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Current Pharmaceutical Design, 2015, *21*, 000-000

Polysaccharide-Based Nanocarriers for Ocular Drug Delivery

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Abstract: Obtaining successful ocular formulations able to support an efficient drug concentration at the target tissue for an appropriate period of time is an interesting challenge for modern pharmaceutical technology. In this sense, nanotechnology is one of the available strategies to obtain a drug carrier system that allows access to different compartments of the eye in order to deliver drugs to the desired site. Biodegradable polymers such as polysaccharides are promising biomaterials for the production of biocompatible and biodegradable nanocarriers (NCs). Different types of polysaccharide NCs are capable of improving the transport of drugs after ocular application and they can be either polysaccharide-matrix carriers or polysaccharide-coated carriers, depending on whether polysaccharide is used as a matrix or as a coating, respectively. This review focuses on recent advances achieved by polysaccharide-based NCs for the treatment of ocular disorders.

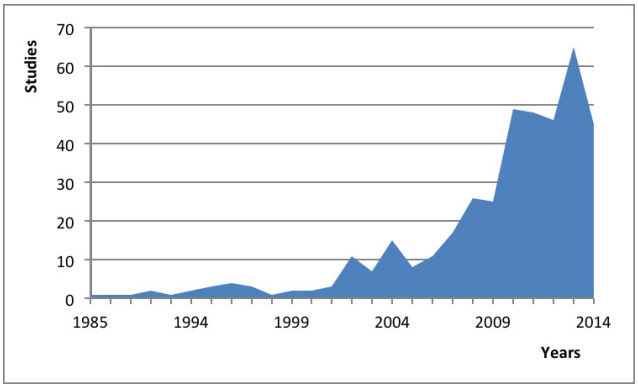
Keywords: Gene therapy, nanocarriers, nanoparticles, ocular barriers, ocular drug delivery, polysaccharides.

1. INTRODUCTION

The purpose of ocular therapeutics is to achieve a desired pharmacological response by maintaining an effective drug concentration at the site of action over an appropriate period of time, without altering the functions of the eye [1]. Tear production, nonproductive absorption and low permeability of the corneal epithelium are related to a transitory residence time on the epithelium with a fast elimination of therapeutic delivery systems resulting in poor bioavailability [2].

Several strategies have been developed for ocular treatments to overcome barriers related to the eye [3]. Increasing the ocular residence time of the drug instilled in the eye is one of the main aims of therapeutic ophthalmology, so ocular delivery systems that act as a reservoir are necessary in order to prolong the residence time of the drug at the site of administration as well as improving the drug release to optimize the therapeutic scheme of administration. An "ideal" model drug delivery system should be biodegradable, biocompatible and supply constant amounts of drugs over a specified period of time. These systems must remain stable over the ocular surface and be free of toxic side effects, and also need to have a long shelf life and be easy to produce and administrate [2, 4].

A good design of an ophthalmic drug delivery system requires extensive knowledge of the therapeutic molecules and the characteristics of ocular barriers [2, 5]. However, obtaining effective topical dosage forms capable of promoting drug penetration and maintaining therapeutic levels with a reasonable frequency of application [6] is a difficult task for modern pharmaceutical technology. Currently, one of the strategies to produce drug carriers systems that allow anatomical barriers to be overcome and drugs be delivered to the desired site as well as minimizing systemic exposure and adverse effects, is the use of nanotechnology. This discipline has become increasingly important impact in ocular therapies [7-9],with the different types of NCs providing interesting tools for ophthalmic drug delivery because they offer the possibility of controlling drug delivery [1]. Consequently, there has been marked increase in the number of articles published about NCs as ocular delivery systems in recent years (Graphic **1**).



Graphic 1. Published studies about NCs as ocular delivery systems by year.

Topical ocular drug delivery may benefit from the particularities of NCs, which can protect encapsulated molecules from environment problems (chemicals and physical drawbacks), improve their transport to the different compartments of the eye [10], optimize tolerance, enhance corneal uptake and increase intraocular concentrations [1, 11]. Moreover, NCs are less inflammatory than regular formulations and do not produce irritable side effects which are related to their small size (approximately 100 nm) [7, 12].

The development of novel nanodevices with a better precorneal retention to improve ocular bioavailability requires extensive research [12, 13]. There is a fine line between nanoparticles (NPs) and NCs. In general terms, NPs are particles varying in size from 10 to 1000 nm that may or may not contain an active molecule depending on the end use, while NCs are NPs designed to transport-

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1381-6128/15 $58.00+.00

ing of active molecules to achieve therapeutic effect [14, 15]. The inherent advantages of nanopharmaceutical systems are related to their colloidal nature [1, 16]. The drug may be attached to a nanoparticle matrix, or dissolved, encapsulated and entrapped, which has led to different terminologies such as NPs, nanospheres or nanocapsules [17]. Additionally, NPs provide excellent tissue penetration persistence, more targeted dosing and longer intervals between doses, which are beneficial to patients [1, 16]. In the article context, NPs will be referred as NCs, being that they will be related to drug delivery systems.

This review focuses on recent developments in topical ocular polysaccharide NCs as drug delivery systems to increase the bioavailability of ophthalmic drugs. The different types of polysaccharide NCs and their contributions to the improvement of the residence time, bioavailability, as well as their ability to transfect cells and inhibit the expression of genes, will be explored.

2. OCULAR BARRIERS TO DRUG DELIVERY

Topical instillation of a drug is the first choice for treatment of ocular diseases, due to the fact that this represents a non-invasive pathway, even when the target tissues are in the posterior segment of the eye. However, the anatomic, physiologic and biochemical barriers protect the eye against the entry of foreign compounds, thereby hindering drug delivery to the desired site. In fact, typically less than 5% of the topically applied drug penetrates the cornea and reaches the intraocular tissues, while a major fraction of the instilled dose is often absorbed systemically via the conjunctival and nasolacrimal ducts [4, 5, 18].

The eye contains several highly different structures with specific physiological functions. The cornea is the most transparent anterior membrane of the eye and continues posteriorly as the sclera [8, 19]. This consists of five to seven layers that resist the passive diffusion of ions and molecules, and maintain the intraocular pressure, with the tight junctions around the epithelial cells complicating their crossing to the corneal stroma. The conjunctiva is a protective membrane that functions as a passive physical barrier, and the vasculature of the conjunctiva may absorb a substantial part of the drug that has passed the epithelium.

The mucus layer secreted by the goblet cells of the conjunctiva behaves as a cover of the corneal and conjunctival surfaces, forming part of the tear film. When the mucin is deployed over the surface, it makes the hydrophobic surface of the epithelium more hydrophilic and thus increases wettability. The lacrimal film has a multifunctional role, since it not only hydrates, nourishes, cleanses, lubricates and has an antibacterial function, but also provides an additional obstacle to drug penetration [19, 20].

The tear is spread over the surface of the eye during blinking. As it is a dynamic fluid that undergoes a constant renewal, it therefore limits the time of residence of the drugs on the surface of the eye. In addition to the physical barriers, ocular tissues contain metabolic enzymes, such as esterases, aldehyde and keton reductases [21], which may degrade and reduce the efficacy of drugs [5]. The bioavailability of an instilled compound is generally low due to anatomical barriers and physiological constraints and on the other hand, systemically administered drugs have poor access to the aqueous and vitreous humor.

3. POLYSACCHARIDES

Polysaccharides are polymers commonly found in nature and synthesized in large quantities by plants and microorganisms [22], with their molecules corresponding to long carbohydrates formed by joining numerous individual monosaccharide units covalently linked as α and β glycosides. Their molecular structure contains carbon, hydrogen, and oxygen, with the general formula $Cx(H_2O)y$, and occur in natural and synthetic forms [23]. Natural polysaccharides are present in plants as pectin and guar gum; in animals as chitosan (CS), hyaluronic acid (HA) and chondroitin; in microbial resources as dextran and xanthan gum and, in algae resources as alginate [3, 24].

Polysaccharides can be produced or recovered from renewable natural resources at a low cost, and are biodegradable, safe, non-toxic, hydrophilic, gel forming, highly stable and sometimes exhibit properties of biorecognition [3, 22, 24]. These polymers have a wide range of molecular weights, several reactive groups and a variable chemical composition. Many are also polyacids, polyalcohols and polyesters, but can occur as polyelectrolytes or non-polyelectrolytes, according to their chemical structure. Additionally, they can exhibit a positive or a negative charge or even be neutral [3, 23, 25, 26]. Polysaccharide derivatives may be obtained by the chemical and biochemical modification of derivable groups on molecular chains. An important group of these polymers may be bioadhesive, due to their ability to form non-covalent bonds among their hydrophilic groups, such as the hydroxyl, carboxyl and amino groups and biological tissues [3, 27].

The potential application of polysaccharides and their derivatives as nanocarrier drug delivery systems has been studied in recent years, as polysaccharide NCs with bioadhesive properties that may prolong the residence time and thus, to improve bioavailability [3]. In fact, many polysaccharides have been used in the development of ocular drug carrier systems, such as CS, HA, chondroitin sulfate (ChS), dextran sulfate (DS), heparan sulfate (HS), pullulan, xyloglucan (XG) and sodium alginate (SA), with the chemical structures of these polysaccharides being shown in Fig. (**1**).

CS and HA are the most widely used polysaccharides for the development of ocular NCs, and many research articles have been published on polysaccharide-based NCs containing these polymers.

3.1. Chitosan

CS and its derivatives are natural polysaccharides derived from chitin. This polymer, a polycationic polymer which contains one amino group and two hydroxyl groups in repeating glucosidic residues, is insoluble in either water or organic solvents but soluble in aqueous dilute acids [28-30]. CS is a useful biomaterial for the production of ocular NCs, due to its biocompatible and biodegradable properties not presenting any risk of accumulation/retention in the body [30, 31]. CS is considered to be a mucoadhesive cationic polymer as a result of the ability of its positively charged amino groups to develop molecular attraction forces by electrostatic interactions with the negative charges of the mucous layer (mucin) [31,32]. This interaction is determined by the formation of either hydrogen bonds or ionic interactions between the positively charged amino groups of CS and negatively charged sialic acid residues of mucin, depending on the environmental pH [33]. CS possesses a crystalline structure due to the inter- and intramolecular hydrogen bonds between the -OH and $-NH_2$ groups with the charge density of CS molecules depending on the degree of acetylation and the pH of the solution [28]. CS is a cationic polysaccharide capable of forming gel in contact with specific multivalent polyanions, such as sodium tripolyphosphate (TPP) [9]. In addition, it is able to penetrate the tight junctions between epithelial cells and is considered to be an antimicrobial and bacteriostatic polymer, due to the interaction of its polycationic functional groups with cell membranes resulting in the enhancement of membranous permeability [31]. CS is rapidly degraded by lysozyme, which is highly concentrated in mucosal surfaces and, in particular, in the ocular mucosa. It can be part of a matrix of NPs or be used to cover them and forming CS-based NPs that allow the association of different types of active compounds and they present a great versatility for the incorporation of other molecules within the nanomatrix structure, such as cyclodextrins, HA and alginate [9]. Furthermore, the size and surface charge of CS-based NPs can be modulated to favor interaction between the NPs and the corneal and conjunctival epithelium.

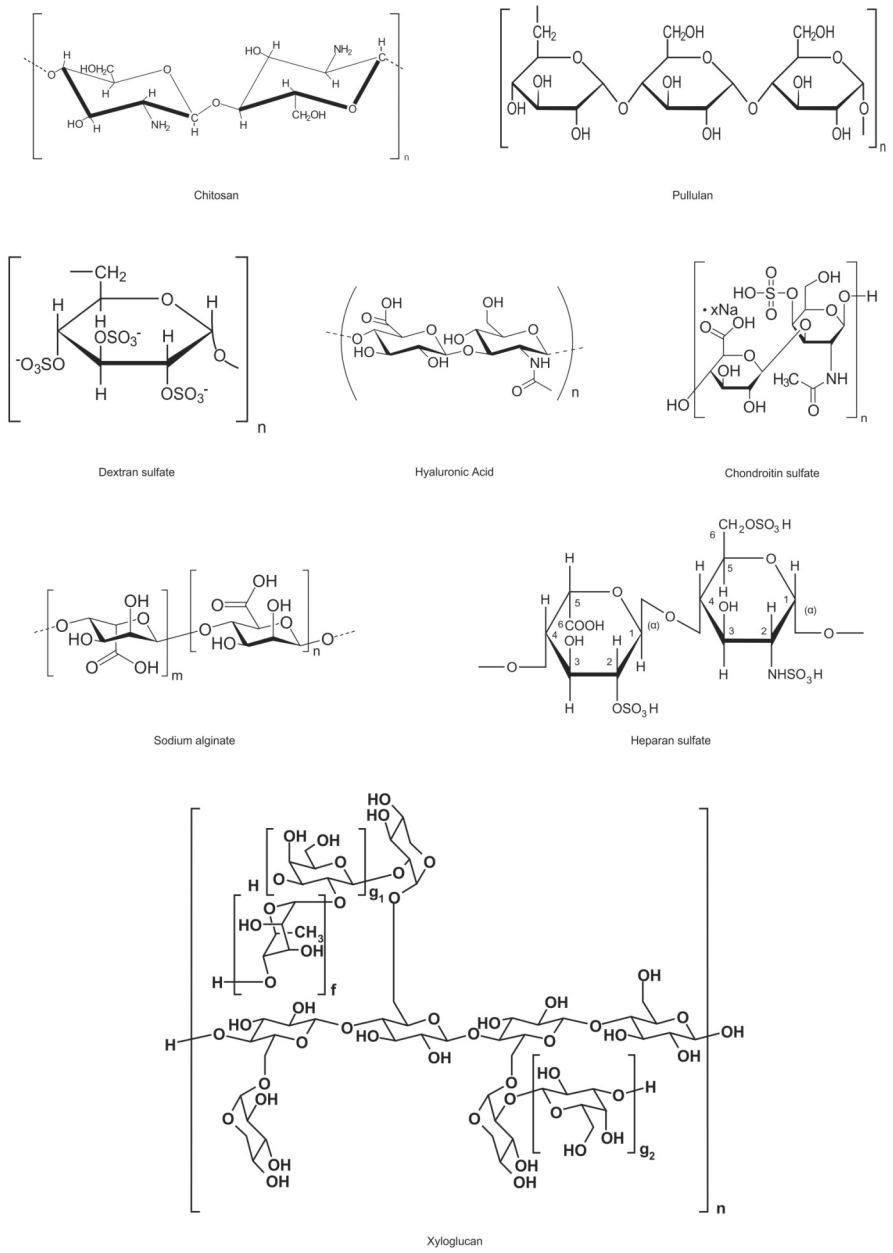

Fig. (1). Chemical structures of polysaccharides used in the design of ocular NCs.

3.2. Hyaluronic Acid

HA or hyaluronan, is a high molecular weight linear glycosaminoglycan composed of repeating disaccharide units of β-1,4-D-glucuronic acid β-1,3-N-acetyl-D-glucosamine [34]. It is an anionic biocompatible, biodegradable, mucoadhesive, non-immuno-genic biopolymer, which is ubiquitously present in mammalian organisms and has significant water binding capacity [9, 35, 36]. HA is an important component of the extracellular matrix, which specifically binds to several plasma membrane receptors [34, 37]. In this way, HA plays an important role in the stabilization and organization of the extracellular matrix and in the regulation of many biological processes, such as tissue hydration, cell adhesion, motility, proliferation and differentiation [38], cell behaviour, tissue repair, several biological processes including cell signalling [39] and in a wide variety of other cellular functions [35, 40]. HA is capable of promoting the adhesion and proliferation in mammalian cells by interacting with the CD44 receptor, which is expressed in human cornea and conjunctive, and thereby facilitates cell internalization of different systems [38, 41]. In addition, HA is capable of entering the cell nucleus and activating transcription [39]. It can also prevent opsonin adsorption by steric repulsion, thus reducing mononuclear phagocyte system uptake [42]. Many investigations have reported HA to be a very interesting polysaccharide, due to its excellent features being encouraging for its application in ocular drug delivery [43-47].

3.3. Chondroitin Sulfate, Dextran Sulfate and Heparan Sulfate

ChS is a polymeric carbohydrate comprising alternating disaccharide units of glucuronic acid/iduronic acid and N-acetyl-galactosamine linked by β-(1→3) glycosidic bonds and sulfated in different hydroxyls [48]. It is a major component of the extracellular matrix of many connective tissues, including cartilage, bone,

skin, ligaments and tendons [49], and is important in maintaining the structural integrity of various tissues and organs, including the eye [50]. ChS is widespread on cell surfaces and within extra/pericellular matrices in the form of proteoglycans, where at least one ChS side chain is covalently attached to a panel of core proteins [51]. It can form a wide range of structures by incorporating different disaccharide repeat units, or by being modified with different numbers/positions of sulfate groups at C-4 and/or C-6 in N-acetylgalactosamine and at C-2 and/or C-3 in glucuronic acid or uronic acid [48].

DS is a biodegradable, biocompatible and semi-synthetic sulfated-polysaccharide composed of major α 1-6 linkages and a minor α 1-3 linkage of sulfated glucose of a branched structure, and is used in the biomedical field among other applications. Both DS and ChS have anticoagulant properties and furthermore, ChS may prevent vein hardening and DS reduce triacylglycerol in plasma [52]. In fact, these polymers have been considered to be very useful in the design of drug delivery systems and also as candidates for hybrid systems [50].

HS is a linear polysaccharide composed of N-acetylated or N-sulfonated glucosamine units and uronic acids. It is a member of the glycosaminoglycan family of polysaccharide [53], and it is present on the cell surfaces and in the extracellular matrix of all animal tissues as a proteoglycan, where the polysaccharide chains are covalently attached to the core proteins. HS can participate in several biological processes including the development, tissue homeostasis, cell adhesion, angiogenesis and inflammation, because it can interact with growth factors, proteases cytokines, and chemokines among other molecules. Furthermore, HS polysaccharides are capable of regulating the immune response through from hematopoiesis, the homing of leukocytes to peripheral tissues and controlling their the elicitation by blocking proinflammatory cytokines, suppressing activation of complements, and inhibiting migration and adhesion of immune cells through their interactions with chemokines [54].

3.4. Xyloglucan, Sodium Alginate and Pullulan

Xyloglucans are branched polysaccharides widely occurring in the primary cell wall of higher plants and in the higher plant seeds with tamarind being the most common plant with XG [22]. XG are 4-linked β-d-glucan backbone, substituted at position O-6 by branches of α-d-xylose or of β-d-galactose-1→2-α-d-xylose disaccharide [55, 56]. They can be biocompatible, mucoadhesive, non-carcinogenic, and they have high viscosity, drug holding capacity and thermal stability [22, 57, 58].

Sodium alginate (SA) is a linear chained anionic polysaccharide composed of l-4 linked/β-D-mannuronic acid (M) and its C-5-epimer α-L-guluronic acid, organized in blocks of consecutive G-residues (G blocks), consecutive M-residues (M-blocks), or alternating M and G (MG-blocks) [59]. The saccharide units of the alginate chain, such as mannuronic and guluronic acid residues, exist in different conformations. SA is a chain polysaccharide having several properties including biocompatibility, non-toxicity and biodegradability, which make it attractive for ocular delivery systems either alone or in combination with other materials. Moreover, SA has a low cost, is easily available and is water soluble [60-62].

Pullulan (PL) is a natural homopolysaccharide produced from Aureobasidium pullulans. The characteristic dimeric segments of pullulan are [→x)-α-d-glucopyranosyl-(1→4)-α-d-glucopyranosyl -(1→] and [→4)-α-d-glucopyranosyl-(1→6)-α-d-glucopyranosyl-(1→], where x may be either 4 or 6 for the (1→4)-linked segment [63]. It is a water-soluble, biodegradable non-toxic, non-immunogenic, non-mutagenic and non-carcinogenic natural polysaccharide [26, 64].

4. POLYSACCHARIDE-BASED NANOCARRIERS

NCs are nanoparticulate systems (nanosystems) used for drug delivery and they can be liposomes, micelles, nanoemulsions, polymeric nanoparticles, and solid lipid particles among others [14, 15]. These drug delivery NPs consist of various safe materials, including synthetic biodegradable polymers, natural biopolymers, lipids and polysaccharides [65], in which the drug is dissolved, entrapped, encapsulated or attached to the surface. They are submicron colloidal particles which present a high surface area and energy, Brownian motion in liquid media and a long shelf-life [16, 17, 66]. NPs may be composed of natural polymers such as CS, HA, gelatine and SA or synthetic biodegradable polymers, including ε-caprolactone, polylactides, methylmethacrylate, among others. They can be obtained by different techniques involving solvent evaporation, spontaneous emulsification/solvent diffusion, salting out/emulsification-diffusion, spray-drying method, ionotropic gelation, and desolvation [18].

Different types of NCs with polysaccharides have shown promising results in ophthalmic drug delivery in recent years. Depending on whether the polysaccharide is in the form of a matrix or a coating in the NCs, it can be a polysaccharide-matrix nanocarrier or a polysaccharide-coated nanocarrier, respectively [9, 65].

Polysaccharide-based NCs have shown to successfully deliver several hydrophilic and hydrophobic drugs and biomacromolecules. The rationale behind the incorporation of polysaccharides into nanosystems mainly relies on promoting the interaction between the NCs and mucus, in order to prolong the residence time on the eye surface.

The following sub-sections will present the features and, latest advances related to polysaccharide-based NCs in ocular delivery applications with the outstanding characteristics and results being included in Table **1**, which has been ordered in the order of description of these NCs over article.

4.1. Polysaccharide-Matrix Nanocarriers

In this type of nanocarrier, one or more polysaccharides are found forming part of internal structure of the nanoparticle [9]. The ratios for each nanoparticle component should be evaluated in order to obtain optimal physicochemical properties that favor interaction with the target tissue. Drug release and degradation of the NPs are considerably influenced by size, the morphology and physical state of the encapsulated drug, the crystallinity of the polymer and the drug loading capacity of system. The surface charge of NPs determines the performance of the nanoparticle system in the body, which can be measured by the zeta potential. In the topical ocular application, positively charged NPs can interact with the negative charges of the mucin, and thus increase residence time and enhance permeation of these NPs across the cornea or conjunctiva [1, 5, 22].

Tropicamide-loaded tamarind seed XG nanoaggregates were prepared and optimized by Dilbaghi *et al.* (2013) [58], where the optimization of component ratios was carried out by applying a face-centred central composite experimental design, using the concentrations of tamarind seed XG and Poloxamer-407 as independent variables. The optimal formulation found was 0.45 % (w/v) of tamarind seed XG and 0.5 % (w/v) of poloxamer, with the results also showing that the presence of poloxamer significantly increases the encapsulation efficiency, while an increase in the tamarind seed XG concentration resulted in larger particle size. Moreover, the corneal permeation studies of tropicamide across the isolated goat cornea revealed that a higher amount of drug permeated from the nanosuspension formulation in comparison to conventional commercial aqueous formulation. As the mucin was adsorbed in 87.35 % of the surface of the tamarind seed XG NPs by a mucin glycoprotein assay, this demonstrated the excellent mucoadhesive properties of these NPs. Furthermore, nanotoxicological studies showed that the tropicamide-loaded tamarind seed XG nanoaggregates were less toxic and non-irritant.

In another study, positively charged controlled-release polymeric NPs of terbinafine hydrochloride were developed by Tayel

Table 1. Outstanding characteristics and results of polysaccharide-based NCs for ocular drug delivery.

N°	Active molecule	Polysaccharides	NC system	Components	Results in ocular delivery	Method	Authors	Year	Refs.
1	Tropicamide	XG	Nanoaggregates	Poloxamer-407	Increased drug permeation	Nanoprecipitation	Dilbaghi *et al.*	2013	[58]
2	Terbinafine hydrochloride	CS	NPs	Eudragit® RS100, and Miglyol® 812, soybean lecithin and Pluronic® F68	Increased drug release	Nanoprecipitation	Tayel *et al.*	2013	[67]
3	Cyclosporine A	CS	NPs	Acetic acid and ethanol	Enhanced ocular penetration and prolonged drug delivery	Spray-drying	Başaran *et al.*	2013	[31]
4	Levofloxacin	CS	NPs *in situ* gel	Poly(lactic-co-glycolic acid), Polyvinyl alcohol	Increased drug retention on ocular surface and prolonged drug release	Nanoprecipitation	Gupta *et al.*	2013	[69]
5	Rhodamine B and Nile Red	CS, DS	Self-assembled NPs	Polyethylene glycol-400	Enhanced mucoadhesiveness	Self- assembly/ polyelectrolyte complexation	Chaiyasan *et al.*	2013	[71]
6	Amphotericin B	CS	Self-assembled NPs	Poly(lactic acid)	Decreased drug clearance, prolonged drug residence time on the ocular surface, sustained drug release	Dialysys/Protection-graft-deprotection	Zhou *et al.*	2013	[72]
7	Chloramphenicol, norfloxacin, pilocarpine hydrochloride, atropine sulfate	CS	Hybrid NPs	Acrylic acid, N-isopropylacrylamide, 2-hydroxyethyl methacrylate	Sustained drug release	Radical-induced co-polymerization	Barbu *et al.*	2009	[11]
8	Rhodamine	CS	Hybrid NPs	Poly vinyl/ Poly(lactic-co-glycolic acid)	Sustained drug release	Modified ionotropic gelation	Jain *et al.*	2011	[76]
9	Gatifloxacin	CS, SA	Hybrid NPs	Pluronic F127	Sustained drug release	Modified coacervation/ionotropic gelation	Motwani *et al.*	2007	[66]
10	Timolol maleate, dorzolamide hydrochloride	CS, HA	Hybrid NPs	TPP	Enhanced mucoadhesiveness, increased drug permeation, increased pharmacological effect	Modified ionotropic gelation	Wadhwa *et al.*	2010	[45]
11	Econazole nitrate	CS	Hybrid NPs	Sulfobutylether-β cyclodextrin	Sustained drug release, enhanced pharmacological effect	Ionotropic gelation	Mahmoud *et al.*	2011	[77]
12	Peptide serine-threonine-tyrosine	CS	Hybrid NPs	TPP	Enhanced pharmacological effect	Ionotropic gelation	Jayaraman *et al.*	2012	[7]
13	Acyclovir	CS	Hybrid NPs	TPP	Prolonged drug release	Ionotropic gelation	Calderón *et al.*	2013	[78]
14	Carteolol	CS	Hybrid NPs	TPP	Excellent mucoadhesiviness, increased drug permeation, enhanced residence time at the ocular surface, prolonged drug release, prolonged pharmacological effect	Ionotropic gelation	Ameeduzzafar *et al.*	2014	[74]

(Table 1) Contd….

N°	Active molecule	Polysaccharides	NC system	Components	Results in ocular delivery	Method	Authors	Year	Refs.
15	Gatifloxacin, prednisolone	HA	Coated NPs	Eudragit RS 100 and Eudragit RL 100	Prolonged residence time, sustained drug release, enhanced drug permeation	Spontaneous emulsification	Ibrahim *et al.*	2010	[81]
16	5-Fluorouracil	CS, SA	Coated NPs	-	Prolonged drug residence time, enhanced drug permeation, sustained drug release	Ionotropic gelation /cross-linking	Nagarwal *et al.*	2012	[82]
17	Cyclosporine A	HA	Coated NPs	Poly ε-caprolactone/ benzalkonium chloride	Enhanced corneal uptake of drug	Nanoprecipitation	Yenice *et al.*	2008	[46]
18	Red fluorescein	HA	Modified core-shell liponanoparticles	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), TPP	Enhanced intracellular uptake, targeted distribution and prolonged intraocular residence of drug	Ionotropic gelation	Gan *et al.*	2013	[34]
19	Indometacin	CS	Coated lipoidal nanosystem	Poly(e-caprolactone), DL-α-disteroylphosphatidyl-choline, DSPC, and L-α-distearoylphosphatidyle-thanolimine	Enhanced permeation, cell uptake and pharmacological activity	Emulsion	Du Toit *et al.*	2013	[1]
20	Coumarin-6	CS	Surface-modified lipid emulsions	Poloxamer 407, stearylamine	Prolonged residence time	High pressure homogenization	Ying *et al.*	2013	[88]
21	Natamycin	CS	Surface modified NPs	Lecithin	Enhanced residence time, decreased clearance, sustained release, high ocular availability of drug	Ionotropic gelation	Bhatta *et al.*	2012	[89]
22	Protamine	HA	Solid lipid NPs	Precirol® ATO 5, N-(1-(2,3-Dioleoyloxy) propyl)-N,N,N trimethyl ammonium, Tween® 80 methyl sulfate	Increased cell transfection	Solvent emulsification-evaporation	Apaolaza *et al.* (2014)	2014	[47]
23	DNA	DS, ChS	Hybrid NPs	Cationized gelatine with SPM	Protection against DNAse, enhanced cell transfection, decreased nanosystem toxicity	Ionotropic gelation	Konat Zorzi *et al.*	2011	[50]
24	plasmid MUC5AC	DS, ChS	Hybrid NPs	Cationized gelatine with SPM	Increased protein expression levels, effective internalization	Ionotropic gelation	Konat Zorzi *et al.*	2011	[98]
25	pDNA	HA, CS	Polysaccharides oligomer -based NPs	Polyamine SPM	Efficient internalization, higher transfection efficiency	Modified ionotropic gelation	Contreras-Ruiz *et al.*	2011	[92]
26	pDNA, siRNA	HA, ChS, DS, HS.	Hybrid NPs	Cationized gelatine with SPM	Targeted delivery, decreased toxicity, efficient release of genetic material, protection against ezymatic degradation	Ionically cross-linked	Parraga *et al.*	2014	[101]
27	DNA	Glycol CS	Non viral NPs	-	Protection against DNAse, targeted delivery	-	Mitra *et al.*	2014	[92]
28	pDNA	HA	Coated polyplexes	Cationic N,N′-cystaminebisacrylamide-4-aminobutanol	Efficient cell uptake and transfection.	Michael-type poly-addition of methoxy-polyetilenglycol amine	Martens *et al.*	2015	[35]

XG: Xyloglucan. CS: Chitosan. DS: Dextran sulfate. SA: sodium alginate. HA: Hyaluronic acid. ChS: Chondroitin sulfate. HP: Heparan sulfate. NPs: nanoparticles. SPM: spermine. TPP: Sodium tripolyphosphate.

et al. (2013) [67], with the aim of prolonging the contact time of the drug with the cornea and conjunctiva. Several drug-loaded formulations were fabricated by the nanopreciptation method using Eudragit® RS100 and CS as positive charge inducers and soybean lecithin and Pluronic® F68 as the emulsifiers. The NPs were prepared with different concentrations of components in order to determine the optimal ratios with results showing that the particle was influenced by the charge inducer type and the charge inducer/ Pluronic®F68 ratio. In addition, small NPs were obtained with a high amount of Pluronic® F68 (a fixed charge inducer ratio) both in the case of CS- and Eudragit® RS100-based NPs. According to these authors, this might have been attributed to the higher emulsification power associated with the use of a high amount of the surfactant. Lower surfactant concentrations might form larger droplets due to the amount of surfactant not being enough to cover the entire organic droplet surface. For this reason, droplets tend to aggregate until the amount of surfactant is able to coat the entire surface of the agglomerate. Larger NPs were obtained when the CS or Eudragit® RS100 ratio was increased at fixed drug concentrations, with the reason for this increase possibly being related to the repulsion between the positive charges of polymers and those of drug molecules. The zeta potential values of obtained NPs ranged between from +20.51 to +40.32 mV, and the interaction between positive charges of NPs and negative charges of sialic acid residues of mucous is known to prolong drug contact time [68]. With regard to the drug entrapment efficiency, this was influenced by the drug/polymer ratio. CS-based NPs showed a lower entrapment efficiency that the NPs prepared with Eudragit® RS 100, with the drug release percentages being dependent on both the drug/Pluronic® F68 and the drug/polymer ratios. Higher drug release percentages from both CS- and Eudragit® RS100- based NPs were observed when they were prepared at a drug/surfactant ratio of 1:3 and a drug/polymer ratio of 1:0.5.

The use of mucoadhesive polymers, which may interact intimately with negatively charged cornea and conjunctiva in the development of NPs, may increase the residence time and concentration of the associated drug at the application site [17, 18]. Drug loaded NPs constitute undoubtedly one of the most versatile drug delivery systems, being able to elude body defence mechanisms, overthrow biologic and physiological barriers and deliver the active molecules to specific cells/tissues or intracellular compartments either by passive or ligand-mediated targeting mechanisms [7, 17]. Polysaccharides based-NCs are developed with the aim of overcoming the drawbacks related with conventional ocular systems by enhancing drug penetrance, prolonging the residence time on the eye surface, increasing the ocular bioavailability of drugs and maintaining the activity at the site of action [1].

Başaran *et al.* (2013) [31] developed cyclosporine A loaded CS-based NPs by the spray-drying method. These NPs were prepared using three types of CS with different molecular weights for increased residence time at the corneal and conjunctival surfaces. As the obtained NPs from CS with medium molecular weight showed higher incorporation and encapsulation efficiencies of the drug and also more uniform release patterns in simulated tear fluid, compared to other formulations, it was selected for *in vivo* studies. An increase in the loaded amount of Cyclosporine A with 10 and 25 % of the polymer did not significantly modify the ocular penetration of drug. Nevertheless, the concentration of Cyclosporine A in both the aqueous and vitreous humour samples was higher after 72 h, indicating an enhanced ocular penetration and prolonged Cyclosporine A delivery for the CS-based NPs.

In another investigation, Gupta *et al*. (2013) [69] optimized levofloxacin loaded poly(lactic-co-glycolic acid)-based NPs to promote their ocular retention. These NPs, which are non-mucoadhesive in nature, were drug loaded in mucoadhesive CS *in situ* gel (NPs laden *in situ* gel). The ocular retention of NPs was evaluated by gamma scintigraphy in rabbits with the results revealing that CS *in situ* gel, poly(lactic-co-glycolic acid)-based NPs and NPs laden *in situ* gel were retained at the corneal surface for a longer time whereas a marketed formulation was cleared very rapidly from the corneal region and attained a systemic circulation through the via nasolachrymal drainage system. The NPs laden *in situ* gel remained for longer time on the eye with a more prolonged release in comparison with the other formulations. In addition, the time-activity curve of NPs laden *in situ* gel exhibited a minimal fall in radioactivity counts whereas the marketed formulation showed a fast fall.

In recent years, new techniques has been used to develop polysaccharides-based NPs. Self-assembled NPs and hybrid NPs have also been made in order to improve the properties of the polysaccharides-based NPs for an improved drug delivery.

4.1.1. Self-Assembled Nanoparticles

Self-assembled NPs are produced after modification of polysaccharide with hydrophobic moieties [9]. Amphiphilic compounds derived from hydrophilic and hydrophobic segments such as glycol, oleoyl or stearic acid residues linked to polysaccharide can form NPs when dispersed in water with a core-shell structure by self-assembly. These self-assembled NPs can be used as carriers for hydrophobic and hydrophilic drugs simultaneously due to their hydrophobic core and hydrophilic shell, with the latter able to greatly reduce macrophage phagocytosis [70]. Modifications in the polysaccharide structure can be made to improve the efficacy of the NPs. The linking of hydrophobic moieties in the polysaccharide structure increases the loading of poorly water-soluble compounds. Likewise, the modification of CS with lipids can favor the interaction of the carriers with cell membranes [9].

Chaiyasan *et al*. (2013) [71] developed NPs by self-assembly of oppositely charged CS and DS in order to enhance the resident time of drugs on the ocular surface. Positively charged spherical and segregated NPs were obtained using polyethylene glycol-400 as a surface stabilizing agent and Rhodamine B and Nile Red as drug analogues. The mucoadhesiviness of these NPs was revealed by imaging the retention of the fluorescein isothiocyanate-labeled CS-DS NPs on the cornea *ex vivo,* and showed retention on the cornea even after 60 min under fluid flow, while solutions of drugs and fluorescein isothiocyanate disappeared rapidly from the corneal surface. Additionally, these authors demonstrated that lysozyme did not affect the properties of NPs and that there were electrostatic interactions between the anionic groups of DS and cationic groups of CS.

Amphotericin B-loaded NPs based on poly(lactic acid)-grafted-CS developed by Zhou *et al*. (2013) [72] showed a strong mucoadhesive force. A decrease in the surface charge of NPs after incubation with mucin for all formulations of NPs with different poly(lactic acid)/Phthaloyl CS feed ratios was observed due to the ionic interaction between negatively charged sialic groups in mucin and the positively charged surface layer of these NPs. The obtained self-aggregated amphiphilic poly(lactic acid)-grafted-CS NPs had a core-shell structure with an average particle size of approximately 200 nm, zeta potentials higher than +30 mV and a high encapsulation efficiency of Amphotericin B in the hydrophobic core of the NPs. The amphotericin B-loaded NPs based on poly(lactic acid)-grafted-CS released their drug content more slowly than the amphotericin B solution and revealed a two-step release pattern. With respect to ocular pharmacokinetics, these were studied using amphotericin B from free amphotericin B solution and amphotericin B loaded self-aggregated amphiphilic poly(lactic acid)-grafted-CS NPs in the New Zealand White rabbits. The area under the concentration-time curve was significantly increased, while the maximum concentration decreased significantly for the amphotericin B loaded self-aggregated amphiphilic poly(lactic acid)-grafted-CS NPs in comparison to the free amphotericin B solution. Clearance was also decreased significantly, and the mean residence time was signifi-

cantly increased in the NPs compared with the free amphotericin B solution. Moreover, after instillation of the NPs, no signs of irritation or damaging effects to the ocular tissues in rabbit eyes were observed.

Self-assembled NPs offer the advantage of combining different properties of polysaccharides obtaining more efficient systems. Thus, desirable features like encapsulation for drugs with different solubility or mucoadhesiveness could be achieved at the same time.

4.1.2. Hybrid Nanoparticles

Hybrid NPs are a new generation of polymeric NPs based on the combination of different polymers in a manner that takes advantage of the useful properties of each polymer [50], and combines polysaccharides with other polysaccharides or with other biomaterials [9].

The ionic cross-linking is another widely used method for preparing NPs, which is generated by auto-aggregation between a polysaccharide and macromolecules of opposite charge, or when an ionic cross-linking agent exists. The most commonly used cross-linking agent is sodium TPP [73], with the ionotropic gelation technique being the method most used for the preparation of these NPs. In this technique the $-NH_2$ groups of CS are protonized to $-NH_3^+$ in acidic medium, which then interact with an anion moiety (the $-PO_4^{-2}$ group of TPP) by electrostatic interaction and form round and homogeneous NPs. On the other hand, covalent cross-linking involves mainly the formation of covalent bonds between the polysaccharide chains and a functional cross-linking agent [74, 75].

Hybrid NPs are non-toxic, biodegradable and favor interaction with mucin. They may allow active molecule to reach the site desired, control its release and achieve a pharmacological effect. For this type of nanoparticle, an assessment of the proportions of the each component of the NPs must be made in order to obtain optimal physicochemical properties. Various authors have observed a significant improvement in bioavailability and a more controlled release of several drugs administered with polysaccharide-based hybrid NPs compared to drug solutions, and also the influence of physicochemical features. Barbu *et al*. (2009) [11] developed nanoparticulate hybrid polymeric hydrogels of less than 70 nm diameter via radical-induced co-polymerization of acrylic acid-functionalized CS with either N-isopropylacrylamide or 2-hydroxyethyl methacrylate. The obtained NPs showed good loading capacities for the broad-spectrum antibacterials chloramphenicol and norfloxacin, and also for the anticholinergics pilocarpine hydrochloride and atropine sulfate. *In vitro* drug release studies demonstrated the potential suitability of these NPs for the delivery of ophthalmic drugs and identified formulations for each active. In addition, lysozyme induced degradation of NPs prepared from CS or from acrylic acid-modified CS with N-isopropylacrylamide or 2-hydroxyethyl methacrylate was found to be dependent on CS content, and N-isopropylacrylamide-based materials containing a low percentage of CS showed the most adhesiveness towards a model mucosal surface.

In another study carried out by Jain *et al.* (2011) [76], poly(lactic-co-glycolic acid)-CS nanoplexes were loaded with rhodamine and evaluated as ocular delivery systems. To develop these nanoplexes, the influence of several factors on the rhodamine-nanoplexes size, and encapsulation efficiency was studied. These were type of organic phase solvent, poly(lactic-co-glycolic acid concentration in the organic phase, CS and polyvinyl alcohol concentration in the aqueous phase and the aqueous phase pH. The optimized nanoplexes had a mean diameter of 115.6 nm, zeta potential of +32.5 mV, encapsulation drug efficiency of 59.4 %, and a sustained release of fluorescent rhodamine from nanoplexes over a period of 48 h. Also, data from *ex vivo* and *in vivo* studies showed that the amount of fluorescent rhodamine in the cornea was significantly higher for nanoplexes than for a control fluorescent rhodamine solution, with these quantities remaining fairly constant up to 24 h. This could have been due to an interaction of poly(lactic-co-glycolic acid) with the cornea by an adsorptive-mediated endocytosis, and also to an electrostatic interaction and mucoadhesion of CS with the corneal epithelium. Confocal microscopy of the corneas revealed that the uptake of the nanoplexes was by a paracellular route, due to movement between the cells through leaky tight junctions, and also by a transcellular route from movement across the plasma membranes of the cells. In addition a histopathological study on the goat and rabbit corneas confirmed the presence of normal ocular surface structures, with cells maintaining their normal morphology, in both control and treated eyes.

Motwani *et al*. (2007) [66] developed gatifloxacin-loaded submicroscopic nanoreservoir systems with CS and SA using a modified coacervation or ionotropic gelation method, by mixing the two aqueous phases at room temperature. In order to screen an appropriate concentration range, so as to allow the formation of turbid solutions but not the aggregates, a number of experiments were performed that varied the concentrations of CS, SA and gatifloxacin in the formulation using a 3-factor, 3-level Box-Behnken statistical design. The formulation selected as the optimal one (CS 0.22 %, SA 0.38 % and drug 0.05 %) had a 79.63 % encapsulation efficiency of gatifloxacin, a particle size of 347 nm and zeta potential of +38.6 mV. The release profiles of this selected formulation obtained in artificial tear fluid showed an initial burst release of about 10-12 % of gatifloxacin, followed by a more gradual and sustained release phase over the following 24 h, even after 24 h, about 5-7 % of the drug still remained in the NPs, regardless of the dissolution media used. In the contrast, the marketed conventional released almost all the drug immediately after start of the study.

HA-modified-CS NPs loaded with timolol maleate and dorzolamide hydrochloride were developed via an ionotropic gelation method employed by Wadhwa *et al.* (2010) [45]. The formulations of NPs were optimized first for different CS:TPP ratios and then for CS:HA ratios. Results showed that 3:1 was the optimum ratio for ocular delivery of timolol maleate and dorzolamide hydrochloride for a the small particle size (143.9 nm), while a 1:0.05 CS:HA ratio was suitable with a particle size of 319.5 nm, a positive zeta potential and entrapment efficiency of 85.1 % in case of dorzolamide hydrochloride and 19.3 % in case of timolol maleate, which are comparable with other internal polymer ratio reported. The *in vitro* release behaviours of dorzolamide hydrochloride from CS NPs and HA-modified-CS NPs over 24 h were 20.5 % and 22.1 %, respectively, while timolol maleate was almost completely released from both formulations within 1 h. HA-modified-CS NPs showed an increase in their mucoadhesiviness to 91.3 % as a result of secondary binding properties of HA and also had a higher transcorneal permeation than CS NPs or the marketed formulation. Additionally, HA-modified-CS NPs showed an improved efficiency of CS NPs, due to a more significant reduction in intraocular pressure level being obtained using HA-modified-CS NPs (with a peak effect at 4 h which remained for up to 12 h) compared to a plain solution of the drug, with a comparable higher reduction in intraocular pressure level was observed compared to CS NPs (with a peak effect at 4 h which remained for up 8 h). For the marketed formulation, the onset of action started within 1 h and the peak effect was obtained at 3 h. These results suggest that HA potentially enhances the mucoadhesiveness and efficiency of CS NPs and thus may be a promising carrier for ocular drug delivery.

Mahmoud *et al*. (2011) [77] also developed CS NPs, using ionic gelation technique, with sulfobutylether-β cyclodextrin as the polyanionic crosslinker and econazole nitrate as the model drug. The authors took advantage of the mucoadhesive cationic CS-based NCs and of the anionic sulfobutylether-β cyclodextrin to develop a one delivery system for the sustained delivery of econazole nitrate to the eye. The influence of the CS concentration, the CS molecular weight (150 kDa vs. 360 kDa), the CS:sulfobutylether-β cyclodextrin ratio and drug loading in the preparation of CS NPs were stud-

ied in order to optimize the formulation, with results revealing that an increased CS concentration as well as a higher sulfobutylether-β cyclodextrin concentration leading to an increase of particle diameters and to agglomeration of the produced particles. Furthermore, low molecular weight CS led to the formation of larger NPs than high molecular weight CS. All the resulting nanosystems ranged in diameter from 90.8 to 461.2 nm, with polydispersity index values between 0.16 and 0.64. Drug *in vitro* release studies of optimized CS/sulfobutylether-β cyclodextrin NPs showed that all the tested formulations of CS NPs exhibited similar controlled release profiles following a zero-order release model, with about 50 % of the drug load released in 8 h, while the econazole nitrate solution had a release profile with 100 % released within 1 h. *In vivo* studies showed that econazole nitrate loaded CS/sulfobutylether-β cyclodextrin NPs provided a greater antifungal effect than that of the drug solution in the rabbit eye. Furthermore, a reduction in the zeta potential values for CS NPs was observed when they were mixed with mucin, which may have been attributed to more NPs being available to interact with mucin. Indeed, according to the authors, these properties allowed the NPs to interact with the ocular mucosa for an extended period of time, thus providing an enhanced and controlled effect of the drug on the ocular surface of the rabbit eye. Finally, the satisfactory antifungal effect of econazole nitrate loaded CS/sulfobutylether-β cyclodextrin NPs and their sustained drug release and mucoadhesive properties that enabled them to interact with the ocular mucosa over a long period of time, make these nanocarrier systems promising for providing ocular drug delivery.

Following the above line, Jayaraman *et al.* (2012) [7] synthetized nanocarrier for retinal delivery of a nano CS-peptide by conjugating the signal peptide serine-threonine-tyrosine (ser-thr-tyr) to water-soluble low-molecular-weight CS by ionic gelation. These authors selected CS because of its excellent features such as its mucoadhesiveness, biocompatibility and antiangiogenic properties, with the latter being an important prerequisite for ocular delivery. A small increase in the diameter (from 150 to 200 nm) of the NPs was obtained for CS NPs when conjugated to the peptide, which may have been due to the rise in the polymer size resulting from the addition of the peptide to the CS backbone. The zeta potential decrease from +34 to +20 mV was due to many of the terminal free $-NH_2$ reacting to form amide bonds between CS and ser-thr-tyr, which lowered the zeta potential when CS was conjugated with the peptide. The technique proved effective in preventing dimerization by blocking the free $-NH_2$ groups in the serine moiety of the signalling protein. Ser-thr-tyr functions as a transduction signalling agent within and between retinal pigmented epithelium cells. Delivering tyrosine to the retinal pigmented epithelium with a carrier such as a nano CS-peptide may significantly enhance phagocytosis by stimulating tyrosine kinase enzyme MerTK, thereby initiating the internalization of photoreceptor outer segments. Once within a cell, ser-thr-tyr may be cleaved and phosphorylated, and the tyrosine can stimulate and activate MerTK. When tyrosine kinase activity was analysed using antiphosphotyrosine antibodies with confocal miscroscopy, the results revealed that after blocking the retinal pigmented epithelium cells with Lavendustin-A, and then fixing the cells with tyrosine antibodies, only the nano CS-peptide, and not nano CS or peptide alone, was able to cause kinase-integrin adherence, binding and engulfment. Therefore, nano CS-peptide can be considered to be a carrier capable of promoting and stimulating phagocytosis in the retinal pigmented epithelium.

The ionotropic gelation technique was also utilized used by Calderón *et al.* (2013) [78] to develop NPs and microparticles containing acyclovir, by using CS cross-linked with TPP. These results showed spherical NPs and some aggregates as a result of the interactions occurring between free amino and hydroxyl groups on the CS surface. The size of the NPs ranged from 30 to 300 nm and exhibited a positive zeta potential (+35.9 mV) due to the presence of amino groups of CS on the surface, which was significantly higher than in the microparticles. These zeta potential values suggest that the residence time of the CS particulate-systems are longer than those obtained with the solution. Moreover, microparticles presented a high encapsulation efficiency (75 %), whereas NPs showed only a value of 16 % for this parameter. The percentage of acyclovir diffused to the receptor chamber over 24 h for each formulation was determined by an *in vitro* acyclovir release assay. The acyclovir release profile from NPs or microparticles had an initial burst period, during which the surface drug was dispensed into the release medium, followed by an induction period in which the drug was released at a gradually decreasing fast rate, and finally a slow release period during which the drug was released at a slow steady rate. Irritation assays based on mucus production carried out for CS-microparticles have shown moderate irritation and mild tissue damage. Although the mechanism of irritant reaction and cytotoxicity of CS-based systems is not well known, several studies have shown a potential toxicity of CS-based systems such as the detected toxic effect of CS NPs reported in the zebrafish embryo model by Hu *et al.* [79], while other authors have shown CS NPs to be well tolerated on the ocular surface and not compromise the cell viability [72, 76, 80].

Ameeduzzafar *et al.* (2014) [74] developed carteolol loaded-CS NPs by the ionotropic gelation method using sodium TPP as the crosslinking agent for ocular drug delivery, which was non-irritant and well tolerated. These NPs were formed by electrostatic interactions between CS and TPP, and the CS concentration, TPP concentration, stirring speed and solution pH were changed in the formulation with the aim of obtaining NPs with optimal features and to be able to evaluate the influence of these parameters on particle size, loading capacity and entrapment efficiency. The obtained CS NPs revealed particle diameters from 88.11 nm to 252.23 nm, with particle size of the NPs increasing with CS concentration, due to more binding sites being available for ionic cross linking of molecules. The mean diameter of the optimized CS-nanoparticle formulation was 168.90 nm and the polydispersity index was 0.212. The mucoadhesive strength or bioadhesive force of the CS NPs was rated as excellent, with adsorption of CS NPs occurring on pig mucin glycoprotein, due to the hydrogen bond formed between a positively charged amino group of CS and the oligosaccharide chains of mucin. The release profiles of carteolol from CS NPs showed an initial fast release followed by a slow release over a period of 24 h. Carteolol loaded-CS NPs revealed more permeation in comparison to the pure aqueous carteolol solution, as a result of the permeation enhancing activity of CS, with this increase possibly being attributed to both the aforementioned interaction of a positively charged amino group of CS with negatively charged sites of mucin and to the nano size of the CS NPs. The *in vivo* study showed that carteolol loaded-CS NPs had a better tolerability and prolonged retention at the corneal site, compared to the aqueous carteolol solution. Furthermore, the formulation reduced intraocular pressure over a longer period of time than to the aqueous carteolol solution, due to an enhanced residence time on the corneal and conjunctival surfaces.

Polysaccharides-based hybrid NPs for ocular gene delivery have been widely researched, and this will be reviewed in detail in the following sections.

4.2. Polysaccharide-Coated Nanocarriers

Polysaccharide-coated NCs present one or more polysaccharides as coverage of nanoparticle surface [9]. Polysaccharide coatings produced an increase in the mean size particle and a shift in the particle charge depending on the charge coverage [81, 82]. Several investigations have shown a significant increase in the mucoadhesiveness, release and bioavailability of different active molecules administered with polysaccharide-coated NCs, compared to uncoated systems. Suspensions of a mucoadhesive nanoparticle loaded Fluoroquinolone/glucocorticoid combination for the treat-

ment of bacterial keratitis were developed by Ibrahim *et al.* (2010) [81] to prolong the release. In this work, the potential of HA coated Eudragit® nanoparticle suspensions containing gatifloxacin and prednisolone bitherapy was evaluated as a long-term extraocular drug delivery, with NPs being prepared by adapting a spontaneous emulsification technique previously described by Bodmeier *et al*. [83]. These were coated with HA in two ways: the first one consisted in coating the performed NPs by HA adsorption onto the NP surface, then mixing a given volume of the resulting suspension with an equivalent volume of 0.025 % w/v HA aqueous solution, while the second one consisted in coating during particle formation by replacing the distilled water with 0.025 % w/v aqueous HA solution. The uncoated NPs with different drug: polymer ratios had mean sizes ranging from 315.2 to 973.65 nm, a low polydispersibility index and positively charged with zeta potential values between +30 and +45 mV. NPs prepared with higher amounts of Eudragit® RL acquired significantly higher zeta potential values because of the availability of more quaternary ammonium groups. All these nanoparticle suspensions showed a significantly prolonged release profile, as compared with free drug release, but without any burst effect. This indicated, according to the authors, that the drugs were homogeneously dispersed in the Eudragit® matrix and that no significant amounts of drug were adsorbed onto the nanoparticle surface. Increasing the drug:polymer ratio significantly retarded the release of gatifloxacin and prednisolone. All the coated NPs with HA were spherical, large and exhibited a broader size distribution than uncoated NPs. Also, a higher polydispersibility index was achieved for the coated NPs than the uncoated systems, indicating that they may aggregate. The zeta potential drastically changed after HA coating, initially being positive due to the cationic Eudragit®, and later becoming negative (around -46 mV), thereby demonstrating the adsorption of the negatively charged HA on the nanoparticle surface by electrostatic interaction. Moreover, coating with HA did not significantly influence the release profile of either drug.

Although, both the HA coated NPs and the commercial gatifloxacin eye drops reached the maximum corneal concentration 1 h after the topical instillation, this concentration was 5.23-fold higher in the case of the nanoparticle suspension according to the gatifloxacin levels recorded in the corneal tissue, with HA coated NPs prolonging the drug effect for more than 6 h, while the effect of the commercial eye drops ended after 2 h. Moreover, the NPs showed a 1.76-fold increase in the C_{max} of gatifloxacin in the aqueous humor in comparison with the eye drops. Thus, NPs coated with HA are promising for drug ocular delivery, due to the significant increase and prolongation of the gatifloxacin concentration in the cornea and aqueous humor, compared with commercial eye drops. Furthermore, the adhesion of NPs to the eye surface also prolonged the residence time of the drug in the conjunctival sac and improved its penetration across the cornea.

NPs loaded with 5-Fluorouracil were prepared by an ionic gelation technique using SA and CS followed by coating with CS, by Nagarwal *et al*. (2012) [82]. The nanoparticle preparation involved, as the first step, the formation of SA-CS NPs through adding CS solution drop by drop to the SA solution containing the drug. The electrostatic interactions between the negatively charged carboxylate groups on alginate and the cationic protonated amino groups on CS produced a spontaneously solidification of the SA shell through cross-linking with CS. The second step involved coating the of SA-CS drug loaded NPs with an additional CS layer through the physical adsorption of CS onto NPs. The NPs obtained revealed that the coating of SA-CS NPs led to a significant increase in the size of them with a consequent change in the morphology. There was a significant change in particle size, with variation in the in mass ratio of CS and SA. The SA-CS and CS coated-SA-CS drug loaded NPs had particle sizes between 329 and 505 nm, with the drug encapsulation efficiency of SA-CS drug loaded NPs being 6.19-26.66 % and with a drug loading capacity of 2.68-18.93 %. In this paper, the author also indicated that no interactions were found between SA-CS drug loaded NPs and mucin, whereas after CS-coating of the SA-CS NPs, a significant rise in the viscosity of mucin was observed, suggesting a considerable interaction between SA-CS drug loaded NPs and mucin, which was able to, enhance the precorneal residence time and hence facilitate an effective sustained drug release.

CS coated SA-CS drug loaded NPs showed a higher burst *in vitro* release in phosphate buffer solution (43.47 %), compared with uncoated SA-CS drug loaded NPs (30.46 %). Over a period of 8 h, uncoated and CS coated SA-CS drug loaded NPs showed release values of 74.15 % and 81.20 % of 5-Fluorouracil, respectively, while for 5-Fluorouracil free solution 99.82 % of the drug was released in 4 h with high a burst effect. Drug loaded NPs exhibited significantly more sustained release in comparison to drug solution. Moreover, CS coated SA-CS drug loaded NPs achieved a higher C_{max} and area under the curve than 5-Fluorouracil solution or uncoated SA-CS drug loaded NPs. On the other hand, a higher permeation of 5-Fluorouracil was observed in CS coated SA-CS drug loaded NPs of 5-Fluorouracil, compared with uncoated SA-CS drug loaded NPs or aqueous solution of drug. In addition, results of *in vivo* study showed that CS coated-SA-CS drug loaded NPs had a significantly higher concentration of 5-Fluorouracil in aqueous humor than SA-CS drug loaded NPs or 5-Fluorouracil solution. Also, uncoated and CS coated SA-CS drug loaded NPs revealed a low concentration of drug in vitreous humour. Related to this, the authors suggested that CS coated SA-CS drug loaded NPs may prolong the retention time due to their positive charge increasing contact between drug molecules and the anterior surface of eye, thereby enhancing ocular absorption via paracellular transport through the tight junctions of the corneal epithelia.

In the same way, Yenice *et al*. (2008) [46] determined cyclosporine A levels in ocular tissues and fluids after topical administration of uncoated and HA-coated poly ε-caprolactone/ benzalkonium chloride nanospheres, and castor oil solution onto healthy rabbit corneas. Nanospheres were prepared by using the nanoprecipitation technique as described previously by Fessi *et al.* (1989) [84], which were then purified by gradient-rate centrifugation. The cyclosporine A levels of nanosphere formulations were 6-8-fold higher than those of the castor oil solution. Similar conjunctival and corneal concentrations of cyclosporine A were found during the initial 4 h when poly ε-caprolactone/benzalkonium nanosphere and HA-coated poly ε-caprolactone/benzalkonium nanosphere were instilled. Then, after the first 4 h, the conjunctival levels of the drug were significantly lower than corneal levels. The corneal levels obtained from poly ε-caprolactone nanospheres were also significantly higher than those obtained using conventional cyclosporine A drops. In addition, the high tissue cyclosporine A concentrations obtained may not have only been due to the use of nanosphere formulations, but also, a result of the presence of benzalkonium as a penetration enhancer as well as the presence of HA as a bioadhesive polymer. The zeta potential of the poly ε-caprolactone/benzalkonium chloride formulation was positive due to the presence of benzalkonium chloride as the surface cationic surfactant. With respect to the distribution of cyclosporine A in ocular tissues, the incorporation of HA as a bioadhesive polymer resulted in a significant increase in the corneal uptake of cyclosporine A whereas this drug in castor oil resulted in a very low corneal uptake over the 24 h period. A constant corneal uptake was achieved by the administration of poly ε-caprolactone/ benzalkonium chloride nanospheres and HA coated poly ε-caprolactone/benzalkonium chloride nanospheres, with nanosphere formulations presenting slower elimination of cyclosporine A from the cornea. Furthermore, the findings inferred that poly ε-caprolactone/benzalkonium and HA-coated poly ε-caprolactone/benzalkonium nanosphere had a slightly increased tendency to penetrate into

the conjunctiva. Nanosphere formulations are thus able to deliver high levels of cyclosporine A into the cornea, with HA-coated poly ε-caprolactone and benzalkonium chloride nanoparticulate systems showing promise for local treatment of immune-mediated corneal disease.

Gan *et al.* (2013) [34] designed HA-modified core-shell liponanoparticles and also studied, in a previous work, the influence of HA on the cellular uptake of these NPs with the aim of improving the treatment efficiency by increasing the retinal pigment epithelium-targeted distribution. HA-modified core-shell liponanoparticle were obtained by covalent linkage of the glucuronic acid moiety of the targeting ligand HA to the primary amine of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in the preformed liposome shell. Core-shell liponanoparticles were prepared previously by hydrating a dry lipid film with a CS NPs solution [85, 86]. The influence of the HA modification density as well as molecular weight were evaluated on a human retinal pigment epithelial cell line (ARPE-19). Quantitative analysis by flow cytometry showed that cellular uptake of HA-modified core-shell liponanoparticles was significantly greater than that of core-shell liponanoparticles, with a higher HA grafting density and a higher molecular weight modification of HA also improving the intracellular uptake of HA-modified core-shell liponanoparticles. The lipid bilayer coated NPs with a higher molecular weight (200-400 KDa) HA modification showed approximately a 40 % greater cellular uptake than HA modified core-shell liponanoparticles with a lower molecular weight (10-100 KDa) HA modification. Moreover, the specific uptake of HA-modified core-shell liponanoparticles to CD44 receptors was investigated by pretreating ARPE-19 cells with a saturable amount of free HA (200-400 kDa) before incubation, with results revealing that cellular uptake of CS NPs and core-shell liponanoparticles was not affected by ligand pretreatment whereas the cellular uptake of HA-modified core-shell liponanoparticles was significantly reduced, indicating that the free ligand competed with HA-modified core-shell liponanoparticles for receptor binding sites. In addition, *in vivo* assays demonstrated that HA-modified core-shell liponanoparticles could specifically target the retinal pigment epithelium cells through interaction between the CD44 receptor and the HA ligand in autoimmune uveitis rats. Even though these findings of Gan *et al*. are not strictly related to the topic of this review, HA-modified core-shell liponanoparticles might still present a promising topical drug delivery system in future to achieve a retinal-pigment-epithelium-targeted distribution and prolonged intraocular residence.

As has been seen in the previous investigation, the polysaccharide coating can also be used to cover NPs composed of lipids. These were able to increase mucoadhesiveness and bioavailability, with an improvement in cellular uptake, cellular targeted distribution and intraocular residence time also reported.

Du Toit *et al*. (2013) [1] compared two specific embodiments of an ocular nanosystem: the CS-poly(ε-caprolactone) nanosystem and the lipoidal-CS-poly(ε-caprolactone) nanosystem. The former is a purely polymeric system, while the latter is based on a composite lipoidal-polymeric nanosystem architecture utilizing phospholipids. The combination of lipoidal-polymeric nanosystems is based on the incorporation of poorly water-soluble drugs, resulting in a prolonged lifetime attributed to the polymeric component, tissue distribution, and inflammatory tissue targeting, according to previous research by the authors [87]. The interaction between the carboxyl or hydroxyl groups of the anionic poly(ε-caprolactone) and the amine groups of CS formed immediate polyionic nanogels, with coating of the nanosystem with the long-chain CS creating a the compatibilizing layer that enabled enclatheration of the nanosystem within a polymeric matrix. An increase in the particle size of the nanogels was produced as a result of surface adsorption of the long chain CS molecules at a perpendicular configuration to the nanosystem surface. Both nanosystems exhibited highlighted an enhanced efficacy in terms of tissue permeation, cell uptake, and anti-inflammatory activity, compared to an indomethacin suspension. Nanosystem formulations permeated the ocular layers more effectively due to the penetration-enhancing capabilities of CS. Lipoidal-CS-poly(ε-caprolactone) nanosystem, thus showing a higher permeation because they possess an amphiphilic shell surface that enables their partitioning across the different barriers and fluids of the eye along the hydrostatic gradient. Incorporating the phospholipids to the nanosystem may lead to an enhanced internalization via the biological processes of phagocytosis or endocytosis, compared to the polymeric nanosystem, due to phospholipids playing an active role in biosynthetic molecular mimicry.

Ying *et al.* (2013) [88] suggested that surface-modified lipid emulsions might be promising vehicles for hydrophobic drug delivery to the ocular posterior segment. This research group explored submicron-sized lipid emulsion with a modified surface using a positive charge inducer (stearylamine) and the functional polymers CS and poloxamer 407 as potential carriers for intraocular drug delivery to the posterior segment via eye drops. Using coumairn-6 as a model drug and fluorescent marker, fluorescence could be observed in the retina after administration of the lipid emulsions. The administration of medium chain triglycerides containing coumarin-6 displayed much lower fluorescence intensity than that resulting from administration of lipid emulsions with the same amount of drug. The authors have suggested that the inner oil property and phospholipid emulsifier did not affect drug delivery efficiency to the retina. Related to this, the fluorescence intensity of coumarin-6 loaded lipid emulsions in the retina was significantly increased by surface modification using a positive charge inducer stearylamine, CS and poloxamer 407, in comparison with unmodified emulsions. Nevertheless, no significant differences were observed between the effects of CS and poloxamer 407 on delivery to the retina. CS-modified lipid emulsions may have electrostatically interacted with the eye surface, while poloxamer 407 possibly increased the lipid emulsion retention time on the eye surface due to its adhesive property.

Another approach was reported by Bhatta *et al*. (2012) [89], who prepared natamycin encapsulated lecithin/CS mucoadhesive NPs to enhance precorneal retention, achieving sustained release, and a high ocular availability at reduced doses and dosing frequency. The obtained NPs showed a mean particle size of 213 nm, an encapsulation efficiency of 73.57 %, with a theoretical drug loading of 5.09 % and zeta potential of +43 mV. These NPs can be considered to be self-organized structures, as a result of the electrostatic interaction between CS and lecithin, due to the presence of negatively charged components in the lipid mixture. A decrease in the zeta potential for a suspension of NPs was observed after 6 h incubation with mucin, possibly resulting from an ionic interaction between the negatively charged mucin and NPs. The turbidity of NPs/mucin aqueous dispersions was also examined, with results revealing a higher turbidity of NPs/mucin dispersions than that of mucin dispersion by itself, indicating an eventual interaction between the NPs and mucin. Furthermore, the NPs formulation exhibited a significant enhancement of the area under the curve (approximately 1.47-fold more), and clearance was significantly decreased (approximately 7.4-fold less) in comparison with a marketed suspension. As the retention time of NPs was significantly higher than the marketed suspension, the positively charged NPs can provide a binding force to the eye surface.

It can be seen from the studies reviewed above, the polysaccharide-based coverage of NPs with a polymeric or lipid core favors the interaction of the system with the ocular surface. The coverage of NPs with different polysaccharides can be used as a strategy to prolong residence time on the ocular surface, enhance permeation with a better release of drugs and, facilitate cell uptake, thereby achieving an efficient ocular therapy.

5. POLYSACCHARIDE-BASED NANOPARTICLES FOR OCULAR GENE THERAPY

Many genes involved in pathological processes and their expression systems have been studied in recent years for the treatment of diseases [90]. Gene therapy is a very promising therapeutic strategy for a wide range of hereditary or acquired diseases, which consists of introducing genetic material (DNA, RNA or antisense sequences) into target cells. The purpose of gene therapy is to regulate the expression of specific altered proteins, thus reversing the biological disorders causing the alterations [91]. The development of effective therapeutic delivery strategies for DNA is a critical research goal [92] and depends on the capacity to manipulate the expression of genes in suitable cells. For gene therapy to become viable, several problems such as poor cellular uptake, rapid *in vivo* degradation, limited transport to the target, and low effective delivery of genetic material to the cell nucleus need to be resolved [39, 47].

A wide range of materials are currently being explored to address the challenges of delivery including, viral vectors, inorganic particles and polymeric-, cationic lipid-, and peptide-based vectors [93]. Although, the viral vectors are the most effective, they have large immunogenicity and oncogenicity problems. On the other hand, non-viral delivery systems are safe, highly reproducible, have low-cost production and no size limit on the DNA to be transported [94]. These features make non-viral vectors attractive systems for use in gene therapy [47]. Gene NCs should be able to entrap nucleic acids, protect genetic material against enzymatic degradation, facilitate cell uptake and be efficiently delivered to the ocular cells. They must also interact with the ocular mucosa and transfect the ocular epithelia under physiological conditions, leading to a desired therapeutic effect [5, 91]. The transfection of the ocular mucosa may be as important in nanocarrier development to treat pathologies which affect the anterior segment of eye as in the treatment of posterior segment diseases [5, 95].

Polysaccharide-based NCs are promising therapeutic systems for ocular diseases, with several investigations having demonstrated that NCs with polysaccharides are capable of protecting and transporting genetic material to target-cells, and can also improve transfection and cellular release. In addition, cationic polymers as CS can condense pDNA by strong electrostatic interactions to form different types of NPs [92, 93]. In general, the cellular internalization of NPs is produced through cellular endocytosis processes, or by the interaction with specific receptors of the cellular membrane as demonstrated for HA-based NPs [47].

Non-viral gene therapy based on solid lipid NPs is also a promising strategy for the treatment of several diseases. Although, solid lipid NPs are safe non-viral vectors, their low efficiency of transfection renders them far from being an ideal vector [90, 91]. At present, the improvement of the efficiency of transfection of non-viral vectors in general, and solid lipid NPs in particular, is a challenge for making further progress in gene therapy. In this sense, Apaolaza *et al*. (2014) [47] designed a new vector composed of solid lipid NPs, protamine and HA of three different molecular weights, and studied its potential utility for gene therapy using pCMS-green fluorescent protein plasmid, which is a mammalian expression vector which can express the gene of interest by cloning. The obtained NPs were stable and exhibited particle sizes ranging from 240 nm to 340 nm, conditioned by the ability of the protamine to precondense the DNA and the space that this peptide takes up. The precondensation of DNA could have caused a reduction in nanoparticle size whereas the occupied space itself might have led to an increase particle size. Protamine is an excellent DNA condenser that reduces the exposition to degradation by different cytoplasmic agents such as DNAses, and helps to transport DNA to the nucleus. It was shown that, DNA condensation affects the transfection capacity and delivery of DNA from solid lipid NPs, the protection of genes from external agents, and DNA topology [96, 97]. The particle charge was positive due to the electrostatic interaction between the positive charge of the protamine and solid lipid NPs, and the negative charge of HA and DNA. Moreover, vectors were able to preserve the pCMS-green fluorescent protein plasmid to protect it against the action of DNAse, and to release it in presence of sodium dodecyl sulphate.

The incorporation of HA and protamine to the solid lipid NPs permitted a versatile vector to be obtained, which was able to efficiently transfect cells with different rates of cellular division without compromising cell viability, thus widening the potential applications of solid lipid nanoparticle-based vectors. The obtained NPs containing protamine, HA and DNA induced almost a 7-fold increase in the transfection capacity of solid lipid NPs in ARPE-19 cells. A significantly higher (50 %) cellular uptake in ARPE-19 cells was obtained for the vectors prepared with HA and protamine than for the DNA-lipid solid NPs vector (30 %), which could have been due to differences in the capacity to bind to the cell surface provided by the capacity of HA to interact with CD44 and other HA-specific receptors to facilitate cell internalization [41]. Similar results were also observed in HEK-293 cells. On the other hand, results of immunochemistry assays also presented a high expression of CD44 receptors in ARPE-19 cell and a significant decrease in the uptake of these vectors in ARPE-19 cells, which might suggest the participation of this receptor in the cell internalization of the vectors.

In recent years, hybrid NPs have been explored as possible carriers to deliver genetic material in the treatment of a wide range of ocular diseases, with the purpose of reaching the target site. Cationized gelatine in the form of hydrogels or complexes has been used for developing NPs with an effective transfection capacity in several types of cells. Konat Zorzi e*t al*. (2011) [50] developed hybrid NPs made from DS, ChS and gelatine cationized with spermine (SPM) by the ionic gelation technique for gene therapy application in the ocular surface. The particle size and zeta potential of the NPs was be modulated through variations in the crosslinking agent and/or anionic polymers ratios related to cationized gelatine. All the nanosystems showed particle sizes less than 300 nm with a spherical particle shape, and the incorporation of DS and ChS to the formulation did not produce any changes in the particle shape in comparison to NPs without polyanions. Furthermore, the superficial charge of NPs changed according to the amount of the polymer. Zeta potentials between -38 to +48 mV were obtained for NPs with ChS, whereas for NPs with DS these ranged from -55 to +39 mV. The ability of NPs to protect pDNA from nuclease degradation was evaluated by the DNAse I protecting assay, by incubating NPs for 1 h in the presence of the enzyme. The results revealed that cationized gelatine with SPM, cationized gelatine with SPM and ChS NPs, and cationized gelatine with SPM and DS NPs significantly protected the associated pDNA from degradation, while naked DNA was completely digested at 5 min after incubation. According to these authors, the protective effect of the NPs may have been attributed to the presence of the SPM attached to the gelatine backbone. This protective ability of polyamines is attributable to the formation of a steric barrier against DNA damaging agents and their ability to condense the DNA. A high association efficiency (around 90 %) was observed in the cell culture, between pDNA with either cationized gelatine NPs, cationized gelatine with SPM and ChS NPs or cationized gelatine with SPM and DS NPs. The interaction between the SPM and DNA can explain the above results. On the other hand, the Cy3-labeled pDNA associated with all types of cationized gelatine NPs, and its respective uptake was evaluated in human corneal epithelia (HCE) cells to determine the influence of ChS and DS on the internalization of these NPs. Free or naked Cy3-labeled pDNA did not show internalization, while an intense green signal demonstrated the effective internalization of the three types of NPs. Additionally, a transfection assay using a model plasmid that encodes enhanced green fluorescent protein

(EGFP), exhibited a maximum expression of EGFP for all of the systems after 48 h. Then the transfection was maintained until at least 72 h post-transfection. The cytotoxicity was also evaluated by incubating the NPs with HCE cell at varying concentrations of NPs using an XTT-based *in vitro* toxicology assay (XTT=2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), with results showing that cytotoxicity decreased as a result of the presence of the natural polyanions. The NPs with cationized gelatine with SPM and the polyanions were capable of decreasing the system toxicity, without compromising transfection efficiency.

The same research group of Konat Zorzi *et al.* (2011) [98], also evaluated the ability of hybrid cationized gelatine NPs containing ChS or DS to transfect ocular epithelial cells, using a plasmid specially designed to encode human MUC5AC. These NPs were developed using the ionic gelation technique, based on cationized gelatine reticulated with TPP as described in their previous investigation. NPs showed particle sizes less 150 nm, a high plasmid association efficiency (>95%) and a positive zeta potential between +20 and +30 mV. Cell viability was measured in the HCE and IOBA-NHC cells 72 h after incubation of the cationized gelatine NPs or cationized gelatine NPs with ChS or DS. The results exhibited no significant differences between controls of both cells with those exposed to the three formulations. An efficient transfection of MUC5AC in HCE and IOBA-NHC cells was produced by the three formulations, influenced by the presence of the anionic polymer in the formulation. Additionally, MUC5AC mRNA expression in HCE cells following incubation with the nanoparticle formulations was 2-fold higher for cationized gelatine NPs than for NPs containing cationized gelatine with polyanions in this cell line. The NPs containing cationized gelatine with ChS showed a 2-fold greater levels of expression in IOBA-NHC cells than those of cationized gelatine NPs, and 3-fold greater than the levels of expression of cationized gelatine with DS NPs. The MUC5AC protein expression was measured by ELISA, and this was significantly higher than that in untreated cells or cells exposed to the nanoparticle formulations. In IOBA-NHC cells, the level of expression was higher for cationized gelatine NPs in comparison with cationized gelatine NPs with ChS or DS, while protein expression in HCE cells was not induced by any of the formulations. The *in vivo* administration of the NPs into the eyes of New Zealand rabbits resulted in a significantly higher MUC5AC expression in the conjunctiva compared to untreated control and naked plasmid. Furthermore, no macroscopic alterations in the ocular structures were found after pMUC5AC-loaded nanoparticle exposure. Even though there was some mild nasal discharge and increased in blinking after instillation, the formulations seemed to be well tolerated. These NPs showed a successful transfection rabbit conjunctiva *in vivo* acting as effective vehicles for gene therapy and candidates for restoring the MUC5AC concentration in the ocular surface.

Contreras-Ruiz *et al.* (2011) [99] determined which internalization pathway is used by corneal-derived and conjunctival-derived cell lines to take up HA-CS oligomer-based NPs. These NPs were made of fluoresceinamine labeled HA and CS-oligomer by a slightly modified ionotropic gelation technique and were loaded with a model pDNA encoding secreted alkaline phosphatase. The ability of NPs made of HA and oligomers of CS to complex with plasmid pDNA, penetrate into cells, and deliver the pDNA was studied in previous investigations by this group [100]. HA-CS oligomer-based NPs revealed a particle size of around 100 nm and a positive zeta potential (+31 mV). The cellular uptake of these NPs was investigated in living human epithelial cell lines derived from the conjunctiva and the cornea by fluorescence microscopy, associating green fluorescence to HA and red fluorescence to the Cy3-plasmid. The results showed that the plasmid-loaded CS oligomer-based NPs were intracellularly localized in both corneal and conjunctival cells, but uptake was always higher in corneal cells compared to the conjunctival cells. According to the fluorescence signals, the plasmid was localized in the nucleus or in the perinuclear region in both cell lines. As the plasmid associated fluorescence could be independently identified from the fluorescence associated to the fluoresceinamine-HA, it could be inferred that the plasmid was separated from the NPs and reached the nucleus. The cell viability did not show significant differences with control when CS oligomer-based NPs were incubated in HCE or in IOBA-NHC cells. The uptake of CS oligomer-based NPs was significantly reduced by incubation at 4 °C and by sodium azide, due to inhibition of active transport processes, whereas uptake of these systems was significantly reduced by Hermes-1 (anti-CD44 Hermes-1 antibody) and excess HA. Filipin also reduced the uptake of NPs by inhibition of caveolin-dependent endocytosis. The authors suggested HA of the HA-CS oligomer-based NPs interacted with CD44 followed by caveolae internalization (lysosome-independent pathway) as a result of these receptors and HA being allied with caveolae. CS oligomer-based NPs can be considered to be potential nanosystems for delivering genetic material to the ocular surface due to them being internalized by an active transport mechanism with higher transfection efficiency and without compromising the cell viability.

In another study, Parraga *et al*. (2014) [101] designed NCs specifically based on the ability of endogenous polyamine SPM to interact with anionic biopolymers, thereby generating ionically crosslinked nanosystems, as has been in previous investigations. The authors developed a wide range of NPs using biopolymers such as HA, ChS, DS, HS, colominic acid as the basis of the cross-linking properties of polyamine SPM, and then they selected suitable component mass ratios. The ability of obtained blank NPs to associate different labile bioactive molecules was evaluated, and results revealed an efficient association of albumin and the growth factor FGFb to NPs. However, they were not able to associate nucleic acids such as pDNA (pEGFP= enhanced green fluorescent protein plasmid) and siRNA (siGAPDH). The functional groups of albumin and the growth factor FGFb can interact through hydrogen bonds and hydrophobic domains with ChS, DS and HA, and the establishment of high repulsive forces of highly negatively charged pDNA and si-RNA and the negatively charged biopolymers used to prepare the NPs, prevented the association. Hybrid NPs were developed through the incorporation of gelatine cationized with SPM to the nanoparticle components in order to obtain nanostructures able to associate genetic material, with the NPs allowing interactions between NPs and the pDNA and siRNA molecules. Optimized NPs were successfully developed, then siRNA and polyanions (ChS or HA) were incorporated to aqueous solutions of cationized gelatine with SPM. These NPs demonstrated an effective association capacity for siRNA that initial prototype platforms did not show. In addition, they exhibited a smaller particle sizes and polydispersity index than the initial nanoparticle prototypes of the HA and SPM. The toxicity assays of optimized NPs revealed that HA/cationized gelatine with SPM NPs presented a higher cytotoxicity in HCE cells compared with those that incorporated ChS. Adding glycolsaminoglycans to the formulation components decreased the toxicity induced by cationic polymers [50], and in this case this effect depended on the composition or specific glycosaminoglycans incorporated, which was more favorable when ChS was included. Also higher proportions of anionic biopolymers in their composition led to better biocompatibilities. NPs prepared with HA disintegrated more rapidly after the internalization process, under the studied conditions, in comparison to those formulated with ChS. This difference was explained by the authors by the greater ability of ChS to interact with the cross-linker agent, and thus generate more compact, robust or stronger nanostructures than using HA. The delivery of siRNA in the cytosol from NPs of gelatine cationized with SPM and ChS was confirmed through NPs with a double label. The gene-interfering potential of internalised NPs formulated with HA or ChS to associate siRNA was evaluated using GAPDH as a model target gene. These results showed a silencing effect that could be interpreted as NPs independent, as regardless of their composition, were

able to release adequate genetic material, protect the siRNA from the degradation by enzymes, escape from the endosomes and release the siRNA in its active form at the target site.

It has been seen in this section that polysaccharide-based NCs can be used as strategies for gene ocular delivery due to their enhancing of cell transfection, achieving efficient internalization, decreasing toxicity and protecting genetic material against enzymatic degradation. However, it should be born in mind that the same characteristics that favor improved therapeutic actions can also have an impact on the toxicity of such particles. [102]

Although intraocular injections have several drawbacks including endophthalmitis, intraocular inflammation, retinal detachment, intraocular pressure elevation and ocular haemorrhage, among others, this route can be used as an alternative to topical ocular administration when the carriers cannot reach successfully the target site [103, 104]. In recent studies, Mitra *et al*. (2014) [92] formulated, characterized, and tested non-viral NPs composed of glycol-CS for gene delivery to the eye administered by the subretinal route. CS polymers alone are ineffective for DNA compaction, so the purpose of chemical CS modification with the glycol was to increase its solubility in the aqueous medium. Nanoparticle compaction with glycol CS and pDNA occurred via strong electrostatic interactions and the obtained NPs had a size of 250 nm and a positive surface potential (+24.17 mV), in contrast with the negative potential of the naked pDNA (-25.90 mV) that did not exhibit aggregation physiological buffer (saline). Glycol-CS NPs resulted stable and resistant to DNAses then incubation of these NPs with DNAse I and then chitosanase, which hydrolyzes β-1,4-linkages and releases the DNA from the nanoparticle. No significant degradation of nanocompacted DNA was obtained, while naked pDNA was completely digested. Furthermore, intact plasmid was released inside the cell with the open-coil and super-coiled DNA conformations being preserved, even after release from the NP network. In fact, DNAse resistance is a useful feature of an effective delivery vehicle. The ability of glycol-CS NPs to drive ocular gene expression *in vivo* pDNA carrying the ubiquitously expressed chicken β-actin-green fluorescent protein expression cassette was compacted and subretinally injected into adult wild-type albino mice at postnatal day. Substantial green fluorescent protein expression was observed exclusively in the retinal pigment epithelium in eyes treated with glycol CS NPs, but not in those treated with uncompacted pDNA or vehicle (saline), at day 14 post-injection. This result suggests that glycol-CS NPs may facilitate gene expression in the retinal pigment epithelium after subretinal delivery, and could be a useful strategy for targeting retinal pigment epithelium-associated disease.

Martens *et al*. (2015) [35] evaluated the use of HA with different molecular weights (22 kDa, 137 kDa, 2700 kDa) as an electrostatic coating of cationic polymeric pDNA gene complexes for nonviral polymeric gene nanomedicines administered by the intravitreal route. The aim of covering nanocomplexes with HA is to provide them with an anionic hydrophilic surface for improved intravitreal mobility. These gene complexes were formed of anionic plasmid DNA and the cationic N,N′-cystaminebisacrylamide-4-aminobutanol (p(CBA-ABOL)). Also PEGylated polyplexes were prepared from the poly-addition of methoxypolyethyleneglycol amine, N,N′-dimethylethylenediamine (DMEDA′) and aminobutanol to cystaminebisacrylamide (CBA-ABOL-DMEDAPEG/pDNA). Results showed that HA uncoated CBA-ABOL/pDNA polyplexes had a size of 108 nm and a positive zeta potential of +29 mV, while the HA uncoated PEGylated polyplexes exhibited a particle sizes of 124 nm and a zeta potential of +10 mV. The HA coated polyplexes had larger sizes and a negative charge on the surface. The complexation of pDNA polyplexes was satisfactory and for the PEGylated polyplexes only a fraction of pDNA was displaced.

The mobility of polycomplexes in intact vitreous humor was evaluated on excised bovine eyes by fluorescence single particle tracking microscopy. HA-coated polyplexes, as in the case of functionalized polyplexes with polyethylene glycol had good mobility in bovine vitreous humor, except for those coated with high molecular weight HA. The results of cell uptake and transfection efficiencies quantified by flow cytometry revealed that HA-coated polyplexes were efficiently taken up *in vitro* in ARPE-19 cells, in contrast with PEGylated polyplexes. Despite the negative charge of the coated polyplexes, the results indicated uptake via CD44-receptor mediated endocytosis. The HA polyplexes were able to induce green fluorescent protein expression in this *in vitro* cell line, without any apparent cytotoxicity. Thus, HA-coating of non-viral gene complexes is an interesting approach for retinal gene therapy by intravitreal administration.

As mentioned above even though intraocular routes are not strictly related to the topic of this review, recent studies where genetic material is administered by these routes have shown encouraging results related to the potential effectiveness of polysaccharide-based NCs in gene delivery.

6. PATENTS ON POLYSACCHARIDE-BASED NANOCARRIERS

Some recent nanosystems developed with polysaccharides have been patented. For example, Alonso Fernandez *et al*. [105] in 2007 patented NPs of CS of low molecular weights and HA for the administration of active molecules. These NPs are considered to be a potential vehicle in gene therapy due to their ability to be incorporated into the cell genetic material, which encodes a protein of interest. The NPs obtained showed an efficient association of biologically active molecules, an easy degradation of the NPs in the biological conditions and an efficient internalization of the NPs in the cells. *In vitro* studies also showed an efficient internalization of the NPs in the cells by cellular endocytosis processes and by the interaction with specific receptors of the cellular membrane. Furthermore, *in vivo* studies revealed high transfection levels of NPs, and the NPs demonstrated their ability to interact with epithelial cells and to promote the transfection of a polynucleotide into a cell.

The NPs of CS of low molecular weight and HA with plasmid DNA pEGFP or pSEAP (Secreted Alkaline Phosphatase plasmid) were evaluated by *in vitro* studies in three different cell lines, including the Human Embrionary Kidney cell line, HCE cell line and Normal Human Conjunctival cell line, as well as *in vivo* studies in the animal ocular epithelium. NPs exhibited very low cell toxicity and a cellular internalization by cellular endocytosis processes and also by interaction with the specific receptors of the cellular membrane. Additionally, the effective delivery of the DNA-plasmid to the target site was achieved through the biodegradation of HA and elimination or biodegradation of CS.

Rodríguez Gascón *et al*. [90] patented lipid NPs for gene therapy in 2011. The lipid nanosystem was made of a lipid component, a cationic surfactant, a non-ionic surfactant, a polysaccharide, and optionally, a positively charged peptide that was useful for the release of pharmacologically active molecules and the transfection of genetic material into target cells and/or tissues. A solid nanoparticle formulation made of solid lipid at room temperature that was selected from acylglycerides, saturated fatty acids with a chain of at least 10 carbon atoms or derivatives thereof and mixtures thereof and a liquid lipid (saturated or unsaturated) at temperatures less than 45˚C. Ammonium salts (tetraalkylammonium salts, alkylbenzyl dimethyl ammonium salts or heterocyclic ammonium salts) were used as the cationic surfactant, while polysorbates, polyethylene glycol and polypropylene glycol copolymers was utilized as a non-ionic surfactant that allowed the particle size and stability of NPs to be controlled. The incorporated polysaccharide into the formulation facilitated the interaction of nanoparticles with the cell surface, and modified the nanoparticle surface charge. The polysac-

charides generally used in the development of the patented nanoparticle are CS, DS, HA, carrageenan, ChS, keratin, colominic acid and xanthan, among others. A positively charged peptide can be part of the structure of NPs or can be adsorbed on the surface. It is selected from nuclear signalling peptides and mitochondrial signalling peptides, RGD peptides (cell surface recognition peptides containing the arginine-glycine-aspartic acid sequence and variants thereof) and cell-penetrating peptides. The biologically active molecule can be a DNA plasmid or a nucleic acid such as DNA, mRNA, iRNA, microRNA, or an antisense sequence. An effective transfection level for transfecting genetic material into cells with a high cell viability was achieved through the incorporation of a polysaccharide into the lipid nanoparticle system, which together with a positively charged peptide favored the transfection process, thereby resulting in higher transfection levels in comparison with those of a nanoparticle system which exclusively contains the polysaccharide or the positively charged peptide. In addition, the NPs containing the polysaccharide and those containing the polysaccharide and positively charged peptide were able to condense the DNA in order to protect it against DNAse. This inventive system has demonstrated that it can be considered to be a potential gene carrier for the treatment of ocular diseases.

More recently, Rodriguez Gascon *et al*. (2013) [91] developed and patented lipid NPs for treating ocular diseases. These included as in the previous patent, a nucleic acid, a lipid component, a cationic surfactant, a non-ionic surfactant, a polysaccharide, and optionally a positively charged peptide. The capacity of this nanoparticle system for effectively transfecting and allowing the expression of the RS1 gene involved in sex-linked juvenile retinoschisis was demonstrated by its inventors. *In vivo* assays showed that these NPs were able to reach retinal cells and thereby protect the genetic material from the action of enzymes. The NPs with polysaccharide-positively charged peptide combination showed higher transfection levels, thus improving cell viability more than with a nanoparticle system that included exclusively the positively charged peptide or polysaccharide, in the same way as in the previous patent. The patented NPs demonstrated that they were capable of transfecting the pCep4-RS1 plasmid into retinal cells (ARPE-l9), causing retinoschisin protein synthesis. The use of these NPs that combine a polysaccharide with a positively charged peptide provided a much higher concentration of retinoschisin, and increased transfection levels more than those produced by NPs without the polysaccharide and the peptide. Furthermore, the greater transfection capacity of the NPs, the inclusion of the polysaccharide, and the combination of positively charged peptide with polysaccharide in the NPs, increased viability with respect to NPs without these components. *In vivo* administration of NPs at the retinal and/or corneal level was performed in adult albino Wistar rats; with the results demonstrating that lipid NPs with pCMS-pEGFP had the ability to induce effective transfection of active molecule in different retinal cell populations, particularly at the level of retinal ganglion cells. The intravitreal injection was more efficient at incorporating NPs in retinal cells that subretinal injection, and the topical administration of lipid NPs with pCMS-pEGFP was also capable of inducing an effective transfection of green fluorescent protein into corneal epithelial cells.

In summary, the polysaccharide-based NCs from the patents described above showed efficient favorable features with regard to their association with biologically active molecules, degradation in biological conditions, internalization in cells and levels of transfection. Even though the development of novel NCs from an initial study to patent registering involves much effort and time, the obtained results justify further study.

7. OTHER POLYSACCHARIDE-BASED OCULAR SYSTEMS

In addition to the development of polysaccharide-based NCs, other topical drug delivery systems for ocular administration containing polysaccharides in their structure have been described in the literature. The use of polysaccharides as biodegradable polymers has been utilized in the preparation of different ocular drug delivery systems including films, hydrogels and lenses loaded with polysaccharide-based NPs, among other systems.

Calles *et al*. (2013) [44] studied the *in vivo* release of timolol maleate of HA-itaconic acid-polyethylene glycol diglycidyl ether modified film. This drug was released quickly from this modified film, and continued its release for up to 8 h. In addition, a significant decrease of intraocular pressure in normotensive rabbits was observed 2 h after administration of the formulation, and after 8 h of administration the film continued to reduce the intraocular pressure by approximately 15 %.

Mucoadhesive CS films were developed by Hermans *et al*. (2014) [106] in order to prolong the ocular delivery of cyclosporine A. The ratios of the film components influenced the mechanical properties and *in vitro* release of cyclosporine A. No significant cytotoxicity was found in any film and furthermore, Cyclosporine A of film formulations remained anti-inflammatorily active and significantly suppressed interleukin-2 secretion.

Huang *et al*. (2013) [107] prepared a type of loaded intra ocular lens with 5-fluorouracil as the antimetabolite drug, whose release could be sustained to prevent posterior capsule opacification. They evaluated its efficacy and safety *in vitro* and *in vivo*. Intraocular lens were activated by a low energy fluorine ion beam followed by surface modification through 5-fluorouracil-CS NPs. The modified intraocular lens surfaces modified were implanted into rabbits' eyes after transparent lens enucleation, in order to evaluate the efficacy of preventing posterior capsule opacification in the anterior chamber. Spheroid particles of different sizes were distributed on the surface of 5-fluorouracil-CS nanoparticle surface-modified intra ocular lens**.** Most of the particles had diameters below 100 nm whereas several large particles had diameters between 100 and 400 nm. Different groups were treated with 5-fluorouracil-CS NPs of different concentrations and cell proliferation efficacies, and the median lethal dose was determined. Values of 1 mg/mL and 0.2 mg/mL as the median lethal dose for the 5- fluorouracil solution and CS NPs group were obtained, respectively. The 5-fluorouracil-CS NPs promoted apoptosis, lowered the necrotic rate and were able to inhibit the human lens epithelial proliferation rate more effectively compared with the 5-fluorouracil solution. *In vitro* assays revealed that the intraocular lens could sustain drug release at 96 h. A small aqueous flare in the 5-fluorouracil-CS nanoparticle intraocular lens group by *in vivo* assays together with a lighter posterior capsule opacification were observed 4 weeks after the surgery.

Garhwal *et al.* (2012) [108] formulated contact lenses with nanosphere-encapsulated ciprofloxacin. The core-shell nanospheres were prepared from a copolymer composed of pullulan and polycaprolactone, and were considered to be a drug delivery platform for ocular treatments since they could be incorporated into a conventional, transparent contact lens and provide for sustained and effective bactericidal activity. The dispersed nanosphere-encapsulated ciprofloxacin had a uniform size with diameters of around 140 nm. On analyzing the results, it was observed that 10 mg of this initial nanosphere-encapsulated ciprofloxacin preparation released 1934 μg of ciprofloxacin over the first 24 h, 390 μg on the second day and 76 μg on the third day of analysis. Nanosphere-encapsulated ciprofloxacin was then incorporated into HEMA-based contact lenses, and antibacterial activity of the nanosphere-encapsulated ciprofloxacin and nanosphere-encapsulated ciprofloxacin incorporated into the HEMA-based contact lenses was tested by using liquid cultures of either Staphylococcus aureus or Pseudomonas aeruginosa. In this way, the proliferation of cultures had been inhibited with less than 2 μg/mL of nanosphere-encapsulated ciprofloxacin when it was inoculated with 10^7 bacteria/mL of Staphylococcus aureus or 10^8 bacteria/mL of Pseudomonas aeruginosa. The HEMA-based contact lenses polymerized with nanosphere-encapsulated

ciprofloxacin were transparent, effectively inhibited the proliferation of greater than 10^7/mL of bacteria added daily over 3 days of culture, and killed up to $5x10^9$ total microbes in a single inoculation. The lens designs included thin or thick lenses incorporating nanosphere-encapsulated ciprofloxacin and also ciprofloxacin-HCl-soaked Acuvue lenses (Acuvue; Johnson & Johnson Vision Care, Inc., Jacksonville, FL). Both types of resulting hydrogels were transparent, had an appropriate size (10 mm in diameter) and shape, and were capable of inhibiting the proliferation of Staphylococcus aureus and Pseudomonas aeruginosa. The antibacterial activity provided was not as prolonged by the nanosphere-encapsulated ciprofloxacin-incorporating lenses as that provided by the Acuvue/ciprofloxacin-soaked lenses. Much of the drug was released over the first 24 h, which was followed by a slower drug release from each lens.

The research reviewed in this section is part of a diverse group of ocular systems containing a polysaccharide as a strategy to enhance their effectiveness in drug delivery to the target site. To continue the development of polysaccharide-based NCs, it is important not only to evaluate these but also other existing polysaccharide-based carriers, in order to optimize and encourage the development of new systems through an integrated and enriched knowledge about the use of polysaccharides for ocular administration.

8. FUTURE TRENDS AND CHALLENGES

The design of a drug delivery system aimed at targeting a particular tissue of the eye is a major challenge for scientists in this field. Topical application of drugs to the eye is the most common and well-accepted route of administration for ophthalmic disorders and the nanotechnology appears to be an interesting tool for overcoming various physiological barriers. The challenges imposed by the ophthalmic therapy are very extensive. In this sense, we can cite gene therapy and a very broad spectrum of chronic diseases including glaucoma or retinal disorders.

In the present article, a large number of systems based on polysaccharides have been described in detail, revealing the great potential of these techniques. It is important to point out that to ensure that these developments achieve a commercial status demands the consideration of toxicological aspects along with several other industry-related requirements including sterilization and scale-up.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Authors thank Dr. Paul Hobson, native speaker, for revision of the manuscript.

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Received: June 1, 2015 **Accepted: August 18, 2015**