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The potential of chitosan in ocular drug delivery

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Abstract

This paper presents an overview of the potential of chitosan-based systems for improving the retention and biodistribution of drugs applied topically onto the eye. Besides its low toxicity and good ocular tolerance, chitosan exhibits favourable biological behaviour, such as bioadhesion- and permeability-enhancing properties, and also interesting physico-chemical characteristics, which make it a unique material for the design of ocular drug delivery vehicles. The review summarizes the techniques for the production of chitosan gels, chitosan-coated colloidal systems and chitosan nanoparticles, and describes their mechanism of action upon contact with the ocular mucosa. The results reported until now have provided evidence of the potential of chitosan gels for enhancing and prolonging the retention of drugs on the eye surface. On the other hand, chitosan-based colloidal systems were found to work as transmucosal drug carriers, either facilitating the transport of drugs to the inner eye (chitosan-coated colloidal systems containing indometacin) or their accumulation into the corneal/conjunctival epithelia (chitosan nanoparticles containing ciclosporin). Finally, the tolerance, toxicity and biodegradation of the carriers under evaluation were reviewed.

Introduction

Even though the history of the polysaccharide chitosan dates back from the 19th century, it has only been over the last couple of decades that this material has received attention in the biomedical and drug delivery fields. Chitosan is a deacetylated form of chitin, which is the second-most abundant polymer in nature after cellulose. The small difference in the chemical structure of chitin and chitosan has, however, extremely important consequences in terms of their utility for drug delivery. Chitin is insoluble in water or in the most common organic solvents used in pharmaceutical technology and, therefore, it is not useful in the development of drug delivery devices. In contrast, chitosan base is soluble in acidic solutions wherein it becomes protonized. This positive charge of the chitosan molecule enables its interaction with polyanions, a process that has been used to obtain complexes as well as micro and nanoparticulate drug delivery systems. Besides its polycationic nature, chitosan has shown excellent film-forming properties (Remuñan-Lopez & Bodmeier 1996). Consequently, its use for the preparation of films, bandages and laminated devices has also opened new applications for this biopolymer.

There are a few review articles regarding the utility of chitosan in drug delivery following different modalities of administration. These articles are a good illustration of how the number of applications of chitosan in the drug delivery field has increased over the last decade (Dodane & Vilivalam 1998; Felt et al 1998; Illum 1998; Paul & Sharma 2000; Janes et al 2001; Singla & Chawla 2001). Even though chitosan has been proposed for parenteral drug delivery, there is no doubt that the main interest in chitosan-based systems has shifted to mucosal drug delivery. This is due to chitosan's unique biological properties such as mucoadhesiveness (Lehr et al 1992; Borchard et al 1996), its ability to enhance transiently the permeability of mucosal barriers (Artursson et al 1994; Borchard et al 1996) and its biodegradability in the rich lysozyme-containing mucus. As we will comment later, these properties are particularly important with respect to the use of chitosan in ophthalmology.

Taking this information into account, the purpose of this review is to provide the reader with an overview of what is currently known about the use of chitosan for the treatment of eye disorders, with particular emphasis on its utility for the design of new

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ocular drug delivery systems. Accordingly, we will first comment on the therapeutic role of chitosan in ophthalmology and then, following a brief presentation of the barriers that need to be overcome in ocular drug delivery, we will illustrate, with a number of examples, the potential that chitosan offers to resolve such limitations. These examples will be classified into the different pharmaceutical presentations of chitosan that are acceptable for ophthalmic application: gels, microspheres and colloidal systems (nanoparticles, nanocapsules).

Chitosan as an active biomaterial in ophthalmology

Chitosan is a very promising biomaterial in ophthalmology not only because of the favourable biological properties indicated above but also because of its inherent biological activity, which may also have an impact in ocular therapeutics. The various forms in which chitosan has been investigated in ophthalmology are indicated in Table 1. Besides being a major component in drug delivery devices, chitosan itself has been shown to have wound healing and antimicrobial activity (Balassa & Prudden 1978; Allan & Hadwiger 1979; Muzzarelli 1983). These effects could be very beneficial for the treatment of a number of ocular diseases. The idea of using chitosan in corneal wound healing came from the recognized acceleration of wound-healing activity of chitosan degradation products (Balassa & Prudden 1978) and the observed success of chitosan as a haemostatic agent for porous vascular grafts (Malette et al 1983). More precisely, based on this previous work it was hoped that chitosan would play an active role, increasing keratocyte migration, thereby leading to a more rapid production of collagen and improved corneal healing. Unfortunately, histological evaluation and measurement of the tensile strength of the rabbit cornea exposed to chitosan (1% solution) did not show an improved corneal wound healing (Sall et al 1987). It is possible that the lack of effectiveness of chitosan in this preliminary work may rely on the type of chitosan selected (not indicated in the publication) or on the protocol of administration. Nevertheless, from our knowledge, there has not been any further work attempting to verify the acceleration of wound healing by chitosan.

Taking advantage of the film-forming properties of chitosan, Markey et al (1989) developed contact lenses with excellent edges and optics. By assuming the wound-healing acceleration (Balassa & Prudden 1978) and antimicrobial activity of the *N*-acetyl-D-glucosamine oligomers (Allan & Hadwiger 1979; Muzzarelli 1983), it was thought that this kind of system could be applied as a protective device for an acutely traumatized eye (e.g. following ocular surgery) or a chronically compromised cornea. Unfortunately, despite the suggested acceptability of these contact lenses, no evidence of their effectiveness has been reported.

It is surprising that this early work on ocular wound healing acceleration has not been further corroborated. It is possible that the lack of success of these initial applied experiments has discouraged researchers from investing efforts in this direction. However, a very important aspect to keep in mind, that stimulates this research field, is the actual availability of rigorously characterized and ultrapurified chitosan. In fact, researchers who specialize in chitosan are well aware of the difficulties in extrapolating or simply comparing early work on chitosan due to the limited information regarding the source, characteristics and purity of this natural material.

In contrast to the lack of confirmation of chitosan's wound-healing activity, its antimicrobial activity was recently corroborated by the findings of Felt et al (2000), who suggested the use of a chitosan solution as an artificial tear formulation. Besides the bacteriostatic activity of chitosan, this proposal was based on its excellent tolerance after topical ocular administration and its ability to spread well over the entire cornea after topical instillation (Felt et al 1999a). Additionally, the prolonged pre-corneal residence time of chitosan solutions reinforced the utility of chitosan for the treatment of dry eye and keratoconjunctivitis sicca.

Barriers to overcome in topical ocular drug delivery

To understand the potential that chitosan offers in improving the treatment of ocular diseases, it is important

Table 1 Forms of chitosan investigated in ophthalmology.

Chitosan form	Application	Drug incorporated	Reference
Contact lenses	Corneal wound healing	—	Markey et al (1989)
Solution	Corneal wound healing	—	Sall et al (1987)
Solution	Dry eye	—	Felt et al (2000)
Solution	Prolonged retention	Tobramycin	Felt et al (1999)
Coated liposomes	Improved corneal retention	Marker	Henricksen et al (1996)
Coated nanocapsules	Improved corneal penetration	Indometacin	Calvo et al (1997)
Coated nanocapsules	Improved/prolonged retention	Marker	De Campos et al (2003)
Microspheres	Improved corneal penetration	Aciclovir	Genta et al (1997)
Microspheres	Improved corneal penetration	Ofloxacin	Di Colo et al (2002)
Nanoparticles	Improved/prolonged retention	Ciclosporin	De Campos et al (2001)
Nanoparticles	Improved/prolonged retention	Marker	De Campos (2002)

to know, first, what the targets for the drugs are and, second, what barriers need to be overcome to reach those targets.

The topical ocular administration of drugs has two different purposes: to treat superficial eye diseases, such as infections (i.e. conjunctivitis, blepharitis, keratitis sicca), and to provide intra-ocular treatment through the cornea for diseases such as glaucoma or uveitis. Despite the efforts dedicated in the 1980s to the design of solid ocular drug delivery systems, the most popular and accepted forms of delivering drugs to the eye are still liquid forms, administered as eye-drops. As shown in Figure 1, one of the major problems encountered with the topical administration of liquid forms is the rapid and extensive pre-corneal loss caused by drainage and high tear turnover. After instillation of an eye-drop, a major fraction of the instilled dose is lost due to the limited capacity of liquid retention of the eye surface and also as a consequence of the blinking process, which is normally stimulated after instillation. Additionally, a certain amount of drug is often absorbed systemically via the conjunctiva and the nasolachrymal duct.

In addition to these anatomical constraints to the retention of drugs at the eye's surface, the second major limiting step for the transport of drugs to the inner eye is diffusion across the cornea. Indeed, although the cornea covers only one-sixth of the total surface area of the eyeball, it is considered to be the main pathway for the permeation of drugs into intra-ocular tissues. Obviously, the extent to which topically applied drugs remain on the eye's surface or penetrate into the inner eye is dependent not only on the physiological characteristics of the corneal barrier, but also on the physico-chemical properties of the drug and the specific behaviour of the vehicle. The importance of these limitations is well illustrated by the known fact that, with classical ophthalmic liquid forms, typically less than 5% of the applied drug penetrates the cornea and reaches intra-ocular tissues (Lang 1995).

The cornea consists of three different sections, the epithelium, stroma and endothelium. The most external section, the epithelium, is composed of a number of well-organized and tightly packed cell layers and represents a selective barrier for the penetration of hydrophilic and ionized compounds. Indeed, the tight junctions between epithelial cells prevent the entrance of molecules by a paracellular route. Contrarily, the stroma beneath the epithelium is a hydrophilic space that represents 90% of the cornea and, consequently, is a selective barrier for those lipophilic compounds that could have easily diffused through the epithelium. As a consequence of this perfect

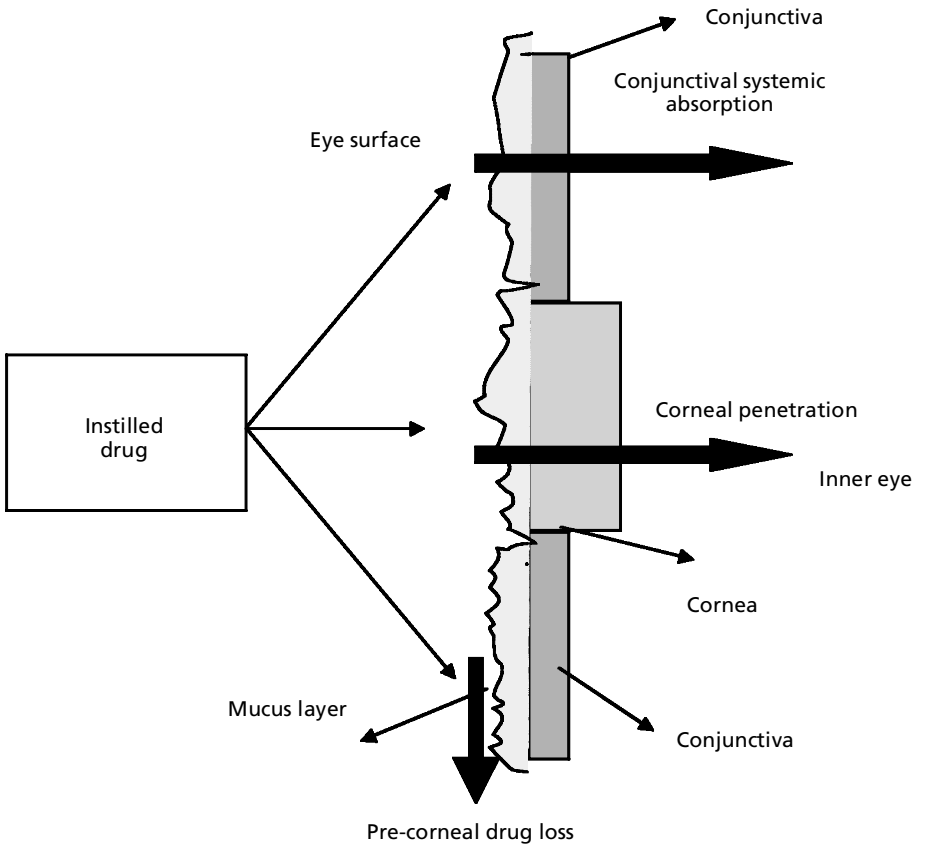

Figure 1 Schematic view of the pre-corneal elimination and transport pathways of a drug applied topically onto the eye.

organization, the transport of hydrophilic compounds is hindered at the epithelial level whereas that of lipophilic compounds is blocked at the hydrophilic gel-like stromal compartment. The stroma may act as a drug reservoir, slowly releasing the drug into the aqueous humour. The endothelium is a well-organized monolayer of epithelial cells and, therefore, does not contribute as a barrier for the penetration of drugs.

A drug that is applied topically and retained on the eye surface may easily reach an important competitive route covering five-sixths of the total surface area of the eyeball – the conjunctiva. The conjunctiva is a thin, vascularized mucous membrane that lines the inner surface of the eyelids and coves the anterior part of the sclera up to the cornea. Owing to the relative surface area and rich blood flow, conjunctival uptake of a topically applied drug from the tear fluid is typically greater than corneal uptake. Ocular penetration via the sclero-conjunctival route is more rapid (for a hydrophilic drug) than via the transcorneal route (Romanelli et al 1994). However, transconjunctival penetration is generally undesirable because most of the drug that crosses the conjunctiva reaches the blood circulation. Consequently, the drug will not only be unable to reach the ocular target site but it might also be responsible for severe systemic side effects.

Covering the conjunctiva and corneal surfaces of the eye is the mucus layer. The tear-film mucus layer is a complex macromolecular structure consisting essentially of mucin, proteins, lipids and DNA. Ocular mucus is mainly secreted by the conjunctival goblet cells (Moore & Tiffany 1981), although corneal and conjunctival epithelium has also been reported to produce mucins (Greiner et al 1985). Mucin is a macromolecular glycoprotein that gives mucus its specific properties, such as viscosity and lubricant and surfactant effects. Mucins are thought to consist of hundreds of short polysaccharide chains attached to a central protein core, having a variable molecular weight and residual negative charges, presumably from sialic acid residues.

After topical instillation of an ophthalmic drug solution, the drug is firstly mixed with the lachrymal fluid and remains in contact with the ocular mucosa for a very short period of time, typically 1–2 min, because of the permanent production of lachrymal fluid. Drainage of lachrymal fluid towards the nasolachrymal duct induces a rapid elimination of conventional dosage forms during blinking.

Researchers have dedicated important efforts to design systems intended to overcome the aforementioned barriers. The specific details of these delivery systems, including solid devices and liquid eye-drops, have already been described in a number of review articles (Sintzel et al 1996; Le Bourlais et al 1998; Felt et al 1999b). Despite the variety of systems designed, at present it is accepted that an optimum ocular drug delivery system would be one that can be delivered in eye-drop form and that would need no more than one or two instillations a day. Most of the new delivery devices have been aimed at improving ocular drug penetration through prolongation of the drug residence time in the cornea and conjunctival sac, as well as slowing drug release from the delivery system and minimizing pre-corneal drug loss. Among the marketed systems, pre-formed gels and in-situ gelling systems are probably the closest to reaching these objectives. However, the performance of these viscous vehicles is limited by several constraints: they do not facilitate the internalization of the drug in the corneal or conjunctival epithelia; they are aqueous systems in which hydrophobic drugs can not be solubilized; and they do not provide a controlled drug delivery.

Therefore, the necessity of exploring novel liquid systems specifically designed for ocular drug delivery still remains. Within this research context, it is currently accepted that the primary objectives indicated above (prolonged retention, enhanced penetration and controlled delivery) can be attained though different strategies that include the use of bioadhesive polymers, penetration enhancers and the advanced design of micro- and nanoparticulate delivery systems. These approaches have been explored independently over the last decade (Greaves & Wilson 1993; Zimmer & Kreuter 1995; Kaur & Smitha 2002) and have led to significant advances in the field. Probably, an adequate combination of them will lead to a new generation of ocular drug delivery systems. As justified in the next section, the position that chitosan is taking within this research field holds great promise. The following sections will address the progress made so far on the design of chitosan-based delivery systems intended for topical ocular administration. References pertaining to the ocular application of these systems are presented in Table 1.

Chitosan: a good candidate for ocular drug delivery

The residence time of a topically applied ophthalmic drug refers to the duration of its contact with the ocular surface. This concept is of particular interest in the formulation of topical ocular drug vehicles, where mucoadhesive polymers are frequently used as an approach to prolong drug residence times. When using a mucoadhesive material, the clearance of the drug is controlled by the mucus turnover rate, which is much slower than the tear turnover rate. This prolonged retention of the drug formulation implies, for a drug with good permeability properties, an enhanced ocular drug bioavailability. Chitosan is in this category of mucoadhesive polymers. The mucoadhesive character of chitosan relates to the attraction between its positively charged amino groups and the negatively charged residues of sialic acid in the mucus (Lehr et al 1992) along with other forces such as hydrogen bonds (Hassan & Gallo 1990). In addition to this special property, chitosan exhibits other attractive features, mentioned in the following paragraphs, which make it a unique candidate for ocular drug delivery.

It has penetration-enhancing properties, which were initially attributed to the modulation of the tight junction barrier between epithelial cells (Artursson et al 1994; Schipper et al 1997; Koch et al 1998) and recently also related to intracellular routes (Dodane et al 1999). More

specifically, using Caco-2 cells these authors found that chitosan 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. This permeability-enhancing property has been used to explain the increased corneal transport of specific drugs (see Chitosan-based ocular drug delivery systems, below)

Chitosan is biodegradable (Pangburn et al 1982; Hirano et al 1989a, 1990), which enables the safe administration and degradation of topically applied ocular chitosan vehicles. As mentioned before, chitosan biodegradation is mediated by the hydrolytic actions of lysozyme and other enzymes (i.e. human chitinase and *N*-acetyl-β-D-glucosaminidases), which produce chito-oligomers and monomers (Muzzarelli 1993, 1997; Nordtveit et al 1994). This susceptibility to enzymatic depolymerization is an exclusive characteristic of chitosan with respect to other polysaccharides. It has been reported that the degradation rate in the presence of lysozyme depends on the degree of acetylation (Nordtveit et al 1994). However, the study of the influence of this parameter has led to contradictory results: Hirano et al (1989b) found an optimum lysozyme degradation rate for 80% *N*-acetylated chitosan, whereas Sashiwa et al (1990) found the highest lysozyme susceptibility for 30% *N*-acetylation. In addition, little is known about some enzymes of human origin (i.e. collagenases and heparinases), even when it is well known that chitosans are more vulnerable than expected to the non-specific actions of a number of enzymes (Muzzarelli 1997). Therefore, these are important issues that need to be further investigated.

Chitosan has excellent ocular tolerance. This has been reported in a rabbit model following topical application of chitosan solutions and using confocal laser scanning ophthalmoscopy combined with corneal fluorescein staining (Felt et al 1999a). Other evidence of the low toxicity and tolerance of chitosan delivery systems will be described later.

Chitosan has favourable rheological behaviour. Chitosan solutions have shown pseudoplastic and viscoelastic properties (Wang & Xu 1994; Mucha 1997). These are very important characteristics, since the pre-corneal tear film has a pseudoplastic character that should not be disturbed by application of liquid formulations. On the other hand, viscoelastic fluids exhibit high viscosity under low shear rate and low viscosity under high shear rate conditions. This behaviour is particularly important in ophthalmic formulations since it facilitates the retention while it permits the easy spreading of the formulation due to the blinking of the eyelids.

On the basis of these favourable biological properties, and also because of its adaptability for designing different delivery systems, chitosan has attracted great attention in the pharmaceutical and biomedical fields. It is, however, surprising that the number of reports on the potential of this cationic polysaccharide in the ophthalmic field is still limited (Felt et al 1998, 1999a). In the following section we will describe, in detail, the characteristics of chitosan and the in-vivo behaviour of chitosan-based ocular drug delivery systems reported so far.

Chitosan-based ocular drug delivery systems

Chitosan solutions

As previously described in this review, the most acceptable dosage forms for topical ocular drug delivery are the liquid forms. The simplest presentation of chitosan in a liquid formulation consists of a chitosan solution, which is sometimes referred to as a hydrogel. Hydrogels are normally defined as polymers that have the ability to swell in aqueous solvents and undergo a liquid–gel transition. However, in ophthalmology, there is not a clear distinction between hydrogels and highly viscous solutions. Chitosan solutions can be prepared in different concentrations and using different types of chitosan (different molecular weight, different salts and different deacetylation degree). Preferably, highly deacetylated chitosan (generally more than 60%) is used since the water solubility decreases with reduction in deacetylation. Chitosan solutions have been well characterized in terms of their pseudoplastic and viscoelastic behaviour (Wang & Xu 1994; Mucha 1997). Furthermore, a synergism between rheological behaviour and mucoadhesion has also been described (Caramella et al 1999). These studies have shown that the viscosity and mucoadhesive properties can be modulated by adjusting the chitosan molecular weight and concentration. Most of the reports on the topical ocular administration of chitosan solutions refer to concentrations in the range 0.5–5% and molecular weight higher than 70–100 kDa (Felt et al 1999a, b). However, it is possible that other molecular weights and concentrations could be used for this specific application.

An alternative way to modulate the viscosity and viscoelastic behaviour of chitosan solutions could be through the incorporation of other hydrophilic polymers that are known to interact with chitosan (e.g. hyaluronic acid). Recently, a number of chemical derivatives of chitosan have been produced, among them PEG-chitosan, which is already commercially available. These approaches involving other polymers are valid insofar as the new polymers are acceptable for ocular administration.

The specific bioadhesive activity of chitosan towards the ocular surface has been confirmed in an ex-vivo study performed using freshly excised cattle cornea and radiolabelled chitosan (Henriksen et al 1996). Some years later, the capacity of chitosan for increasing pre-corneal drug residence times was clearly shown in a rabbit model, using gamma scintigraphy. For this purpose, chitosan gels containing tobramycin and the commercial drug solution were labelled with ^{99m}Tc-DTPA and instilled onto the cornea of conscious rabbits. The pre-corneal retention time was assessed by determining the eye-associated radioactivity, using a gamma camera. The results showed a 3-fold increase of the corneal residence time of the chitosan solution as compared with that of the commercial drug solution (Felt et al 1999a). In addition, at 10 min post-intillation of the commercial solution, all the radioactivity was concentrated in the lachrymal duct, whereas in the case of chitosan formulations, 25–50% of the radioactivity remained associated with the cornea. The results of pre-corneal drainage were very similar irrespective of the chitosan concentration (0.5–1.5%)

or molecular weight (160–1930 kDa). On the basis of these results, the authors suggested that the improvement in retention time using chitosan might be due to a saturable bioadhesive mechanism. Hence, they concluded that the use of a low concentration of low-molecular-weight chitosan would be sufficient to provide a significant enhancement of the residence time. However, no comment was made on the differences in deacetylation degree (which was in the range 59–87%) of the polymers tested, a parameter that could also have some effect on the behaviour of chitosan in-vivo.

As indicated above, besides their utility as drug delivery vehicles, chitosan solutions have also been investigated as tear substitutes in the management of dry eye disorders (Felt et al 2000). These studies revealed that chitosan solutions are easier to manipulate and provide a more accurate and reproducible administration, as well as a lower incidence of blurred vision and discomfort than conventional hydrogels used at the ocular level. In addition, the antibacterial activity of chitosan could be useful to prevent, as mentioned above, the frequent secondary infections associated with this disorder.

Chitosan microspheres

Chitosan microspheres have mainly been prepared using two basic methodologies: the water-in-oil solvent evaporation technique and the spray-drying technique (Kas 1997). In both cases, chitosan is dissolved in an aqueous medium (either acidic or neutral) and then emulsified in an oily phase, where the evaporation takes place, or simply sprayed-dried to accelerate the evaporation process. These particles are finally collected and stored as powders.

Genta et al (1997) developed aciclovir-loaded chitosan microparticles of a size below 25 μm, using the water-in-oil solvent evaporation technique. In-vitro release profiles revealed that these chitosan microparticles slightly reduced the drug dissolution rate. Following in-vivo administration to conscious rabbits of the drug-containing microparticles, it was found that the ocular drug bioavailability increased as compared with that of the drug suspension (control formulation). More specifically, these systems provided a 4-fold increase in the AUC value with respect to that of the control drug suspension. The authors explained these positive results in terms of the mucoadhesive character of chitosan. However, no further mechanistic details were explored.

More recently, chitosan microspheres were incorporated within a poly(ethylene oxide) (PEO) ocular insert with the idea of improving the release behaviour of the system, while taking advantage of the known beneficial effects of chitosan (Di Colo et al 2002). Chitosan microspheres containing the drug ofloxacin were prepared using the spray-drying technique and then added to the PEO gel. The in-vivo results showed no clear benefit from the incorporation of the chitosan microspheres into the insert. However, it was found that the t_{max} of the aqueous humour ofloxacin concentration-vs-time profile was increased for the formulations containing chitosan – a result that was attributed to the corneal-permeability-enhancing effect of chitosan.

The scarce number of reports on the use of chitosan microspheres for ophthalmic purposes could be justified by the limited acceptability of large particles. In fact, it has been indicated that the size of the particles is a critical parameter in their ocular tolerance and acceptability. This may be the reason why research in this field has basically shifted to the design of colloidal systems (less than 1 μm), including liposomes, submicron emulsions, nanocapsules and nanoparticles. The preparation, characteristics and potential of chitosan-based colloidal drug carriers in ocular drug delivery are discussed in the following section.

Chitosan-based colloidal drug carriers

Introduction of colloidal carriers in ocular drug delivery Overall, the research carried out over the last 15 years has led to the conclusion that colloidal drug carriers, such as liposomes, submicron emulsions, nanocapsules and nanoparticles, are promising systems for improving the ocular retention of topically administered drugs. The first proof of efficacy of liposomes in ophthalmic therapy was shown for idoxuridine, used in the treatment of herpetic keratitis in a rabbit model, in 1981 (Smolin et al 1981). Some years later, Fitgerald et al (1987a) demonstrated, using gamma-scintigraphy, that liposomes have a reduced pre-corneal clearance. In addition, they found that this behaviour was positively affected by the small size and a positive surface charge. Despite the number of articles directed to this end (Maurice 1993), some inherent disadvantages of liposomes, such as their low drug-loading capacity and compromised stability, have probably hindered further development in this area. In this sense, it is important to keep in mind that, due to the low retention of the eye-drops on the eye surface, the drug loading of the colloidal vehicles is a crucial parameter. An alternative colloidal vehicle – nanoparticles/nanocapsules made of biodegradable polymer – was initially proposed with the idea of controlling the release and, simultaneously, facilitating the interaction of the drug with the ocular mucosa (Wood et al 1985; Fitgerald et al 1987b). Surprisingly, using polyalkylcyanoacrylate nanoparticles, it was soon observed that these colloidal carriers were able to enter the corneal epithelium via endocytosis (Zimmer et al 1991). Unfortunately, some damage to the cell membrane was also observed, which was attributed to the polymer degradation products. The transport of the colloidal systems across the corneal epithelium was later corroborated by our research group using poly-E-caprolactone (PECL) nanocapsules (Calvo et al 1994). However, in our study, no evidence of membrane alteration was detected. Furthermore, in a later work we observed that microparticles were not as efficient as nanoparticles or nanocapsules at increasing the corneal transport of the model drug indometacin (Calvo et al 1996) and thus concluded that size has a key role in the ability of these particles to work as ocular drug carriers.

Chitosan-coated colloidal drug carriers A second generation of ocular colloidal drug carriers is represented by those coated with mucoadhesive polymers. Following the

observation of the improvement in transport of drugs associated with nanocapsules and nanoparticles, we decided to explore whether or not a chitosan coating would further improved their efficacy as ocular drug carriers. The rationale for designing chitosan-coated systems was to combine the advantages of nanoparticles as ocular drug carriers with the mucoadhesive and permeability-enhancing properties of chitosan. We first reported the preparation of these novel systems in 1997 (Calvo et al 1997a). The systems can be composed of a solid core (nanoparticles), which is made of a biodegradable polymer, or an oily core (nanocapsules). These systems can be prepared using solvent-displacement or solvent-emulsion evaporation techniques (Alonso 1996). A negatively charged phospholipid is introduced into the colloidal carrier to facilitate the attachment of chitosan onto the surface of the particles. The size of the particles is slightly increased, and their zeta potential shifts from negative to positive values, due to the chitosan coating. These chitosan-coated systems are very versatile in regard to their drug-loading capacity and release properties. Indeed, by varying the core composition and judiciously adjusting the preparation technique it is possible to load either hydrosoluble or hydrophobic compounds. For example, hydrosoluble proteins such as tetanus toxoid have been loaded into chitosan-coated poly(lactic acid/glycolic acid) using the double emulsion-solvent evaporation technique (Vila et al 2002). On the other hand, lipophilic drugs, such as indometacin and diazepam, have been very efficiently incorporated into chitosan-coated oily droplets, otherwise called chitosan nanocapsules (Calvo et al 1996, 1997a).

Despite the versatility of chitosan-coated nanostructures and preparation techniques, only those providing a high loading and a relatively rapid release (from minutes to a few hours) are expected to have a potential application in ophthalmology. For example, we have studied the in-vivo efficacy of these systems using indometacin as a model drug. The ocular drug disposition was determined after a single instillation of ^{14}C-indometacin-loaded systems to conscious rabbits and subsequent quantification of the radioactivity levels in cornea and aqueous humour (Calvo et al 1997b). The results showed that chitosan-coated nanoparticles increased the drug levels in cornea and aqueous humour to a significantly greater extent than either the commercial drug preparation or drug-loaded uncoated systems. This initial work led to the conclusion that a chitosan coating adds a clear benefit to the potential of colloidal systems as ocular drug carriers. To investigate whether this benefit was solely due to the positive charge, or could be additionally related to the inherent properties of chitosan, we compared the behaviour of chitosan-coated formulations with that of poly(L-lysine)-coated formulations. The lack of effect of the poly(L-lysine)-coating stood for the intrinsic beneficial effect of chitosan. Hence, the mechanism that explains the increased ocular drug penetration achieved with the chitosan-coated systems could be a combination of an improved interaction with the corneal epithelium, followed by the penetration of the particles in the corneal epithelial cells (Calvo et al 1994). This mechanism was later corroborated, as discussed hereafter. In addition to their efficacy, we could observe that these chitosan-coated systems have a good ocular tolerance (low ocular lesion index).

The effect of a chitosan coating on the ocular retention of liposomes has also been investigated in the anaesthetized rat model (Henriksen et al 1996). In this study, the authors chose to label the liposomes with ^{125}I-bovine serum albumin (BSA) and monitored the eye-associated radioactivity over time. Even though the ocular retention of ^{125}I-BSA was apparently more pronounced for the chitosan-coated liposomes, the difference from the non-coated liposomes was not statistically significant. In the interpretation of these results we should, however, take into account that the measurements were performed on ^{125}I-BSA and a certain amount of protein could have been released from the system following in-vivo application. More studies need to be performed to verify the limited success of this experiment.

The importance of the coating of nanoparticles with a mucoadhesive has been noted with other polymers, besides chitosan. For example, in a study performed by Zimmer et al (1995), it was observed that coating albumin nanoparticles with mucoadhesive polymers, hyaluronic acid among others, led to a prolongation of the response of the drug associated with them (pilocarpine).

Chitosan nanoparticles

As mentioned in the introductory section, the cationic nature of chitosan has been conveniently exploited for the development of particulate drug delivery systems. An interesting property of chitosan is its ability to gel on contact with specific polyanions. Based upon this principle, a few years ago we developed chitosan nanoparticles (Calvo et al 1997c). This technique involves the addition, at room temperature, of an aqueous phase containing sodium tripolyphosphate into an aqueous phase containing chitosan. Nanoparticles are formed immediately upon mixing of the two phases through inter- and intramolecular linkages created between the phosphate groups of sodium tripolyphosphate and the amino groups of chitosan. The conditions for the formation of high-yield nanoparticles with a particular nanometric size may vary significantly depending on the purity, acid salt and molecular weight of chitosan employed. Consequently, the formulation parameters should be optimized for each individual chitosan type. More recently, we adapted this gelation technique to produce depolymerized chitosan nanoparticles (Janes et al 2003). This idea came from different studies suggesting that the molecular weight could affect a number of key properties of chitosan, such as biocompatibility (Richardson et al 1999), permeability enhancement (Schipper et al 1996) and mucoadhesion (Henriksen et al 1996). The conclusion from this study was that depolymerized chitosan retains its ability to form low-molecular-weight chitosan nanoparticles via ionotropic gelation.

The described nanoparticles can be made entirely of chitosan or a combination of chitosan with other hydrophilic polymers and macromolecules. The introduction of a second ingredient in the nanoparticle formulation

increases their versatility in terms of the association and delivery of drugs and their susceptibility to interact with biological surfaces. Interesting properties have been observed with nanoparticles made of chitosan and a diblock copolymer of ethylene oxide and propylene oxide (PEO-PPO) (Calvo et al 1997d). An alternative strategy aimed at modifying the surface properties of chitosan nanoparticles has been based on the use of PEG-conjugated chitosan. Despite the change in the solubility properties of the copolymer, nanoparticles consisting of chitosan-PEG could also be produced by adjusting the gelation conditions.

More recently, we have adapted the ionic gelation technique to produce particles consisting of two polysaccharides with potential interest for ocular drug delivery. Using an experimental design, we were able to identify the formulation conditions for obtaining chitosan–hyaluronic acid nanoparticles. The interest in these new nanoparticles comes from the fact that hyaluronic acid is already being used in ocular drug delivery (Felt et al 1999b).

Because of the particularly mild conditions required for the formation of these chitosan-based nanoparticles (chitosan alone or in combination with other hydrophilic polymers), they are particularly attractive for the association and delivery of sensitive macromolecules. Proteins, such as tetanus toxoid, and the peptide insulin are examples of macromolecules that have been efficiently associated with these nanoparticles (Calvo et al 1997d; Fernandez-Urrusuno et al 1999). Protein loading reached values as high as 50% (50 mg of protein per 100 mg of nanoparticles), which is, to our knowledge, the greatest loading capacity reported so far for a nanoparticulate protein carrier. More recently, we adapted the nanoparticle gelation procedure to encapsulate lipophilic compounds such as ciclosporin. In this case, it was necessary to incorporate a small amount of a polar solvent such as acetone, ethanol or acetonitrile to dissolve the hydrophobic peptide. This approach allowed us to precipitate the peptide in the form of nanocrystals within the gelled nanoparticles (De Campos et al 2001).

With respect to the in-vitro release behaviour of these nanoparticles, the results reported to date indicate that the release rate is highly affected by whether the active ingredient is entrapped in a solid form or molecularly dispersed within the chitosan matrix and, in the latter case, on the nature of the interactive forces between the active molecule and chitosan. Obviously, where the active molecule is entrapped in a solid form, its dissolution rate rather than its interaction with chitosan is the factor that determines the release process (De Campos et al 2001). The in-vitro release behaviour of chitosan nanoparticles was also found to be affected by the introduction of auxiliary ingredients in the nanoparticles. For example, the incorporation of PEO–PPO into the nanoparticles led to a significant increase in the release rate of BSA (Calvo et al 1997d). Consequently, these results show that it is possible to modulate the drug release rate simply by adjusting the composition of the chitosan nanoparticles.

Bearing in mind the interest of these nanoparticles for mucosal drug delivery, we have very recently investigated their stability in simulated fluids containing mucus components (i.e. lysozyme and mucin). We found it important to assess the stability of these nanoparticles, given the recognized role of the particle size in their ability to interact with mucosal surfaces in general and, in particular, with the ocular mucosa (De Campos et al 2002.). The results showed there to be a slight size reduction and maintenance of the zeta potential upon incubation of chitosan nanoparticles in the presence of lysozyme. On the other hand, no noticeable change in the viscosity of a mucin dispersion was seen after incubation with chitosan nanoparticles. Therefore, the conclusions from this study were that the stability of chitosan nanoparticles is not compromised by the presence of lysozyme in the tear fluid and that the viscosity of the suspension of nanoparticles is not expected to change upon contact with the ocular mucosa. However, these conclusions should be taken cautiously due to the absence of blinking, tear turnover and other physiological variables that are present in-vivo.

We have recently investigated the efficacy of chitosan nanoparticles in prolonging the delivery of drugs to the eye surface. We chose ciclosporin as a model drug that could benefit from a controlled release behaviour at the eye surface due to its potential indication for the treatment of severe dry eye. With this idea in mind, we compared the ocular disposition of three different formulations of ^{3}H-ciclosporin, following topical instillation to conscious rabbits: ^{3}H-ciclosporin-loaded chitosan nanoparticles, a nanosuspension of ^{3}H-ciclosporin in a chitosan solution and an aqueous nanosuspension of ^{3}H-ciclosporin. Interestingly, chitosan nanoparticles are able to provide a selective and prolonged drug delivery to the ocular mucosa without compromising inner ocular tissues avoiding systemic absorption (De Campos et al 2001). More specifically, following topical instillation of a suspension of ^{3}H-ciclosporin-loaded chitosan nanoparticles, it was possible to achieve significant levels of ciclosporin in cornea and conjunctiva for at least 48 h; these levels were up to 2- to 10-fold higher than those provided by the chitosan solution containing ^{3}H-ciclosporin and the aqueous ^{3}H-ciclosporin suspension (Figure 2). In addition, it was found that the access of ciclosporin to the intraocular structures and blood circulation was restricted by the nanoparticle formulation. These results led us to conclude that chitosan nanoparticles may represent an interesting vehicle for drugs whose target is the ocular mucosa.

The specific localization of ciclosporin in the external ocular structures could be related to the mechanism of action and biodistribution of chitosan nanoparticles (discussed in next section) and also to the inherent properties of this peptide. Indeed, the high accumulation in the cornea must be certainly due to a facilitated interaction of the drug with the corneal epithelium. However, this accumulation may also be favoured by the hydrophobic nature of ciclosporin and, hence its inability to overcome the corneal stroma. The most surprising result was the important accumulation of ciclosporin in conjunctiva together with the lack of systemic absorption. This specific drug retention could be justified by the mechanism of interaction of the particles with the conjunctival epithelium, described later.

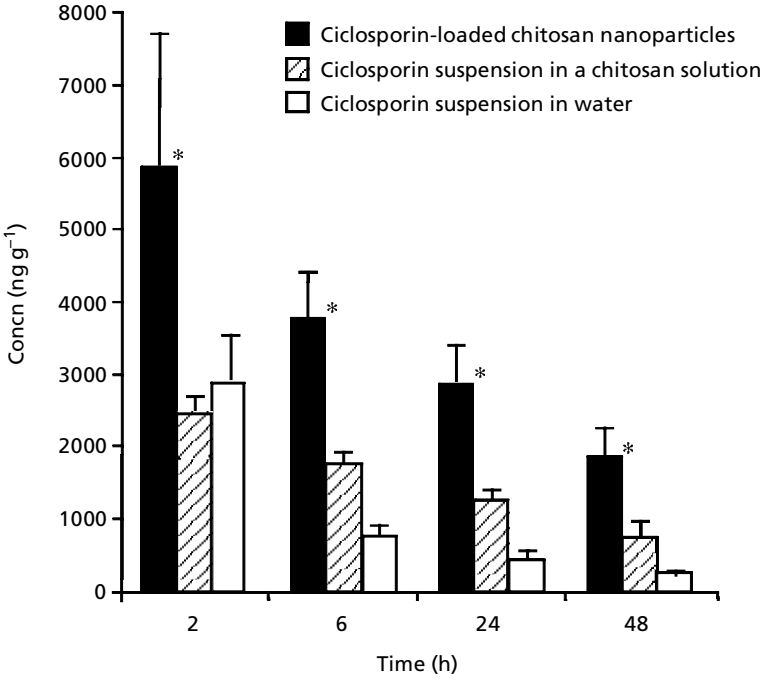

Figure 2 Ciclosporin concentration in the cornea (expressed as ng ciclosporin per g cornea; mean ± s.d.) after topical administration in rabbits of ciclosporin-loaded chitosan nanoparticles and control formulations consisting of a ciclosporin suspension in a chitosan aqueous solution and a ciclosporin suspension in water (*$P < 0.05$ vs controls) (from De Campos et al (2001), with permission).

Mechanism of action of chitosan-based systems upon contact with the eye surface

Two types of study have been performed to elucidate the mechanism of action of chitosan-based systems: those based upon the use of radioactivity and further evaluation by gamma-scintigraphy and those based upon the use of fluorescent markers and further evaluation by fluorimetry (quantitative analysis) or confocal laser scanning microscopy (CLSM) (qualitative analysis).

The work aimed at studying the mechanism of action of chitosan solutions, following topical ocular application, has used gamma emitters as markers (Henriksen et al 1996; Felt et al 1999a). These studies led to the conclusion that chitosan solutions have a prolonged retention at the ocular surface, behaviour that has been attributed to the mucoadhesive character of chitosan. However, this type of analysis did not provide information about the possible interaction between the polymer and the biological surface.

The ocular retention of chitosan nanoparticles, compared with that of chitosan solution, was, very recently, investigated in-vivo (De Campos et al 2002). For this purpose, chitosan was previously labelled with fluorescein acid, which was covalently linked to chitosan. These fluorescein-labelled chitosan nanoparticles were administered by topical instillation to rabbits and their interaction with the cornea and conjunctiva analysed quantitatively, by spectrofluorimetry, and qualitatively, by confocal microscopy. The first general observation was that chitosan nanoparticles had a greater corneal and conjunctival retention than chitosan solutions. Moreover, it was found that the retention of the nanoparticles was more important in the conjunctival tissue than in the cornea. Overall, these results indicated that the affinity of chitosan for the ocular surface (either cornea or conjunctiva) is greater when it is in a particulate form.

The more important retention of the chitosan nanoparticles as compared with the chitosan solution could be justified by a different mechanism of interaction of the soluble and particulate forms of chitosan with the ocular mucosa. To elucidate this mechanism, we examined cross sections of the corneal and conjunctival epithelia by CLSM. The corneal epithelium exhibited a strong fluorescent signal at the boundary region between corneal epithelial cells and a lower fluorescent signal inside the cells. This observation suggests that the nanoparticles may enter the corneal epithelium by a paracellular/transcellular pathway. This behaviour appears to be different from that of other types of nanoparticles, such as poly(alkylcyanoacrylate) (Zimmer et al 1991) and PECL nanoparticles (Calvo et al 1994), which were found to cross the corneal epithelium by a transcellular pathway. Nevertheless, the paracellular transport of chitosan nanoparticles could be explained by the presence of soluble chitosan molecules in the formulation. In fact, observation of the cornea exposed to chitosan solution also showed there to be significant fluorescent signals surrounding the cells, thus suggesting its paracellular transport. Consequently, these results represent preliminary evidence of the mechanism of interaction of chitosan nanoparticles and chitosan solutions with the corneal epithelium. However, more detailed studies are required.

Interestingly, the confocal microscopy images of cross sections of the conjunctival epithelium exposed to chitosan nanoparticles were quite different to those of the corneal epithelium. The nanoparticles were localized inside some specific cells. This uneven distribution could be due to the heterogenous nature of this epithelium. Indeed, this epithelium contains not only regular epithelial cells, but also goblet cells and antigen-presenting cells. Therefore, there is a possibility that chitosan nanoparticles are taken up differently by the various types of cells. The affinity for some specific epithelial cells could explain the more important retention of chitosan nanoparticles in the conjunctiva as compared with the cornea.

We have further investigated the mechanism of interaction of chitosan nanoparticles with conjunctival epithelial cells using normal human conjunctival cell (NHC) cultures (Enriquez de Salamanca et al 2002). The confocal images of NHC cells exposed to FITC-BSA-labelled nanoparticles showed a great number of fluorescent particles inside the cells, thus corroborating our previous in-vivo observations. An additional interesting result from these studies was that the particles exhibit very low toxicity.

The mechanism of interaction of chitosan-coated systems, more specifically chitosan-coated PECL nanocapsules, has also been investigated in-vitro and in-vivo (De Campos et al 2003). For this purpose, chitosan-coated systems were labelled with rhodamine B (Rd), a hydrophobic probe that was introduced in the oily core of the system. Then, the quantitative analysis of the Rd transport across the cornea was performed using a diffusion chamber. Rd transport across the cornea was significantly increased ($P < 0.05$) by the physical presence of chitosan-coated nanocapsules (Figure 3), thus suggesting that these systems have absorption-enhancing properties. However, despite the recognized effect of the physical presence of the nanocapsules, a greater Rd transport was observed when the marker Rd was encapsulated into the nanocapsules. This observation led us to suggest that these systems work as ocular carriers rather than as penetration-enhancement vehicles.

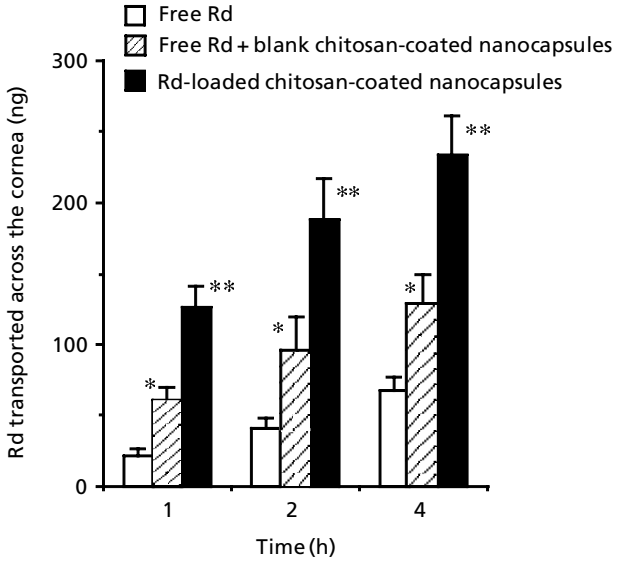

Figure 3 Amount of rhodamine (Rd) transported across the cornea during the ex-vivo studies (mean ± s.d., n = 4). *$P < 0.05$ vs free Rd; **$P < 0.05$, vs free Rd and free Rd plus blank chitosan-coated nanocapsules (from De Campos et al (2003), with permission).

The corneal disposition of chitosan-coated systems was further investigated by examining cross sections of the rabbit cornea, previously incubated with the systems, by CLSM. The images of the cross-sections showed a great amount of fluorescent spots that were uniformly distributed inside the cells, thus suggesting that the nanocapsules penetrate the corneal epithelium through a transcellular pathway, as previously reported for uncoated PECL nanocapsules (Calvo et al 1994). Furthermore, the images of cross-sections at different depths of the corneal epithelium showed that, even after prolonged exposure, these chitosan-coated systems were not able to reach more than a 20-μm depth. This penetration depth was much lower that previously observed for PECL and PEG-PECL nanocapsules (De Campos et al 2003). Systems coated with chitosan exhibited an important interaction but a low penetration depth into the corneal epithelium. Therefore, these results suggest that both the extent of interaction and penetration depth of colloidal systems with the cornea are highly affected by the surface composition of the system.

To verify whether or not the mechanism of transport of chitosan-coated nanocapsules was affected by the extreme conditions of the ex-vivo study (corneal diffusion chamber), Rd-loaded chitosan-coated nanocapsules were instilled into the eye of conscious rabbits and cross sections of the corneal epithelia were examined by CLSM. Figure 4 compares the confocal microscopy images of cross sections of the corneal epithelium previously exposed to the colloidal systems ex-vivo during a 4-h period and sections of the corneal epithelium at 4 h post-instillation of this formulation in-vivo. Following in-vivo administration, the nanocapsules were found to cross the corneal epithelium by a transcellular pathway, as previously observed in the ex-vivo studies.

Overall, the studies performed so far that have been aimed at investigating the mechanism of action of chitosan-based systems led to the conclusion that, irrespective of their presentation (gels and colloidal systems), they have an increased and prolonged residence time on the ocular mucosa. Furthermore, all of them were shown to penetrate the epithelium either by a cellular or paracellular mechanism of transport. Whether this transport is affected by the way chitosan is presented (as a soluble coating or as a solid particle) requires further investigation.

Tolerance and toxicity of chitosan-based systems

Before any excipient is accepted for drug administration in man it is necessary to demonstrate its low toxicity and adequate biocompatibility. Furthermore, if the excipient

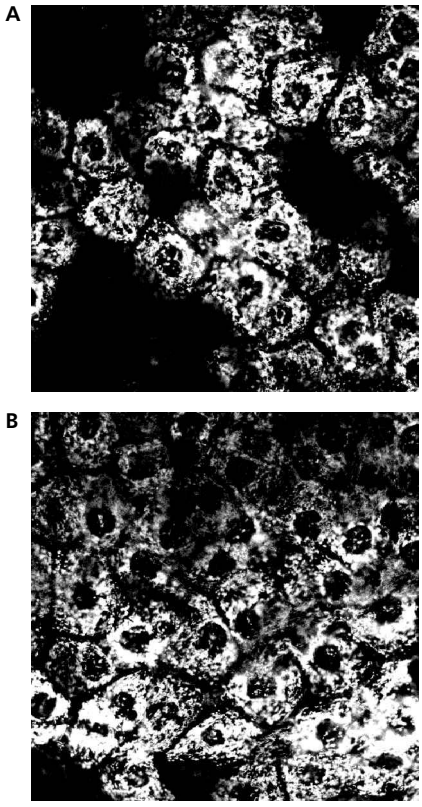

Figure 4 Confocal images of a cross section of the rabbit corneal epithelium after 4 h of incubation ex-vivo (A) or at 4 h post-instillation in-vivo (B) with chitosan-coated poly-E-caprolactone nanocapsules (from De Campos et al (2003), with permission).

is a polymer of a high molecular weight and is expected to interact intimately with biological surfaces, as in the case of chitosan, the proof of biodegradability becomes of primary importance. A number of reports have claimed the low toxicity and good biocompatibility of chitosan following intravenous, oral (Hirano et al 1989a; Knapczyk et al 1989) and nasal (Aspden et al 1997) administration. Similarly, the ocular tolerance and toxicity of chitosan has been investigated following topical administration to rabbits and also in cell cultures. More specifically, the ocular tolerance of chitosan gels was tested by Felt et al (1999a) using CLSM and corneal fluorescence staining. These studies provided evidence of the low irritation caused by chitosan after repeated topical administration to the corneal surface of rabbits (4 instillations a day for a period of 3 days). Similarly, an acute ocular tolerance test was also performed for chitosan-coated PECL nanoparticles (Calvo et al 1997b). After repeated administration of these systems (2 instillations per hour for a period of 6 h), no irritation or appreciable disruptions in the epithelial cells was observed using a slit-lamp and a histological assay.

In the previous section, it was shown that chitosan-based systems are able to enter epithelia. Given this intimate interaction, it is important to assess whether or not chitosan can be conveniently biodegraded and eliminated from the organism. With respect to the biodegradation of chitosan, it has been shown that chitosan is rapidly degraded by lysozyme (Hirano et al 1989b; Aiba 1993). This degradation mechanism could have, a-priori, a certain relevance in ophthalmics since lysozyme is highly concentrated in mucosal surfaces and, in particular, in the ocular mucosa (Rohen et al 1992). We have recently verified the susceptibility of the chitosan coating of colloidal carriers to lysozyme (Vila et al 2002). More specifically, after incubation of chitosan-coated PLGA nanoparticles with lysozyme, we observed a neutralization and further inversion of the zeta potential (from positive to negative values). This observation indicated that lysozyme interacts with the chitosan coating. However, more detailed work is necessary to determine the degradation rate of these carriers. Nevertheless, in this regard, it is also important to keep in mind that chitosan can also be depolymerized in-vitro to obtain the adequate-molecular-weight fractions (Janes & Alonso 2003) and thus to reduce the necessity of being degraded following in-vivo application. On the other hand, it is known that the degradation by lysozyme increases with a reduction of the degree of deacetylation (Hirano et al 1989b). Therefore, it could be expected that by adjusting the molecular weight and deacetylation degree of the polymer a specific degradation rate will be achieved.

Finally, preliminary studies performed in conjunctival cell cultures have shown the low toxicity of chitosan nanoparticles (De Campos et al 2002; Enriquez de Salamanca et al 2002). More detailed studies are underway to fully understand the intracellular fate of these new drug delivery systems.

Concluding remarks

The information reported until now has provided evidence of the ability of chitosan-based systems to improve the surface ocular retention and to enhance the transport of drugs across the cornea. Furthermore, some studies indicated that the in-vivo ocular fate of these chitosan-based systems is dependent on the intrinsic characteristics of the system. This is particularly appealing, since chitosan can be presented as a simple solution, as a soluble coating around oily droplets or solid nanoparticles, and also in the form of solid chitosan nanoparticles. Furthermore, initial experiments have indicated the adequate tolerance and low toxicity of these new ocular drug carriers. Therefore, while more work is necessary to fully understand all factors that are determinant for the mechanism of action and efficacy of these carriers, we can certainly conclude that chitosan-based systems have a promising future in ocular drug delivery.

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