Expert Opinion

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# Chitosan nanoparticles for drug delivery to the eye

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The purpose of this review is to provide the reader with an overview of the advances made in ocular delivery of bioactive molecules by means of chitosan-based nanosystems, and their potential relevance in clinical use. The studies described clearly emphasise that chitosan-based nanostructures are versatile systems that can be tailor-made according to required compositions, surface characteristics and particle size. Such parameters, which are known to influence their *in vivo* performance, can be modulated by adjusting the formulation conditions of the nanotechnologies responsible for their formation, by incorporating additional materials in the preparation steps, and/or by using synthetically modified chitosan. Moreover, this review illustrates how the advances achieved in the understanding of the interaction of nanosystems with the ocular structures should result in the coming years, logically, into challenging innovations in ocular nanomedicines with significant impact on clinical practice.

**Keywords:** chitosan, gene delivery, nanomedicines, nanoparticles, ocular delivery

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## 1. Introduction

Topical instillation is the method of preference for the administration of drugs used in the treatment of ocular disorders, affecting the ocular surface and both the anterior and posterior segment of the eye. It presents several advantages over the invasive routes, such as an easy administration and better patient compliance. However, topical drug delivery to the eye is often impaired by the innate protective characteristics of the eye against the entry of foreign compounds. Due to the removal mechanisms (blinking, tears) and to the complex composition and dynamic character of the lachrymal fluid [1] the bioavailability of an instilled compound is generally low, with only a small fraction reaching the target site. Therefore the major problem in ocular therapy is to provide and maintain an adequate concentration of the drug at the site of action. With traditional dosage forms most of the instilled drug is lost within the first 15 – 30 sec after instillation and less than 5% of the applied drug penetrates the cornea and reaches the intraocular tissues [2]. Limited drug absorption is mainly due to short pre-corneal residence time related to tear turnover, rapid nasolachrymal drainage of instilled drugs and non-productive absorption through the nasal duct and conjunctiva [3].

In the case of disorders that affect the internal structures of the eye, an additional inconvenience is the limited penetration of the biomolecules. Topically applied drugs can reach the intraocular tissues by either the corneal and/or the non-corneal (conjunctival–scleral) pathways. The cornea, one-sixth of the ocular surface, is the main route of access to the posterior segment of the eye. Nevertheless, due to its well-organised structure, it is considered to be one of the most specialised barriers in the organism and significantly hampers the penetration of drugs, irrespective of their solubility properties. Therefore, over the last decades intensive research

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has been oriented to increase the penetration of drugs into the cornea [4-8], thus forming a reservoir from which they may diffuse to the posterior segment of the eye [9-10]. In contrast, the conjunctiva is a more permeable epithelium, but, due to its high vascularisation, it generally contributes to the non-productive absorption of the drug and consequently to the appearance of side effects. This is the main reason why conjunctival drug transport research has remained relatively unexplored, as compared with the important research undertaken to study the transport across the cornea. However, the conjunctival pathways are gaining an increasing interest with regard to their implication in the access of the drug to the inner eye and, thus, for the treatment of disorders that affect the internal structures of the eye [11-12].

The anatomy, physiology and biochemistry of the eye make this organ even more impervious when we consider therapeutic macromolecular drugs, such as peptides, proteins and gene medicines. Over the past years, researchers have identified several macromolecules which may be of benefit in treating different disorders of the eye, and represent an increasingly important segment of the therapeutic arsenal in ophthalmology [13-18]. However, their potential is still limited by their instability and inability to interact with the ocular surface and/or cross the ocular epithelia [19]. Therefore, the design of an appropriate delivery system for these compounds would be essential for their effective use in therapy [20-23].

Considering the disadvantages of the conventional ocular dosage forms, a great effort in developing new drug delivery systems that are able to efficiently incorporate drugs as well as control their release rate, while facilitating their interaction with the ocular tissues, has gained increasing attention over the last years. The ideal ocular drug delivery system should be able to protect the drug from the biological environment, while facilitating its delivery to the ocular epithelia or its transport through biological barriers. Moreover, it should be dispensed in the form of eye drops, causing no blurred vision or irritability, and would need no more than one or two administrations per day [21]. Nanometric delivery systems could be the answer to this ideal system. Therefore the purpose of this review is to provide the reader with an overview of the great potential offered by nanosystems in terms of improving the efficacy of drugs used in ocular therapies, with special emphasis on the advances achieved in chitosan-based colloidal delivery systems.

## 2. Nanometric delivery systems in ophthalmology

Colloidal delivery systems have become a focus of attention in the field of biomedicine due to the potential benefits in providing wide improvements in drug delivery and targeting. They have been successfully used to:

1. deliver drugs in a controlled fashion
2. minimise drug degradation
3. prevent harmful side effects
4. increase drug bioavailability
5. increase the fraction of the drug accumulated in the site of action
6. enhance drug permeation

A general classification distinguishes three different systems as a function of their composition: (i) nanoemulsions; (ii) nanospheres or nanoparticles; and (iii) nanocapsules.

Nanoemulsions are submicrometric emulsions, adequate for the encapsulation and further delivery of both hydrophobic and hydrophilic drugs [24-25]. However their limited stability represents an important drawback [26-27]. Despite this, some formulations containing the polypeptide cyclosporine A are under clinical evaluation, as for example a cationic nano-emulsion for the treatment of dry-eye syndrome (Novagali Pharma, France), or even on the market, as Restasis® (Allergan, CA, USA), approved for the treatment of keratoconjunctivitis sicca.

Nanoparticles and nanocapsules are colloidal delivery systems formed, in the first case, by a solid matrix of polymers or lipids (nanoparticles or nanospheres) and, in the second case, by an oily or aqueous core coated with a polymeric corona (nanocapsules). A wide variety of biomaterials, as well as technologies, may be applied for the development of nanoparticulate delivery systems. This versatility makes them adequate nanocarriers for the delivery of drugs with different physicochemical properties, including peptides, proteins and genes [28-30]. The most relevant and appealing characteristic of nanoparticles is their intrinsic capacity to adhere to the ocular surface and interact with the epithelium, as first observed by Wood *et al.* in 1985 [31] and corroborated by a number of successive studies [32-34]. The degree of interaction of particles with the mucosa was found to be dependent on their size [35], nanometric carriers being the most efficient ocular delivery systems [36]. Therefore, particle size has a key role in the ability to interact with the ocular mucosa and work as ocular drug carriers. Moreover, much of the published data suggest that in the case of ophthalmic drug delivery, an appropriate particle size and a narrow size range ensure low irritation, adequate bioavailability and compatibility with ocular tissues [37-38].

Apart from size, other biophysical characteristics, such as stability in the biological environment and bioadhesiveness, are known to modulate the performance of drug nanocarriers. The importance of these characteristics has been extensively discussed in other reports [39-42].

From a rheological point of view, nanometric suspensions are characterised by a very low viscosity that makes feasible their administration as eye drops, but with the critical difference of an intimate interaction with the ocular surface, a higher penetration into the deeper layers of the ocular structure and a controlled delivery of the associated drug [21]. The possibility of prolonging the release of a drug makes these vehicles very attractive systems for ocular delivery, as they can greatly decrease the frequency of drug administration.

The performance of a nanocarrier as ocular delivery vehicle and its ocular tolerability can be deeply affected by the material used for their elaboration. Therefore a rational selection of the biomaterials used for the nanoparticles preparation must be made. Furthermore, many pathological conditions of the eye (for example dry-eye syndrome, diabetic retinopathy and age-related macular degeneration) require a chronic administration of the drug. Therefore, biodegradability would be a basic requirement to avoid accumulation of these nanocarriers in the ocular structures. This aspect is particularly important given the limited knowledge of the long-term accumulation of nanoparticulated biomaterials in the body [43-44]. Thus the potential of several polymers, including acrylic polymers, polyesters and polysaccharides, as ocular delivery systems has been investigated over the last decades. Among them, the polysaccharide chitosan stands out for its unique biopharmaceutical properties, ocular tolerability and biodegradability, thus representing a highly promising biomaterial for the production of biocompatible and biodegradable nanocarriers.

## 3. Chitosan and its use in ophthalmology

Chitosan exhibits several favourable biological properties that make it an interesting polymer for use in pharmaceutical formulations, as demonstrated by the number of scientific reports published. It is a mucoadhesive and biodegradable polymer that possesses penetration-enhancing properties and an adequate toxicity profile [45-51]. All these excellent biopharmaceutical characteristics make it a unique material for the design of drug delivery vehicles, not only for parenteral administration of drugs [52], but also for their transmucosal delivery [42], including ocular delivery.

The history of its use in ophthalmology started with its employment as a viscosity and permeation agent, evolving to its use as the main constituent of different nanometric delivery systems. Interestingly, the pharmaceutical presentation of chitosan deeply influences its behaviour in ocular therapy. When topically instilled to rabbits, chitosan nanoparticles loaded with the polypeptide cyclosporine were able to provide a selective and prolonged delivery of the drug to the cornea and conjunctiva, with drug levels up to 2- to 10-fold higher than that provided by a suspension of the drug in a chitosan solution. Moreover, the access of the drug to the intraocular structures and blood circulation was restricted by the nanoparticle formulation [53]. This specific drug retention could be justified by an interaction of the particles with the ocular epithelium that will be further described in the following sections. The same results were obtained in another study in which chitosan was labelled with fluorescein and topically instilled to rabbits, as a solution or in the form of nanoparticles [54]. The measured fluorescence intensities in both the cornea and the conjunctiva were much higher in the case of chitosan nanoparticles with respect to the chitosan solution, thus indicating a greater affinity of the polymer for the ocular surface when is in particulate form.

Special mention must be made of the use of chitosan as a complexing agent of therapeutic macromolecules, particularly genes and protein molecules. This polymer has been extensively investigated for formulating complexes, which are formed only for the tropism existing between the positively charged chitosan and negatively charged macromolecules. Although such complexes are extremely easy to synthesise, their physicochemical characteristics, such as particle size and density of surface charge, are very difficult to control. This lack of control of the properties of the complexes represents the main difference between them and the nanoparticle systems described in section 4.1. However, some confusion exists in the nomenclature of these systems, as complexes are so often called nanoparticles due to their nanometric size. It has been demonstrated that chitosan can effectively bind both DNA and RNA molecules and protect them from nuclease degradation [55-58]. However, to our knowledge, neither chitosan complexes nor nanoparticles have been studied as delivery vehicles for gene therapy in the eye [59].

In the following section, we will report in detail the characteristics and the *in vivo* behaviour of chitosan nanoparticles (CS NP) as ocular delivery systems for the treatment of diseases mainly affecting the surface of the eye. Despite the progress made in the design of CS-based nanotechnologies, the topical administration of these nanosystems has not yet resulted in significant improvements in the therapy of diseases affecting the posterior segment of the eye. Therefore a more invasive drug administration, such as the intravitreal or intrachameral injection, among others, is still required for the treatment of pathologies affecting the inner structures of the eye. Even in this case the use of polymeric nanoparticles may be of benefit, as they can circumvent the problem of frequent administrations by providing a controlled release of the encapsulated drug and thus reducing the clinical complications generally associated with these invasive modalities of administration [60]. However, to our knowledge, other polymers are generally preferred for the development of nanoparticles for intraocular drug delivery and very limited experience exists with chitosan to date.

## 4. Chitosan nanocarriers

Over the last few years, the characteristics and the potential of CS nanocarriers for the transmucosal delivery of drugs have been extensively explored. Different types of CS nanosystems have shown a great capacity to improve the transport of drugs upon administration by transmucosal routes [42] and more specifically by the ocular mucosa. Overall, a variety of hydrophilic and hydrophobic drugs and biomacromolecules have been successfully delivered to the eye upon inclusion in CS-based nanosystems. A general classification of chitosan nanocarriers distinguishes between CS-coated and CS-based nanosystems, depending on whether CS is in the form of a coating or a nanomatrix. Besides these, new generations of CS-based nanocarriers with improved properties for ocular

delivery have also been designed. They include: i) self-assembled nanosystems, obtained after modification of CS with hydrophobic moieties, intended to improve the interaction of the carriers with biological membranes [61]; and ii) hybrid nanoparticles that combine chitosan with other biomaterials, thus creating systems with new and improved properties. For illustration, a schematic representation of the various nanostructures which are the object of this review has been included in the manuscript (**Figure 1**). In the following sections, the authors will present the progress made in the design of chitosan nanocarriers as ocular delivery vehicles. Highlighted features are included in **Table 1**.

### 4.1 Chitosan nanoparticles

Chitosan is a promising biomaterial for the production of biocompatible and biodegradable nanocarriers. The interesting features of chitosan nanoparticles for ocular administration of drugs especially lie in: i) the mild conditions required for their preparation; ii) the possibility to obtain homogeneous particles population and modulate their size and surface charge in an easy way; iii) a great capacity for the association of different types of active compounds, including peptides, proteins and nucleic acids; and iv) a great versatility for the incorporation of other molecules (i.e., poloxamers, glucomannan, cyclodextrins, hyaluronic acid, alginate) within the nanomatrix structure, thus generating hybrid nanoparticles that will be extensively described in section 4.3.

Chitosan is a cationic polysaccharide able to gel when in contact with specific multivalent polyanions, such as sodium tripolyphosphate (TPP). Based upon this principle, a few years ago our group took the challenge of developing a simply way to produce CS nanoparticles (NP) by ionotropic gelation [62]. Nanoparticles are spontaneously formed upon the mixing of chitosan and TPP solutions, through the formation of inter- and intra-molecular linkages between the phosphate groups of TPP and the amino groups of chitosan. More recently, this gelation technique was adapted for the production of depolymerised chitosan nanoparticles [63]. This idea came from different studies suggesting that molecular weight could affect a number of key properties of chitosan, such as biocompatibility [64], permeability enhancement [65] and mucoadhesion [66]. Despite the hydrophilicity of this polysaccharide, CS NP could be optimised for the encapsulation of both hydrophilic and hydrophobic drugs, for example doxorubicin [67], as well as macromolecules like peptides [53,68] and gene medicines [69-70]. Moreover, nanocarriers with improved properties in terms of stability in the biological environment, bioadhesiveness and targeting could be obtained by either the rational modification of the chitosan structure or the incorporation of other biomaterials within the nanocarriers, as discussed in the next sections.

As mentioned before, the pharmaceutical presentation of chitosan can deeply influence its behaviour in ocular therapy. The differences observed between a soluble and a particulate form of chitosan could be justified by a different mechanism of interaction with the ocular mucosa. With the aim of elucidating this mechanism, fluorescent labelled CS was topically instilled to rabbits either in solution or in the form of nanoparticles, and the intensity of the fluorescence found in both the cornea and the conjunctiva, eventually analysed by confocal microscopy and spectrofluorimetry [54]. The results showed that CS NP were able to intimately interact with the corneal and conjunctival epithelia, therefore showing a greater corneal and conjunctival retention in relation to the CS solution. Another interesting observation was that CS nanoparticles were able to penetrate through the corneal epithelium, as supported by the strong fluorescent signal exhibited at the boundary region between the corneal epithelium, in addition to a weaker fluorescent signal observed inside the cells. Overall, these results corroborate a greater affinity of chitosan for the ocular surface when is in nanoparticulate form, and suggest a mechanism of entry of the nanoparticles into the ocular epithelium that combines the paracellular/transcellular pathways. This behaviour appears to be different from that of other types of nanoparticles, such as poly(alkylcyanoacrylate) [71] and poly-ε-caprolactone (PECL) nanoparticles [33], which were found to cross the corneal epithelium only by transcellular pathway. Nevertheless, the paracellular transport of chitosan nanoparticles could be explained by the presence of soluble chitosan molecules in the formulation. A second analysis of the disposition of CS nanoparticles on interaction with the ocular surface was later performed by Enriquez de Salamanca using CS nanoparticles encapsulating a fluorescent model biomacromolecule (fluorescein isothiocyanate [FITC] bovine serum albumin [BSA]) [72]. Sections of the eyes treated with CS nanoparticles revealed fluorescence localised throughout the cytoplasm of corneal and conjunctival epithelial cells, thus corroborating previous *in vivo* observations. Additionally, the mechanism of interaction of the CS NP was explored *in vitro*, using for that purpose a normal human conjunctival cell line, IOBA-NHC. Confocal images showed that FITC-BSA-labelled nanoparticles were actively taken up by the conjunctival cells.

Given the intimate interaction of CS nanoparticles with the ocular mucosa and their ability to enter the epithelia, 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 [73-74], which is highly concentrated in mucosal surfaces and, in particular, in the ocular mucosa [75]. It was found that one of the key factors controlling the degradation rate of chitosan is its degree of deacetylation (DD): the lower the DD the faster the enzymatic hydrolysis [76]. The biodegradability of chitosan enables the safe administration and degradation of topically applied ocular vehicles. As stated before, this aspect plays a crucial role, particularly in all those ocular pathologies that require chronic pharmacological treatment. Therefore, biodegradability would be a basic requirement to avoid the accumulation of these nanocarriers in the ocular structures.

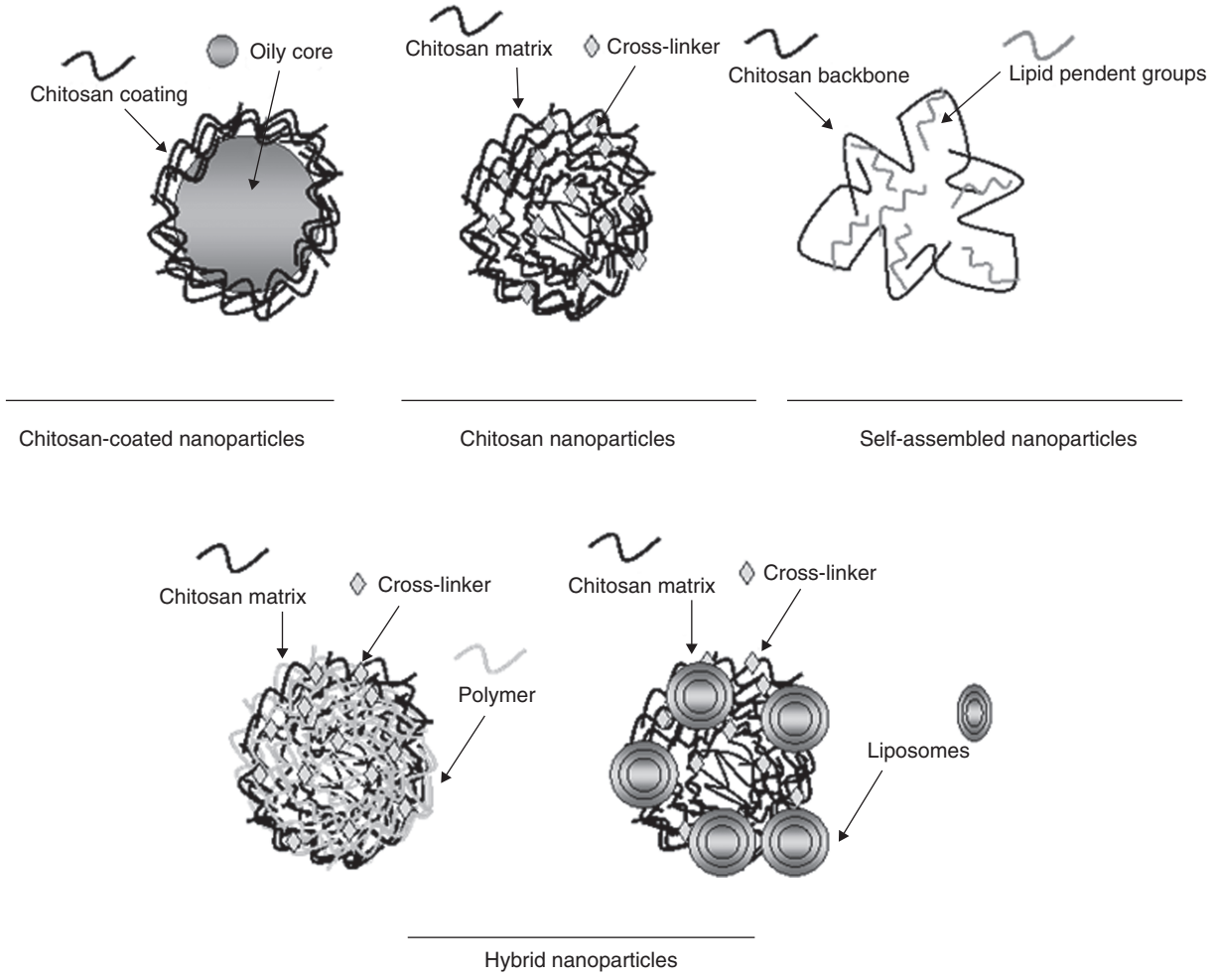


**Figure 1. Schematic representation of CS-based nanosystems specifically designed for the administration of drugs and biomacromolecules onto the eye surface.**

This aspect is particularly important given the limited knowledge of the long-term accumulation of nanoparticulated biomaterials in the body. A number of previous reports claim the low toxicity and good biocompatibility of chitosan following intravenous, oral [47,77] and nasal administration [78]. Similarly, CS nanoparticles exhibit very low toxicity when topically administered to the eye of rabbits [72,79]. In this study no clinical or pathological differences between the eye treated with CS NP and the control were observed. The absence of abnormal inflammatory cells in the cornea, conjunctiva and lids was also consistent with the lack of clinical signs after exposure to the nanoparticles. Although rabbit and human ocular surfaces are not equivalent, a good correlation between rabbit and human eye irritation data exists when low volumes are used [80]. These results correlate well with an *in vitro* study where CS NP showed negligible toxic effect [72]. In contrast, a recent report claimed the appearance of an immune response after the injection of CS nanoparticles into the vitreous chamber. However, it has been hypothesised that the immune reaction could be induced by some impurities of the CS and not by the polymer itself [81].

### 4.2 Chitosan-coated nanocarriers

As mentioned in previous sections, nanoparticles are able to interact and penetrate the ocular epithelia, while providing increased bioavailability of the associated molecules. However, their efficacy as an ocular delivery vehicle is critically related to their physicochemical characteristics. A relationship between the intensity of this transport and the size and surface properties of the carrier, in terms of surface charge and composition, has been found [35,82]. Various authors have observed a significant improvement of the bioavailability of different drugs administered with CS-coated nanoparticles, as compared to the uncoated vehicles. For example, we have studied the *in vivo* efficacy of CS-coated nanoparticle systems using indomethacin as a model drug. The ocular drug disposition was determined after a single instillation of $^{14}$C-indomethacin-loaded systems to conscious rabbits and subsequent quantification

**Table 1. Highlighted features observed upon topical instillation of chitosan-based nanocarriers.**

| Nanocarrier | Particularity | Biomolecule | Relevant features | Ref. |
|---|---|---|---|---|
| Chitosan nanoparticles | - | CyA | Up to 10-fold increase of the drug levels detected in the ocular mucosa versus CyA suspended into CS solution | [53] |
| | - | - | Demonstrated the capacity of the NP to penetrate the ocular epithelium, showing an improved corneal and conjunctival retention as compared to CS in solution | [54] |
| | - | FITC-BSA | Demonstrated biocompatibility of the CS NP upon interaction with the ocular surface | [72] |
| Chitosan-coated nanoparticles | Oily core | Indomethacin | Increased drug levels in the anterior segment of the eye in relation to uncoated systems | [83] |
| | Oily core | Indomethacin | Increase in the concentration of drug detected in the anterior segment and the vitreous versus indomethacin eye drops. | [36] |
| | Oily core | - | Increased transport across the cornea of the encapsulated marker rhodamine B | [34] |
| | Poly(lactic acid) core | RAPA | Improved immunosuppressive effect in the treatment of corneal allografts versus RAPA eye drops | [86] |
| Hybrid chitosan nanoparticles | Liposomes | FITC-BSA | Demonstrated interaction with the ocular mucosa, showing a pattern that is dependent on the lipidic composition, and a good ocular tolerance | [131] |
| | Carbopol | Pilocarpine | Sustained release and prolonged therapeutic effect of drug-loaded NP when compared with eye drops or pilocarpine-loaded into gels and liposomes | [89] |
| | Poly(acrylic acid) | Pilocarpine | Sustained release and prolonged therapeutic effect versus pilocarpine eye drops | [90] |
| | Hyaluronan | pDNA | Important interaction of the NP with the ocular epithelia, leading to the transfection of the tissues and expression of the reporter protein for up to 1 week, without causing any damage or inflammation to the ocular structures | [120] |
| Self-assembled chitosan nanoparticles | Cholesterol group | CyA | Important retention of the NP at the precorneal area, for up to 2h | [133] |
| | Palmitoyl group | Prednisolone (Allergan, CA, USA) | 10-fold increase of the drug levels detected in the aqueous humour versus those observed with the commercial suspension | [134] |

CS, chitosan; CyA, cyclosporin A; FITC-BSA, fluorescein isothiocyanate-bovine serum albumin; NP, nanoparticles; RAPA, rapamycin.

of the radioactivity levels in cornea and aqueous humour [83]. The results showed that CS-coated nanoparticles gave rise to significantly higher drug levels both in the cornea and in the aqueous humour than either the uncoated systems or the commercial drug preparation. This work led to the conclusion that a chitosan coating adds a clear benefit to the potential of colloidal systems as ocular drug carriers. In the same way, CS-coated vesicles caused a marked hypoglycemic effect after topical delivery of insulin to the eye [84], whereas CS-coated timolol-loaded niosomes showed an efficient and long-lasting control of the intraocular pressure [85]. The clear benefit that a chitosan coating adds to the potential of nanoparticulate systems, as ocular drug carriers, is due to the inherent properties of chitosan and not to the positive surface charge impaired to the nanoparticles. In fact, comparing the behaviour of chitosan and poly(L-lysine)-coated formulations of similar surface charge, a lack of effect for the poly(L-lysine)-coating was observed, thus demonstrating the intrinsic beneficial effect of chitosan. Comparative studies in which chitosan or carbopol were used as coating materials of liposomes and niosomes reiterated the superiority of CS for topical administration to the ocular mucosa [84,85]. Thus, surface modification of colloidal delivery vehicles with chitosan can improve the interaction and the penetration of the carrier in the corneal epithelium [33]. However, the behaviour of CS-coated nanosystems is not only due to a possible penetration enhancement effect, but also to a real ability of these systems to work as ocular carriers. In fact the transport of the hydrophobic fluorescent probe rhodamine B (Rd) across the cornea, evaluated *ex vivo* by means of a diffusion chamber, was significantly higher when the marker was encapsulated into the oily core of CS-coated PECL nanocapsules, rather than when physically mixed with the nanocarriers [34]. This observation led us to suggest that these systems work as ocular carriers rather than as penetration enhancement vehicles. The corneal disposition of CS-coated systems was further investigated by examining cross-sections of the excised rabbit cornea, previously incubated with the systems, by confocal microscopy. The images showed a great number of fluorescent spots 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 [33]. To verify whether or not the mechanism of transport of CS-coated nanocapsules was affected by the extreme conditions of the *ex vivo* study, Rd-loaded CS-coated nanocapsules were instilled onto the eye of conscious rabbits. Following this *in vivo* administration, the nanocapsules were found to cross the corneal epithelium by a transcellular pathway, thus corroborating the results obtained with the *ex vivo* studies. Moreover, it was found that after repeated administration of these systems, neither irritation nor appreciable alterations of the epithelial cells were observed.

Apart from their colloidal size and mucoadhesive character, CS-coated nanoparticles have the inherent advantage of their versatility in terms of drug-loading capacity and release properties. In fact, by an opportune selection of the core composition, it is possible to encapsulate both hydrophobic and hydrophilic compounds, including biomacromolecules. For instance, hydrophilic proteins such as tetanus toxoid have been loaded into CS-coated poly(lactic-co-glycolic acid) using the solvent evaporation technique [41]. On the other hand, lipophilic drugs, such as indomethacin and diazepam, have been very efficiently incorporated into CS-coated nanocapsules [27,36].

In a different work, the hydrophobic polymer poly-lactic acid, PLA, was used for the preparation of nanoparticles, eventually coated with CS modified with cholesterol (CS-CH), overall regarded as a strategy to increase the hydrophobicity of the resulting nanocarriers and therefore the loading capacity of the highly hydrophobic immunosuppressive agent rapamycin (RAPA) [86]. With respect to the interaction of the CS-CH/PLA nanoparticles with the ocular mucosa, it was shown that they congregated at the conjunctival sac, in contrast to the behaviour observed for CS-CH NP, which easily spread onto the complete ocular surface, as described in section 4.4. This distribution is reported to be due to the high viscosity of the CS solution. In spite of this, a similar pattern was previously reported for PECL nanoparticles due to their hydrophobic character [87]. The efficacy of RAPA-loaded CS-CH/PLA nanoparticles was eventually investigated for the treatment of corneal allografts, and the results showed an excellent immunosuppressive effect of the RAPA-loaded nanoparticles in relation to RAPA eye drops, as can be observed in **Figure 2**.

Considering all this information, it can be stated that CS-coated nanosystems have high potential for the administration of drugs to the eye surface, as an increase in the therapeutic efficacy has been widely observed. The nanoparticles coated with chitosan exhibited an important interaction with the corneal epithelium, being able to enter the epithelial cells and accumulate in the tissue. Therefore, these results suggest that, apart from acting at the ocular surface, CS-coated nanoparticles could either facilitate the transport of the associated drug to the inner structures of the eye, or act as a reservoir after delivering the drug in the cornea [9-10].

### 4.3 Hybrid chitosan nanoparticles

As stated before, one of the interesting features of CS nanoparticles is the possibility of introducing other biopolymers within their structure. The introduction of a second ingredient in the nanoparticles composition greatly increases their versatility, leading to vehicles with improved properties, not only in terms of association and delivery of drugs, but also with respect to their ability to interact with biological surfaces. Interesting properties have been observed with the incorporation of a di-block copolymer of ethylene oxide and propylene oxide (PEOPPO) [88]. The presence of this copolymer led to a significant increase in the release rate of the encapsulated protein BSA, thus confirming the possibility of modulating

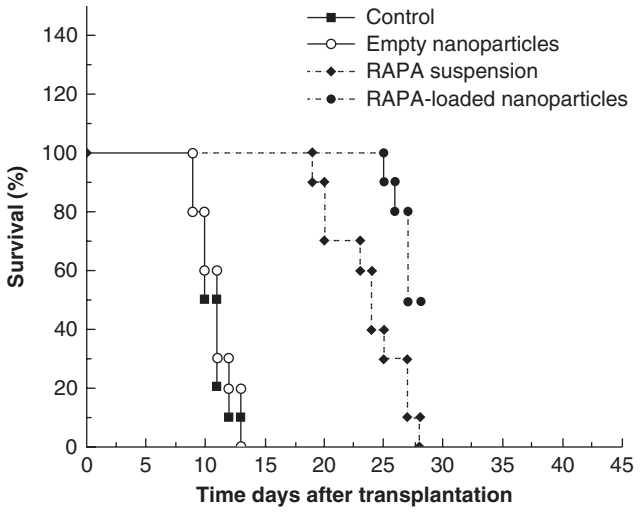


**Figure 2. Survival curves of corneal allografts in rabbits treated with RAPA-suspension, RAPA-loaded CS-CH/PLA NP and empty CS-CH/PLA NP.** The groups were treated twice a day for 4 weeks following surgery. In the control group the grafted eye was not treated throughout the observation period. Adapted with permission from [86].

the release rate of the drug simply by adjusting the composition of the nanoparticles. Since then, a variety of biocompatible, biodegradable and mucoadhesive polymers, widely used in ophthalmology, have been successfully included within the polysaccharidic matrix of chitosan nanoparticles, giving rise to a new generation of hybrid systems. The combination of CS with carbopol, poly(acrylic acid) and alginate led to the development of sustained delivery formulations. More specifically, Kao *et al.* evaluated the potential of pilocarpine-loaded CS/carbopol nanoparticles as an alternative sustained release formulation for clinical use. The efficacy of the nanocarriers was evaluated in rabbits with a miotic test and compared with a solution of the drug and with other two formulations incorporating pilocarpine (gel and liposomes) [89]. Results confirm the potential of the developed nanoparticles, as they elicited the most significant long-lasting decrease in the pupil diameter of rabbits. A sustained release of pilocarpine was also obtained with chitosan-poly(acrylic acid) (CS-PAA) nanoparticles, prepared using a template polymerisation of acrylic acid (AA) in a chitosan solution [90]. Pilocarpine was successfully loaded into the resulting nanostructures, and both *in vitro* and *in vivo* studies revealed that the prepared nanoparticles suspension were better at sustaining the release of pilocarpine than commercial eye drops. Similarly the incorporation of alginate in CS-based nanoparticles through ionotropic gelation led to a prolonged topical ophthalmic delivery of the antibiotic gatifloxacin for up to 24 h [91]. Overall these results show that it is possible to modulate the release rate of an encapsulated drug simply by adjusting the composition of the chitosan nanoparticles. Alginate is a polysaccharide that has been previously used in ocular delivery both alone, in the form of hydrogels or films, and in combination with other materials, such as chitosan [91-95]. The incorporation of alginate in CS-based nanoparticles led to the formation of a carrier that is biocompatible, non-toxic and able to effectively protect the associated biomolecules [96-97]. However, to the best of our knowledge, *in vivo* studies to demonstrate the efficacy of these alginate/chitosan systems in ocular delivery remain to be done.

A particularly interesting formulation for the ocular delivery of macromolecules has been recently disclosed by our group, upon incorporation of the natural occurring glycosaminoglycan hyaluronic acid (HA) into the conventional CS nanoparticles [98]. HA is a very interesting polysaccharide that finds a number of applications in drug delivery, and more specifically in ophthalmology, due to its unique biopharmaceutical properties. HA is a mucoadhesive, biocompatible, non-immunogenic and biodegradable polymer [99-103]. In addition it is one of the components of the extracellular matrix of connective tissues in vertebrates, such as the vitreous and aqueous humour in the eye. Many of the physiological and biological functions of HA are based on its specific interactions with cell surface receptors, such as CD44 [104-106]. Indeed, several different delivery systems that incorporate HA in their structure have been developed, as for example hydrogels and microparticles [107,108]. It has also been used for the surface modification of colloidal systems, with the objective of improving their adhesive properties or targeting the CD44 hyaluronan receptor [109-110]. As stated before, HA/CS nanoparticles have been recently developed in our group [98]. They were obtained by a slightly modified ionotropic gelation technique and using TPP as a cross-linking agent. A very interesting finding is that the physicochemical properties of the nanoparticles could be easily modulated by adjusting the ratio of the two major components, HA and CS, as well as the crosslinker. Furthermore, nanoparticles can be prepared starting from HA and CS of different molecular weights [112], thus increasing their versatility, as it is well known that molecular weight can deeply affect the properties of both polymers [63,113-115]. In addition, HA/CS nanoparticles were demonstrated to possess a high drug loading capacity, irrespective of the solubility properties of the encapsulated macromolecules. In fact, not only hydrophilic macromolecules such as BSA and plasmid DNA, but also hydrophobic polypeptides, such as cyclosporin A (CyA), were efficiently entrapped in the nanostructures [98,112]. Apart from their use as ocular drug delivery vehicles in general, these nanoparticles are particularly attractive for the delivery of nucleic acids, due to the involvement of HA in several cellular processes that promote the transfer of genes to the target cells [116-117]. To this end, the conditions for the transfection of two ocular cells lines, derived from the corneal and conjunctival epithelium (immortalised human corneal epithelial cells [HCE] and normal human conjunctival cells [IOBA-NHC]) were explored using plasmide-enhanced green fluorescent

protein (pEGFP) as a model plasmid. Results show that the nanoparticles present a high capacity for transferring genes to both ocular cell lines. Moreover it was observed that the molecular weight of chitosan as well as the amount of HA incorporated in the nanosystems greatly influence the transfection efficiency. A reduction of the molecular weight of chitosan gave rise to an increase of the levels of the green fluorescent protein expressed, with the higher levels observed in the nanoparticles composed of oligomers of CS (CSO), that is HA/CSO nanoparticles. In the same way, when the amount of HA was increased, a higher transfection and a marked decrease in the *in vitro* toxicity of the nanoparticles was observed. These results were eventually confirmed in non-dividing cells, using for that purpose a culture model of the corneal epithelium [118]. As stated before, one of the major advantages of the incorporation of HA in a nanosystem is its potential role as a target ligand. For this reason, the capacity of HA/CS nanoparticles to interact with CD44 receptors and the influence of this interaction on the transfection efficiency were further explored. The results showed that the uptake of the nanoparticles in HCE cells was partially mediated by their interaction with CD44, in agreement with previous studies performed with HA-coated liposomes and HA-coated polyethylenimine (PEI) complexes [111,119]. Particularly, the very positive behaviour of these nanoparticles was partially attributed to a specific interaction with the CD44 receptor, as the efficacy of the transfection was significantly reduced upon blocking of the receptor by means of either a monoclonal antibody or an excess of HA in solution [118], as can be easily appreciated in **Figure 3**.

Considering these encouraging achievements, further studies aimed to study the nanoparticles' interaction with the ocular surface. Fluorescent nanoparticles show a great capacity to enter the corneal and conjunctival epithelium in a very effective way upon instillation onto the ocular surface of rabbits. Moreover, the visualisation of specimens of the cornea under the confocal microscope, at different time points after instillation, revealed a progressive decrease in the fluorescence intensity, thus indicating that somehow these nanoparticles are bioassimilated by the ocular tissue in about 12h. It was also observed that the rate of nanoparticles bioassimilation depended on the molecular weight of chitosan, as the fluorescence decayed faster for the systems HA/CSO [120]. Based on these results, the accumulation of the nanocarriers in the ocular structures is not expected. In addition, the examination of the ocular structures as well as the ocular surface function, after acute administration of the nanoparticles, shows no signs of damage, irritability or dysfunction related to the nanocarriers [121].

After topical instillation of pEGFP-loaded HA/CSO nanoparticles onto the ocular mucosa of rabbits, expression of the green fluorescent protein was detected in the corneal epithelium for up to one week, in contrast to the lack of expression observed with the naked plasmid DNA. With respect to the conjunctiva, a second model plasmid was used, due to the autofluorescence associated with this tissue. The results show expression of the reporter protein, β-galactosidase, after topical instillation of pβgal-loaded HA/CSO nanoparticles [120].

Overall these results show the positive behaviour of HA/CS nanoparticles in terms of transfection efficiency and open up new possibilities in the therapeutic application of nucleic acids in ophthalmology. Several ocular disorders would benefit from this therapy, as for example dry-eye syndrome, a common pathology affecting more than 11.0% of the general adult population [122]. In view of the mucin involvement in dry-eye conditions, the transfection of the conjunctiva with nanoparticles encapsulating a plasmid coding for mucin could restore a normal mucin layer over the ocular surface, thus making possible its proper wetting [123]. As a matter of fact, in the last years the activity within the field has exponentially increased, and consequently important advances have been made [124-126]. Despite this, the delivery of genes to the ocular structures is severely limited by several constraints that must be solved before gene therapy becomes a reality in clinical practice. First of all, the nucleic acids are degraded due to the presence of endonucleases in the lachrymal fluid, mucosa and in the extracellular matrix. Secondly, the genetic material should be able to interact with the biological membranes, and further internalised by the cells of interest. Moreover in the case of plasmid DNA, there is an additional barrier represented by the access to the cell nucleus for transcription [127-130]. All these processes – internalisation, distribution and metabolism of the administered nucleic acid – are key parameters that must be considered for the rational design of carriers able to promote the transport of genes to the ocular structures. Even if we are conscious that the potential of CS nanoparticles in ocular gene therapy remains to be explored and that more studies are needed in the field, the preliminary results obtained with HA/CS nanoparticles as pDNA delivery systems hold great promise.

A great deal of attention has been directed to the preparation of hybrid colloidal carriers obtained from polysaccharides and lipids. This approach does not require any chemical modification of chitosan, as discussed in section 4.4, for self-assembled CS nanoparticles, but only the rational combination of hydrophilic and hydrophobic components, leading to the formulation of nanocarriers with new and interesting properties. This approach has been successfully used for the preparation of nanocomplexes between preformed CS nanoparticles and liposomes (LCS-NP). They were evaluated as carriers to the ocular surface, both *in vitro* and *in vivo* [131]. Uptake experiments in human conjunctival cells and primary cultures of the conjunctival epithelium show the internalisation of the nanocomplexes, as indicated by the fluorescent signal clearly identified inside the cells. This internalisation could not be attributed to a disruption of cellular membranes because the LCS-NP complexes did

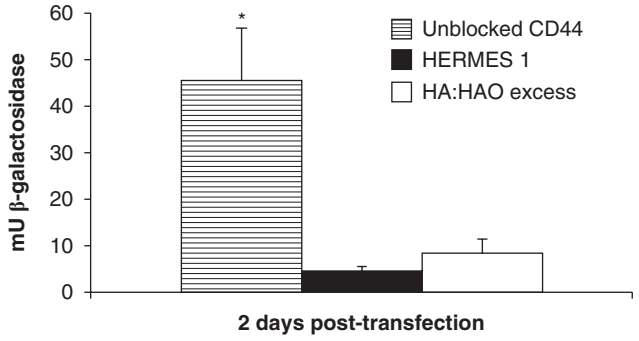


**Figure 3. Transfection efficiency levels measured after transfection of human corneal epithelial (HCE) cells with plasmid DNA-loaded HA/CSO nanoparticles.** The hyaluronan (HA) receptor CD44 was unblocked, or previously blocked with either the monoclonal antibody Hermes-1 or an excess of a mixture of HA of different molecular weights (HA:HAO). The expressed β-galactosidase was quantified by the ONPG reaction (mean ± SD, $n = 3$).

*: significant difference, $p < 0.05$.

Adapted with permission from [118].



not cause significant changes in neither cell viability nor in cell morphology. In fact, after exposure to LCS-NP, the cell viability was even higher than that observed after exposure to CS nanoparticles. Regarding the primary cultures of the conjunctival epithelium, the internalisation of the different LCS-NP was apparently delayed by the presence of mucus to a degree strictly dependent on the composition of the liposomes. This finding may be of great interest in the design of specific carriers capable of modulating the retention time of a particular encapsulated drug at the ocular surface. With respect to the *in vivo* tolerance of LCS-NP formulations, no alterations in the ocular surface structures of rabbit were observed. The clinical macroscopic sign score was compatible with a non-irritated ocular surface; moreover, no histological alterations were observed and there was absence of abnormal inflammatory cells in cornea, conjunctiva and lids. Consequently, all these observations led to the conclusion that LCS-NP were non-irritating for the ocular surface and account for a high biocompatibility of the system.

### 4.4 Self-assembled chitosan nanoparticles

Nanostructures based on synthetic derivatives of CS have been explored in the field of drug delivery. Modifications in the CS structure are expected to improve the efficacy of the nanoparticles. For example, the attachment of hydrophobic moieties is intended to increase the loading of poorly water soluble compounds. Moreover, it has been hypothesised that the modification of CS with lipids could favour the interaction of the carriers with cell membranes [61].

Polymeric amphiphiles, consisting of hydrophilic and hydrophobic segments (that is glycol, oleoyl or stearic acid residues linked to chitosan), have received increasing attention in the field of drug delivery because they can form self-assembled nanoparticles with unique physicochemical characteristics, in terms of structure and thermodynamic properties [132]. However, only a few reports address the use of self-assembled systems of chitosan as drug carriers for ophthalmic therapy. In this sense, amphiphilic nanoparticles were prepared upon covalent attachment of cholesterol to CS [133]. These self-assembled nanoparticles demonstrated to be effective carriers for the encapsulation of the hydrophobic polypeptide CyA. Eventually, the ocular distribution of fluorescent and radio-labelled CS-CH nanoparticles was investigated by single photon emission computed tomography (SPECT) and scintillation counter. The CS-CH nanoparticles showed a good spread over the entire precorneal area immediately after the topical administration and a residence time of 2h on the precorneal area, whereas no permeation of the nanoparticles into the posterior segment of the eye was observed, as indicated by the scintillation counter measurements. According to previous data of unmodified CS nanoparticles [53], radioactivity in the aqueous humour and blood was barely detected.

Another interesting system was obtained after the attachment of palmitoyl groups to a quaternised chitosan [134]. This modification led to the formation of self-assembled polymeric micelles, quaternary ammonium palmitoyl glycol chitosan (GCPQ) micelles, due to the ability of the polymer to form multiple inter- and intra-polymer hydrophobic and hydrogen bond association. The formation of hydrophobic domains made possible the encapsulation of low water soluble drugs, as for example, prednisolone. These nanoparticles were topically administered to rabbits, and the vitreous and aqueous humour collected for the evaluation of drug levels. The results corroborated the efficacy of the nanoparticles, as drug levels in aqueous humour were similar to those found with a 10-fold higher dose of a commercially available prednisolone suspension (**Figure 4**). In contrast, undetectable levels were found in the vitreous in both cases.

## 5. Conclusion

All the information reported to date has disclosed the great potential that chitosan offers in improving the treatment of ocular diseases. Thus, it can be accepted that the use of chitosan nanoparticles in ophthalmic pharmaceutical formulations is an attractive area, offering great potential to overcome the inherent difficulties associated with ocular drug delivery.

## 6. Expert opinion

As described above, significant improvements in ophthalmic formulations intended to increase the retention and residence time of drugs at the ocular surface have been made. A

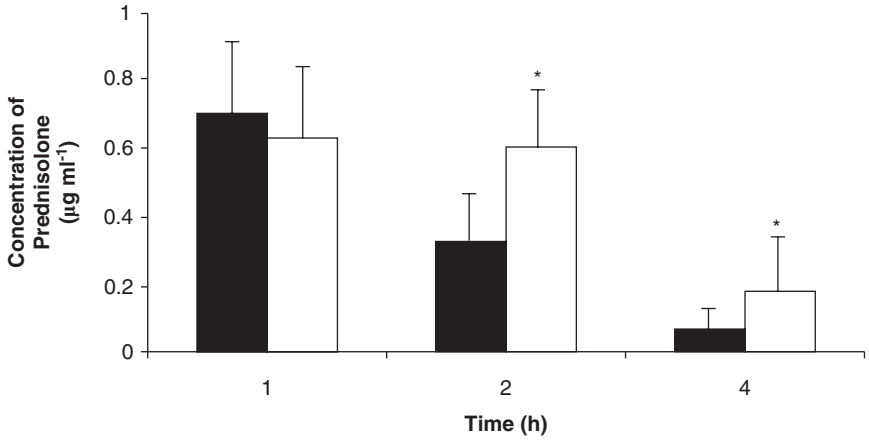


**Figure 4. Prednisolone concentrations found in the aqueous humour of rabbits at 1, 2 and 4 h after topical administration of 35 μl of prednisolone in suspension (Prednisolon Forte, Allergan) at a concentration of 10 mg/ml (white bars), or prednisolone-loaded GCPQ micelles at a concentration of 1 mg/ml (black bars) (mean ± SD, *n* = 4).**

*: significant difference, $p < 0.05$.

Adapted with permission from [134].



greater unmet need, though, is the necessity of new vehicles for the selective delivery of drugs to the different ocular structures after topical administration. The arguments in favour of the potential of nanosystems are strong, however significant formulation improvements will have to be made in order to overcome the low clinical impact achieved using conventional nanoparticles. Within this framework, CS-based nanoparticles have emerged in the last few years as a challenging alternative to drug delivery systems previously suggested for topical ocular administration. The rationale for designing these systems has been to combine the potential of nanoparticles as ocular drug carriers with the mucoadhesive and permeability-enhancing properties of chitosan. The different approaches derived from such rationale have been possible thanks to parallel progress in the engineering of colloidal systems with perfectly controlled characteristics, which have conveniently exploited the cationic nature of chitosan for the development of nanoparticulate drug and gene delivery systems. With respect to the latter point, besides its great potential, gene therapy has to date failed to deliver on its promise and remains a very ambitious goal. We are still far from the perfect gene carrier suitable for clinical use and much more work is needed for effective gene transfer *in vivo*, as the biological systems present numerous barriers that have to be overcome for successful gene therapy. However, rapid improvements in the understanding and use of CS-based delivery nanotechnologies could reverse this situation, making possible the effective delivery of gene mainly at an ocular level, where gene therapy is much more affordable than at a systemic level, and where we can gain important knowledge to be applied in the more ambitious systemic application of gene medicines.

In spite of the great interest of the different CS-based nanostructures described in this chapter, their potential therapeutic application and final relevance in clinical use could be hampered by undesirable effects arising from the specific nature of chitosan. Indeed, we should keep in mind cytotoxicity and immunostimulant effects associated with its use, even if the information on its biocompatibility is still insufficient. Considering the aforementioned concerns and being aware of the importance of composition in the design of safe drug carriers, the ionic gelation technique has been conveniently adapted in order to develop nanostructures in which chitosan is combined with biomaterials well known for their safety. Cyclodextrins, alginate and hyaluronic acid are examples of synthetic and natural products which have led to a new generation of drug delivery systems when combined with chitosan. The final composition and surface characteristics of these new systems can be controlled by adjusting the formulation conditions and by judicious selection of the components. The control of such characteristics provides unique opportunities to modulate the *in vivo* performance of these composed nanostructures while still benefiting from the potential of chitosan. In comparison with conventional nanoparticles made of chitosan, the introduction of additional ingredients in the nanoparticles formulation increases their versatility and their opportunities to interact with biological surfaces.

These new nanoparticulate compositions will certainly further alter the landscape of drug delivery and contribute towards the development of more efficient therapeutic systems. Concretely, it could be anticipated that the judicious choice of the biopolymeric components, as well as the ability to control the structural architecture of the new nanosystems, will help in the future design of more sophisticated nanocarriers

intended for very definite purposes. Nevertheless, the development of the 'magic bullet' concept at ocular level will require a more advanced comprehension of the biological disorders causing ocular diseases and the therapeutic targets, as well as a more extensive understanding of the parameters affecting the interaction of nanoparticulate carriers with the ocular structures.

## Declaration of interest

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