous CyA suspension. From these results, we can conclude that CS nanoparticles may represent an interesting vehicle in order to enhance the therapeutic index of clinically challenging drugs with potential application at extraocular level.

Keywords: Chitosan; Nanoparticles; Ocular; Topical; Cyclosporin A; Dry eye; Drug delivery

1. Introduction

Most efforts in ophthalmic drug delivery have been devoted to increasing the corneal penetration of drugs with the final goal of improving the efficacy of treatments of different ocular diseases. These attempts include the use of colloidal drug

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delivery systems, such as liposomes (Smolin et al., 1981) and biodegradable nanoparticles (Losa et al., 1991) and nanocapsules (Losa et al., 1993). However, the short residence time of these colloidal systems in the ocular mucosa represents a limitation in the therapy of extraocular diseases, such as keratoconjunctivitis sicca or dry eye disease.

Extensive investigations carried out over the last decade support the view that the local immunosuppression caused by cyclosporin A (CyA), is effective for the management of extraocular disorders, i.e. keratoconjunctivitis sicca or dry eye disease (Power et al., 1993; Kaswan, 1994; Stern et al., 1998). Despite the evidence that the target sites for the treatment of these diseases are the cornea and conjunctiva (Gündüz and Özdemir, 1994; Acheampong et al., 1999), the CyA delivery systems investigated so far (i.e., oils, emulsions, collagen shields, liposomes and nanocapsules) have not been successful. The most popular oil-based vehicles have serious limitations that include the slow partition rate of CyA into the corneal epithelium (Acheampong et al., 1999), the intraocular and/or systemic absorption of CyA (Foets et al., 1985; Bellot et al., 1992), and the local side effects associated with the use of oils (symptoms of irritation, blurred vision, itching, transient epithelial keratitis and toxic effects at corneal level; Kaswan et al., 1989; Benítez del Castillo et al., 1994). These side effects are less important in the case of liposomes, nevertheless, the formulation of CyA-loaded liposomes reported in the literature was not able to achieve adequate CyA levels in the ocular mucosa (Nussenblatt, 1988). Collagen shields were found to provide a sustained delivery of CyA to the surface of the eye. However, the use of such a system is limited by the ocular irritation and blurring of vision that it causes (Dua et al., 1996). Finally, in a previous attempt by our group to improve the ocular penetration of CyA, we developed CyA-loaded poly-ε-caprolactone nanocapsules. These new delivery systems were efficient at improving the transcorneal transport of CyA while reducing systemic absorption but they did not provide significant CyA at the ocular mucosa for extended periods of time (Calvo et al., 1996). Consequently, the design of a system with improved drug delivery properties to the ocular surface would be a promising step towards the management of external ocular diseases, such as keratoconjunctivitis sicca or dry eye disease.

Taking into account this information and also the fact that the cornea and conjunctiva have a negative charge, it was thought that the use of mucoadhesive polymers which might interact intimately with these extraocular structures would increase the concentration and residence time of the associated drug. Among the mucoadhesive polymers investigated until now, the cationic polymer chitosan (CS) has attracted a great deal of attention because of its unique properties, such as acceptable biocompatibility and biodegradability (Knapczyk et al., 1989; Hirano et al., 1990) and ability to enhance the paracellular transport of drugs (Artursson et al., 1994). Moreover, CS has recently been proposed as a material with a good potential for ocular drug delivery. More specifically, CS solutions were found to prolong the corneal residence time of antibiotic drugs (Felt et al., 1999), whereas CS-coated nanocapsules were more efficient at enhancing the intraocular penetration of some specific drugs (Calvo et al., 1997a; Genta et al., 1997). More recent work by our group has shown the interaction and prolonged residence time of CS nanoparticles at the ocular mucosa after their topical administration to rabbits (De Campos and Alonso, in preparation). Indeed, following topical instillation of fluorescence-labelled nanoparticles, we observed that these colloidal drug carriers remain attached to the cornea and the conjunctiva for at least 24 h. Therefore, these data led us to accept that these CS nanoparticles may have potential as drug delivery systems to the ocular mucosa.

Based on these considerations, the major goals of this study were to associate the hydrophobic peptide CyA to these hydrophilic nanoparticles and on the other hand, to investigate the potential of these new vehicles for delivering CyA to the outer ocular structures.

2. Materials and methods

2.1. Chemicals and animals

The polymer Chitosan SeaCure 123 (CS) was purchased from Pronova Biopolymer AS (Norway). Sodium tripolyphosphate (TPP) was supplied by Sigma Chemical Co. (USA). CyA was a gift from Novartis (Switzerland). [Mebmt-b-^{3}H]CyA (specific activity of 250 μCi/ml) was purchased from Amersham Ibérica SA (Spain). Tissue solubilizer (BTS-450) was purchased from Beckman (USA). The liquid scintillation cocktails Ecolite™ (+) and Aquasol II were purchased from ICN (Spain) and Beckman (USA), respectively. Ultrapure water was obtained with MilliQ equipment (Waters, USA). Other materials were reagent grade chemicals.

Male albino New Zealand rabbits weighing between 2.0 and 2.5 kg were used in the in vivo study. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed.

2.2. Preparation of chitosan nanoparticles

In the first step, CS was purified by dialysis against ultrapure water, at room temperature during 72 h. The purified product was freeze-dried and stored at room temperature.

CS nanoparticles were prepared according to the procedure previously developed by our group (Calvo et al., 1997a). Nanoparticles were obtained upon the addition of a TPP aqueous solution (0.5 ml, 0.2% w/v) to a CS aqueous solution (4 mg, 0.2 or 0.5% w/v) under magnetic stirring at room temperature. The formation of the particles was a result of the interaction between the negative groups of the TPP and the positively charged amino groups of CS (ionic gelation). A variable volume (125 or 250 μl) of a CyA solution in an acetonitrile/water mixture (1:1; 2.5 mg/ml) was incorporated either into the CS solution or the TPP solution, prior to the formation of the nanoparticles. The [^{3}H]CyA-loaded nanoparticles were prepared by adding trace amounts of [^{3}H]CyA to the CS–CyA solution, before the incorporation of TPP. Nanoparticles were purified by centrifugation at 9000 × *g* in a glucose bed for 30 min. Supernatants were discarded and nanoparticles were resuspended in pure water.

For the in vivo studies we prepared two control formulations consisting of a CyA suspension in water and a CyA suspension in a CS aqueous solution (CS–CyA) with trace amounts of [^{3}H]CyA. The CyA suspension in water was obtained by adding the CyA solution (acetonitrile/water 1:1, 2.5 mg/ml) to water. The CS–CyA formulation was prepared following the procedure used to prepare the nanoparticles, but without the addition of TPP.

2.3. Characterization of the nanoparticles

The morphological examination of the CS nanoparticles and CyA-loaded CS nanoparticles was performed with a transmission electron microscope (TEM; CM12 Philips, USA). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar films for viewing by TEM.

The size and the zeta potential of the nanoparticles were analysed by photon correlation spectroscopy and laser doppler anemometry, respectively, using a Zetasizer, 3000 HS (Malvern Instruments, UK). For the determination of electrophoretic mobility, samples were diluted with 0.1 mM KCl and placed in the electrophoretic cell, where a potential of 150 mV was established. Each batch was analysed in triplicate.

2.4. Cyclosporin A loading of nanoparticles

For the determination of the loading efficiency of the process, the nanoparticles were first separated from the aqueous suspension medium by ultracentrifugation at 18 000 × *g* for 45 min. The amount of CyA loaded into the nanoparticles was calculated as the difference between the total amount used to prepare the nanoparticles and the amount that was found in the supernatant. These amounts were determined by counting the [^{3}H]CyA using Ecolite™ (+) as a scintillation cocktail (LS 6000 LL, Beckman Instruments).

The CyA loading capacity of the nanoparticles and the CyA association efficiency of the process were calculated as indicated below:

$$\text{Loading capacity} = \frac{\text{Total amount CyA} - \text{Free CyA}}{\text{Weight of nanoparticles}} \times 100,$$

$$\text{Association efficiency} = \frac{\text{Total amount CyA} - \text{Free CyA}}{\text{Total amount CyA}} \times 100.$$

Given the fact that CyA precipitates are in the form of nanocrystals upon incorporation to the CS solution and, in order to verify whether these nanocrystals are associated to the CS nanoparticles or suspended independently, a control experiment was performed. In this control experiment, a suspension of CyA nanocrystals was ultracentrifugated in the above conditions and, then, the amount of CyA that formed a sediment was determined. The results showed that only 10% of the nanocrystals sedimented upon ultracentrifugation. Consequently, we can induce that the amount of CyA that sediments with the CS nanoparticles is, indeed, associated to them.

2.5. In vitro release studies

Aliquots of the suspension of nanoparticles containing 10 μg of CyA and traces of [^{3}H]CyA were diluted in 10 ml of purified water and incubated, under agitation, at 37 °C, in order to assess sink conditions during the release studies. At different time intervals, samples were centrifuged (18 000 × g for 45 min) and the CyA released determined by liquid scintillation counting, as described above.

2.6. In vivo blood, plasma and ocular distribution studies

Studies were performed on fully-awake male New Zealand rabbits. The following formulations were tested: CyA-loaded CS nanoparticles and two control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water. The animals were divided into three groups ($n \geqslant 4$–6) and 10 μl of the formulations containing 320 μg/ml of CyA and traces of [^{3}H]CyA were placed in the cul-de-sac of both eyes of animals of each group. Four additional instillations were given at 10-min intervals. At different times post-instillation (2, 6, 24 and 48 h) rabbits were sacrificed. Immediately before sacrifice blood samples were collected at these times from the marginal ear vein in tubes containing heparin and immediately centrifuged at 37 °C, 4500 rpm for 4 min and the plasma fraction was then quickly separated. In addition, the eyes were proptosed and rinsed with normal saline. The aqueous humour was withdrawn from the anterior chamber with the aid of a 25-gauge needle fitted to an insulin syringe. Cornea, conjunctiva and iris/ciliary body were subsequently dissected in situ. Each tissue was rinsed with normal saline, blotted dry in order to remove any adhering drug and transferred to pre-weighed counting vials. The vials were re-weighed and the weight of the tissues was calculated. Blood and ocular tissues were digested at 37 °C until completely dissolved in 1 ml of tissue solubilizer (BTS-450) and discoloured by adding 30 μl of hydrogen peroxide. Afterwards, 30 μl of acetic acid and 10 ml of Ecolite™ (+) scintillation cocktail were added to each vial. The aqueous humour samples (100 μl) were dissolved directly in 10 ml of Ecolite™ (+). Plasma was treated with 30 μl of hydrogen peroxide and 4 ml of Aquasol II was added to the samples.

Finally, the samples were allowed to stand for at least 24 h in the dark to minimize chemiluminiscence before counting in a liquid scintillation counter equipped with automatic quench correction as described above.

2.7. Statistical analysis

The statistical significance of the differences between the concentrations of CyA following administration of the nanoparticles and the corresponding controls was tested, at each time point, 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 $P < 0.05$.

3. Results and discussion

3.1. Formation and characterization of cyclosporin A-loaded chitosan nanoparticles

The preparation of CS nanoparticles, based on an ionic gelation process, involves the mixture of two aqueous phases at room temperature. One phase contains a solution of CS and the other contains a solution of the polyanion TPP. Because of its hydrophobic nature, a number of experiments had to be performed in order to determine the appropriate conditions for the incorporation of the hydrophobic peptide CyA into the CS nanoparticles. A successful entrapment was achieved by dissolving the hydrophobic peptide in an acetonitrile/water mixture (1:1) prior to its incorporation into the CS solution, followed by the addition of the TPP solution. The appropriate formulation conditions were decided from the results of preliminary studies aimed at investigating the effect of the acetonitrile/water mixture volume and the concentration of the CS solution on the physico-chemical characteristics of the nanoparticles. Results presented in Table 1 indicate that the particle size is, as previously reported (Calvo et al., 1997b), dependent upon the CS concentration, the minimum size corresponding to the lowest CS concentration. On the other hand, the size of the particles was affected by the acetonitrile/water volume, the size being the smallest for the highest acetonitrile/water volume assayed in this study. This could simply be attributed to the dilution of the CS solution caused by the addition of the acetonitrile/water mixture. Nevertheless, it is reasonable to speculate that the incorporation of the organic solvent acetonitrile affects the entanglement of the CS chains following the gelation process. On the other hand, the zeta potential of nanoparticles was not modified by the CS concentration nor by the incorporation of the acetonitrile/water mixture in the CS solution, as expected. Based on these results, the concentration of CS selected for the preparation of the CyA-loaded CS nanoparticles was 0.2% and the acetonitrile/water mixture volume was 125 μl.

TEM of the CyA-loaded CS nanoparticles showed that nanoparticles have a solid dense structure and a round shape (Fig. 1). In addition, using high magnifications it was possible to observe very small particles, which were later identified as CyA nanocrystals entrapped in the CS matrix. Ford et al. (1999) had previously shown that CyA dissolved in water miscible organic solvents can be precipitated in an aqueous phase as discrete spherical particles. This precipitation process was found to occur in our preparation. In fact, following addition of the acetonitrile/water CyA solution to the CS solution, a Tyndall effect was observed. A similar effect was also observed upon addition of the acetonitrile/water CyA solution to a water phase, thereby forming a suspension of nanocrystals of CyA. Therefore, as we understand the nanoparticles formation process, after incorporation of the counter ion TPP to the CS solution containing the CyA nanocrystals, CS gels in the form of discrete nanoparticles simultaneously entrapping the CyA nanocrystals suspended in the medium. Hence, the final product consists of a suspension of CS nanoparticles containing CyA nanocrystals entrapped within the CS molecules. The mean size of these CyA-loaded

Table 1
Mean particle size and zeta potential of CS nanoparticles prepared using different concentrations of CS solution and acetonitrile/water (1:1) mixture volumes

CS solution concentration (% w/v)	Acetonitrile/water mixture volume (μl)	Mean particle size (nm)	Zeta potential (mV)
0.2	0	278 ± 03	+38.6 ± 1.6
0.2	125	283 ± 24	+38.2 ± 1.8
0.2	250	281 ± 05	+38.0 ± 1.6
0.5	0	644 ± 29	+36.2 ± 4.7
0.5	125	620 ± 37	+36.5 ± 2.3
0.5	250	534 ± 21	+36.8 ± 1.6

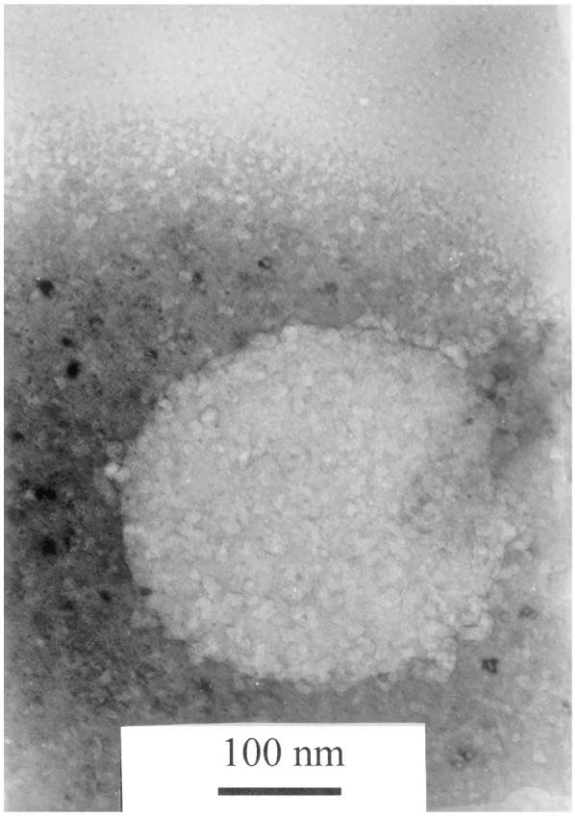

Fig. 1. Transmission electron micrograph of the CyA-loaded CS nanoparticles developed.

CS nanoparticles was 293 ± 9 nm, the polydispersity index 0.34 and the zeta potential $+37.5 \pm 0.9$ mV.

CS nanoparticles displayed a high CyA association efficiency (73.4%) leading to final CyA loading values as high as 9.1%. These values are particularly high if we take into account the hydrophobic character of CyA and the hydrophilic nature of CS.

3.2. In vitro release of cyclosporin A from the nanoparticles

Fig. 2 displays the release profile of CyA from CS nanoparticles under sink conditions. Results showed that CyA is rapidly released from nanoparticles (62% released in 15 min) followed by a very slow drug release. The initial fast release phase may be due to the rapid dissolution of the CyA nanocrystals associated to the CS nanoparticles upon dilution under sink conditions. This rapid dissolution process suggests that, due to the hydrophilic nature of CS, the release medium penetrates into the particles and dissolves the entrapped CyA. Therefore, it could be proposed that the major factor that governs CyA release is its dissolution rate in the release medium. In this sense, and taking into account the sink conditions in which this study was performed, and the extremely small size (a few nanometres) of the CyA nanocrystals, it is not surprising that the dissolution process occurs so rapidly. The absence of such a significant dilution process upon instillation suggests that this fast release should not occur in vivo.

3.3. In vivo studies

In order to investigate the role of CS nanoparticles in the ocular disposition of CyA, we tested three different formulations: CyA-loaded CS nanoparticles, a CyA suspension in a CS aqueous solution and a CyA suspension in water. The last two control formulations were prepared under the same conditions as the CyA-loaded CS nanoparticles, but the counter anion TPP, in the case of the CS aqueous solution, and both TPP and CS, in the case of the CyA suspension in water, were omitted.

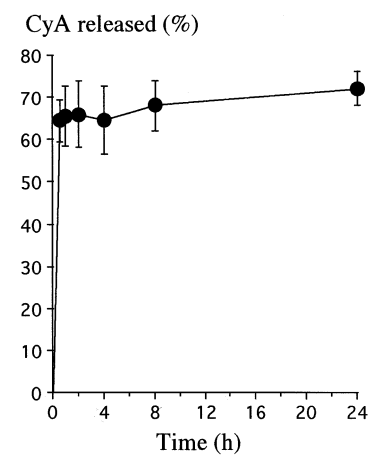

Fig. 2. CyA release profile from CyA-loaded CS nanoparticles.

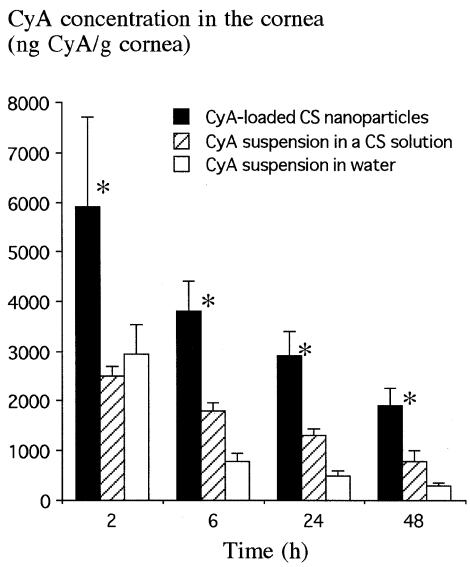

Fig. 3. CyA concentration in the cornea after topical administration in rabbits of CyA-loaded CS nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (* denotes statistically significant differences, $P < 0.05$).

The concentration of CyA in the cornea and conjunctiva after topical administration of the three formulations is shown in Figs. 3 and 4, respectively. The animals treated with CyA-loaded CS nanoparticles had significantly higher corneal and conjunctival drug levels ($P < 0.05$) than those treated with a suspension of CyA in a CS aqueous solution (2–6-fold increase). Surprisingly, no significant effect was observed for the CS solution containing CyA in terms of conjunctival and corneal retention under the experimental conditions of this study. Indeed, no statistically significant differences were found between CyA levels in animals treated with a CyA suspension in a CS aqueous solution and a CyA suspension in water. It was also interesting to note that for all three formulations the corneal and conjunctival levels showed a maximum at 2 h post-administration and decreased gradually afterwards. The key difference was, however, that at 24 h post-instillation of the CS solution, the CyA conjunctival concentration descended to subtherapeutic levels whereas, using CS nanoparticles, these levels were sufficiently high to adequately modulate the local immune response and to suppress inflammatory processes (50–300 ng/g levels; Kaswan, 1988; Acheampong et al., 1999) during at least 24 (conjunctiva) or 48 h (cornea) post-administration.

Consequently, using the CyA-loaded CS nanoparticles, therapeutic concentrations of CyA were maintained in the cornea and conjunctiva throughout the duration of the study. This suggests that these clinically relevant ocular tissues may act as a reservoir for CyA-loaded nanoparticles. The reason for this improved interaction of CyA-loaded nanoparticles with the cornea and the conjunctiva could be found in the mucoadhesive properties of CS (Lehr et al., 1992). However, the limited effectiveness of the CS solution as compared to the CS nanoparticles indicates that the fact that CS is in the form of nanoparticles plays a key role in improving their interaction with the ocular surface. In fact, this facilitated interaction of the CS nanoparticles with the cornea and the conjunctiva has recently been visualized by confocal microscopy (De Campos and Alonso, in preparation). Moreover, this interaction has not been seen as totally dependent on the mucoadhe-

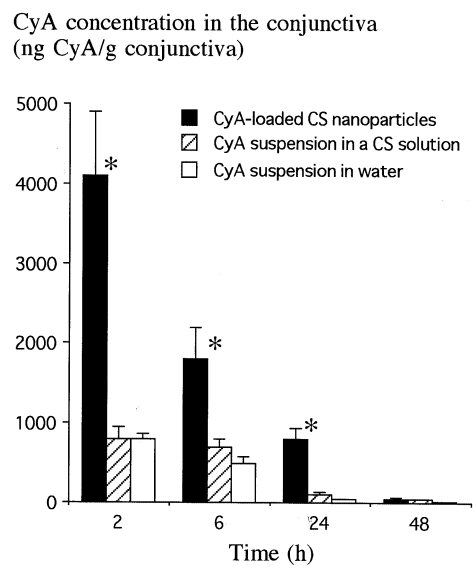

Fig. 4. CyA concentration in the conjunctiva after topical administration in rabbits of CyA-loaded CS nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (* denotes statistically significant differences, $P < 0.05$).

sive character of the polymer since it has already been reported for colloidal carriers made of non-mucoadhesive polymers (Calvo et al., 1994). Therefore, it is our hypothesis that it might not be the mucoadhesive character of CS molecules but the electrostatic interaction between the positively charged CS nanoparticles and the negatively charged corneal and conjunctival cells that is the major force responsible for the prolonged residence of CyA in these epithelia. This mechanism of interaction is important in dry eye disease, since the ocular surface mucin content in dry eye disease is quite variable (Gündüz and Özdemir, 1994; Danjo et al., 1998).

Another interesting observation from Figs. 3 and 4 is that the CyA levels achieved in cornea were higher than those in conjunctiva (significant differences at 6, 24 and 48 h for the three formulations, $P < 0.05$) and that these CyA levels drop faster in the conjunctiva than in the cornea. This could be attributed to the uptake of CyA-loaded nanoparticles by the antigen presenting cells (Langerhans cells and macrophages; Baudouin et al., 1997) and/or the diffusion of the drug to the blood and lymphatic vessels underlying the fine and leaky conjunctival epithelium. Indeed, the systemic absorption of CyA cannot be discarded; however, according to the results shown in Fig. 5, the blood levels observed for the three formulations tested are much below the described toxic levels (300 ng/ml; Bowers and Canafax, 1984). Moreover, the plasma concentrations were found to be below detectable levels (9.85 ng/ml) during the study period. Therefore, we can conclude that a selective and prolonged CyA delivery was achieved using CS nanoparticles, without compromising inner ocular tissues and minimizing systemic drug absorption.

Finally, Figs. 6 and 7 show the CyA concentrations in the aqueous humour and iris/ciliary body, respectively, after topical administration of the three formulations. Only minimal intraocular concentrations were attained throughout the duration of the study and CyA levels in aqueous humour went down below the limit of detection at 48 h post-administration. The extremely low CyA levels at these inner ocular structures are justified by the limited intraocular penetration of free CyA, because of its retention in the corneal stroma (BenEzra and Maftzir, 1990). Nevertheless, these results also suggest that the CS nanoparticles do not enhance the penetration of CyA, which remains on the ocular surface. Indeed, the higher CyA levels in the aqueous humour, at 2 h post-administration, achieved for CS nanoparticles should be attributed to the higher corneal CyA levels corresponding to this formulation, as compared to the control formulations. More specifically, the ratio of CyA concentrations in cornea/aqueous humour/iris–ciliary body provided by the nanoparticles was approximately 6000/9/65, whereas for the CyA control formulations it was 2500/0.1/25. Subsequently, no significant differences in the CyA intraocular levels ($P < 0.05$) were observed, thereby confirming that CS nanoparticles favour the accumulation of CyA on the external tissues without compromising intraocular structures.

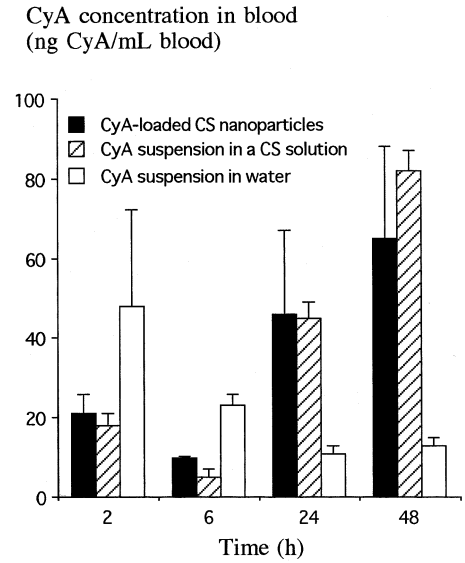

Fig. 5. CyA concentration in blood after topical administration in rabbits of CyA-loaded CS nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (* denotes statistically significant differences, $P < 0.05$).

4. Conclusions

In the present study, we investigate the potential of CS nanoparticles for the specific delivery of drugs to the ocular mucosa, using the immunosuppressive peptide CyA as a model drug. The advantages of these systems in ocular drug delivery include their ability to contact intimately with the corneal and conjunctival surfaces, thereby increasing delivery to external ocular tissues without compromising inner ocular structures and systemic drug exposure, and to provide these target tissues with long-term drug levels. Consequently, these systems show great promise with regard to the circumvention of the present limitations in the management of external inflammatory/autoimmune ocular diseases, such as keratoconjunctivitis sicca or dry eye disease.

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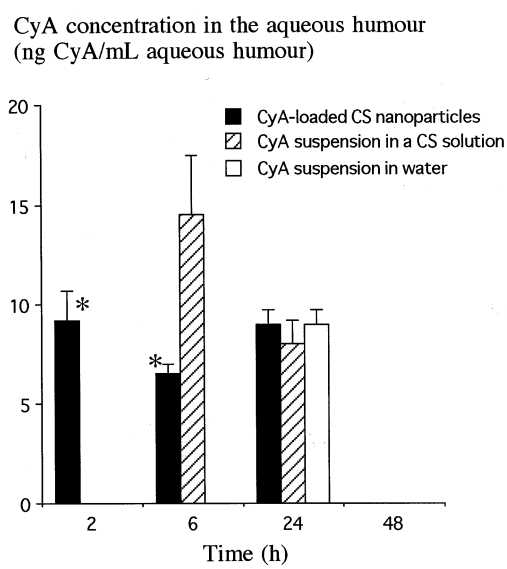

Fig. 6. CyA concentration in the aqueous humour after topical administration in rabbits of CyA-loaded CS nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (* denotes statistically significant differences, $P < 0.05$).

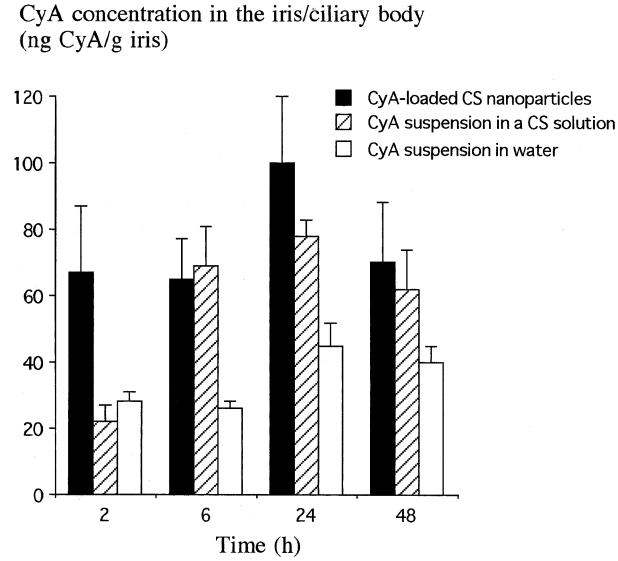

Fig. 7. CyA concentration in the iris/ciliary body after topical administration in rabbits of CyA-loaded CS nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (* denotes statistically significant differences, $P < 0.05$).

References

Acheampong, A.A., Shackleton, M., Tang-Liu, D.D., Ding, S., Stern, M.E., Decker, R., 1999. Distribution of cyclosporin A in ocular tissues after topical administration to albino rabbits and beagle dogs. Curr. Eye Res. 18, 91–103.

Artursson, P., Lindmark, T., Davis, S.S., Illum, L., 1994. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm. Res. 11, 1358–1361.

Baudouin, C., Brignole, F., Pisella, P.J., Becquet, F., Philip, P.J., 1997. Immunophenotyping of human dendriform cells from the conjunctival epithelium. Curr.Eye Res. 16, 475–481.

Bellot, J.L., Alió, J.L., Moreno, J.M.R., Artola, A., 1992. Corneal concentration and systemic absorption of cyclosporin-A following its topical application in the rabbit eye. Ophthalmic Res. 24, 351–356.

BenEzra, D., Maftzir, G., 1990. Ocular penetration of cyclosporin A. Invest. Ophthalmol. Visual Sci. 31, 1362–1366.

Benítez del Castillo, J.M., Aghila, C., Duran, S., Hernandez, J., Sanchez, J.G., 1994. Influence of topically applied cyclosporin A in olive oil on corneal epithelium permeability. Cornea 13, 136–140.

Bowers, L.D., Canafax, D.M., 1984. Cyclosporin: experience with therapeutic monitoring. Ther. Drug Monit. 6, 142–147.

Calvo, P., Thomas, C., Alonso, M.J., Vila-Jato, J.L., Robinson, J.R., 1994. Study of the mechanism of interaction of poly(e-caprolactone) nanocapsules with the cornea by confocal laser scanning microscopy. Int. J. Pharm. 103, 283–291.

Calvo, P., Sanchez, A., Martinez, J., Lopez, M.I., Calonge, M., Pastor, J.C., Alonso, M.J., 1996. Polyester nanocapsules as new topical ocular delivery systems for cyclosporin A. Pharm. Res. 13, 311–315.

Calvo, P., Vila-Jato, J.L., Alonso, M.J., 1997a. Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. Int. J. Pharm. 53, 41–50.

Calvo, P., Remuñan-Lopez, C., Vila-Jato, J.L., Alonso, M.J., 1997b. Novel hydrophilic chitosan–polyethylene oxide nanoparticles as protein carriers. J. Applied Pol. Sci. 63, 125–132.

Danjo, Y., Watanabe, H., Tisdale, A.S., George, M., Tsumura, T., Abelson, M.B., Gipson, I.K., 1998. Alteration of mucin in human conjunctival epithelia in dry eye. Invest. Ophthalmol. Visual Sci. 39, 2602–2609.

De Campos, A.M., Alonso, M.J., in preparation. Chitosan nanoparticles as novel topical ocular drug delivery systems: evaluation of the in vitro stability and the in vivo fate. Pharm. Res.

Dua, H.S., Jindal, V.K., Gomes, J.A.P., Amoaku, W.A., Donoso, L.A., Laibson, P.R., Mahlberg, K., 1996. The effect of topical cyclosporin on conjunctiva-associated lymphoid tissue (CALT). Eye 10, 433–438.

Felt, O., Furrer, P., Mayer, J.M., Plazonnet, B., Buri, P., Gurny, R., 1999. Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. 180, 185–193.

Foets, B., Missotten, L., Vanderveeren, P., Goossens, W., 1985. Prolonged survival of allogenic corneal grafts in rabbits treated with topically applied cyclosporin A: systemic absorption and local immunosuppressive effect. Br. J. Ophthalmol. 69, 600–603.

Ford, J., Woolfe, J., Florence, A.T., 1999. Nanospheres of cyclosporin A: poor oral absorption in dogs. Int. J. Pharm. 183, 3–6.

Genta, I., Conti, B., Perugini, P., Pavaneto, F., Spadaro, A., Puglisi, G., 1997. Bioadhesive microspheres for ophthalmic administration of acyclovir. J. Pharm. Pharmacol. 49, 737–742.

Gündüz, K., Özdemir, O., 1994. Topical cyclosporin treatment of keratoconjunctivitis sicca in secondary Sjögren's syndrome. Acta Ophthalmol. 72, 438–442.

Hirano, S., Seino, H., Akiyama, I., Nonaka, I., 1990. Chitosan: a biocompatible material for oral and intravenous administration. In: Gebelein, C.G., Dunn, R.L. (Eds.), Progress in Biomedical Polymers. Plenum Press, New York, pp. 283–289.

Kaswan, R.L., 1988. Intraocular penetration of topically applied cyclosporin. Transplant Proc. 20, 650–665.

Kaswan, R.L., Salisbury, M.A., Ward, D.A., 1989. Spontaneous canine keratoconjunctivitis sicca. A useful model for human keratoconjunctivitis sicca: treatment with cyclosporin eye drops. Arch. Ophthalmol. 107, 1210–1216.

Kaswan, R.L., 1994. Characteristics of a canine model of keratoconjunctivitis sicca: effective treatment of topical cyclosporin. Adv. Exp. Med. Biol. 350, 583–594.

Knapczyk, J., Krówczynski, L., Krzck, J., Brzeski, M., Nirnberg, E., Schenk, D., Struszcyk, H., 1989. Requirements of chitosan for pharmaceutical and biomedical applications. In: Skak-Braek, G., Anthonsen, T., Sandford, P. (Eds.), Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. Elsevier, London, pp. 657–663.

Lehr, C.M., Bouwstra, J.A., Schacht, E.H., Junginger, H.E., 1992. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharm. 78, 43–48.

Losa, C., Calvo, P., Castro, E., Vila-Jato, J.L., Alonso, M.J., 1991. Improvement of ocular penetration of amikacin sulphate by association to poly(butylcyanoacrylate) nanoparticles. J. Pharm. Pharmacol. 43, 548–552.

Losa, C., Marchal-Heussler, L., Orallo, F., Vila-Jato, J.L., Alonso, M.J., 1993. Design of new formulations for topical ocular administration: polymeric nanocapsules containing metipranolol. Pharm. Res. 10, 80–87.

Nussenblatt, R.B. 1988. The use of cyclosporine in ocular inflammatory disorders. Transplant Proc. 20, 114–121.

Power, W.J., Mullaney, P., Farrell, M., Collum, L.M., 1993. Effect of topical cyclosporin A on conjunctival T cells in patients with secondary Sjögren's syndrome. Cornea 12, 507–511.

Smolin, G., Okumoto, M., Feiler, S., Condon, D., 1981. Idoxuridine–liposome therapy for herpes simplex keratitis. Am. J. Ophthalmol. 91, 220–225.

Stern, M.E., Beuerman, R.W., Fox, R.I., Jiamping, G., Mircheff, A.K., Pflugfelder, S.C., 1998. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. Cornea 17, 584–589.