Synthetic nanocarriers for the delivery of polynucleotides to the eye

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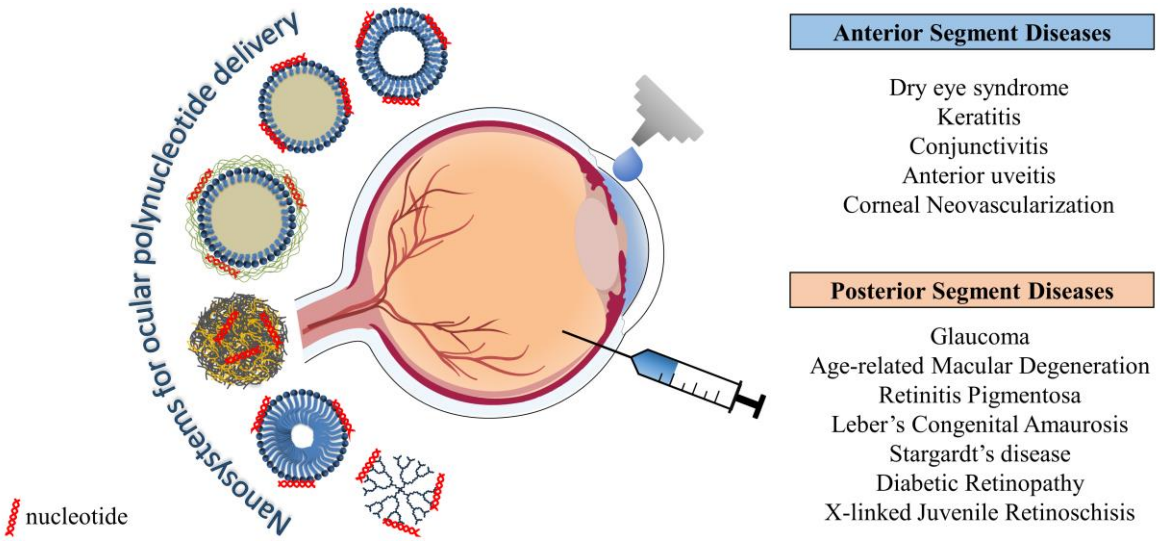
Abstract

This review is a comprehensive analysis of the progress made so far on the delivery of polynucleotide-based therapeutics to the eye, using synthetic nanocarriers. Attention has been addressed to the capacity of different nanocarriers for the specific delivery of polynucleotides to both, the anterior and posterior segments of the eye, with emphasis on their ability to (i) improve the transport of polynucleotides across the different eye barriers; (ii) promote their intracellular penetration into the target cells; (iii) protect them against degradation and, (iv) deliver them in a long-term fashion way. Overall, the conclusion is that despite the advantages that nanotechnology may offer to the area of ocular polynucleotide-based therapies (especially AS-ODN and siRNA delivery), the knowledge disclosed so far is still limited. This fact underlines the necessity of more fundamental and product-oriented research for making the way of the said nanotherapies towards clinical translation.

Keywords: polynucleotides; nanocarriers; ocular polynucleotide delivery; anterior segment; posterior segment; eye.



1. Introduction

According to the World Health Organization (WHO), 285 million individuals (4.25% of the world's population) suffered from visual impairment in 2010, of which 246 million had low vision and 39 million were blind (Pascolini and Mariotti, 2011). Furthermore, it is predicted that by 2020, 76 million individuals will presumably suffer from blindness mainly due to cataract, glaucoma and age-related macular degeneration (AMD) (Pizzarello et al., 2004). This scenario underlines the necessity of more innovative and effective ocular therapy strategies. Nowadays approaches based on polynucleotide ocular delivery hold great promise since they may alter gene expression without affecting the structure and sequence of the gene.

The eye is an attractive organ for the development of polynucleotide-based therapies due to the fact that the target tissues are accessible without the need of systemic administration. However, apart from this, the eye is protected by extraordinary barriers, which are very difficult to circumvent, especially in the case of hydrophilic and high molecular weight molecules such as polynucleotides. These barriers are illustrated in Fig. 1, and are briefly described as follows:

In the anterior segment, the first barrier encountered by topically applied molecules is the tear film that is composed of three layers consisting of lipid, aqueous fluid and mucus layers. The presence of different enzymes and mucins in the tear film as well as its constant turnover protect the eye against external pathogens. This is followed by the glycocalyx which is formed by cell surface mucins and covers the surface of the corneal and conjunctival epithelia (Spurr-Michaud et al., 2007). The corneal barrier consists of a transparent and avascular multiple layer epithelium, a collagenous layer (stroma) and an internal endothelium. The corneal epithelium continues with the conjunctiva, a transparent and vascularized epithelial membrane that contains goblet cells which are responsible for the production of the mucin MUC5AC (Ruponen and Urtti, 2015). In addition, the presence of tight junctions in both tissues constitutes an obstacle for permeation of drugs, especially through the cornea (Yoshida et al., 2009). The aqueous humor is also part of the anterior segment and it is mostly composed of water and electrolytes, low molecular weight compounds and proteins (de Berardinis et al., 1965; Tripathi et al., 1989).

The posterior segment is protected by the sclera, which represents the continuation of the cornea, and it is formed by the vitreous humor, retina, choroid and optical nerve. The vitreous humor is a highly dense matrix mainly composed of collagen, hyaluronic acid (HA) and also proteoglycans that contain negatively charged glycosaminoglycans (GAGs), that can hinder the diffusion of drugs to the retina, even when they are directly injected into this compartment (Peeters et al., 2005). However, it can also serve as a reservoir for the sustained release of drugs (Bourges et al., 2003). The retina encompasses different cell layers consisting mainly of nerve cells (ganglion cell layer (GCL)), photoreceptors and retinal pigment epithelium (RPE).

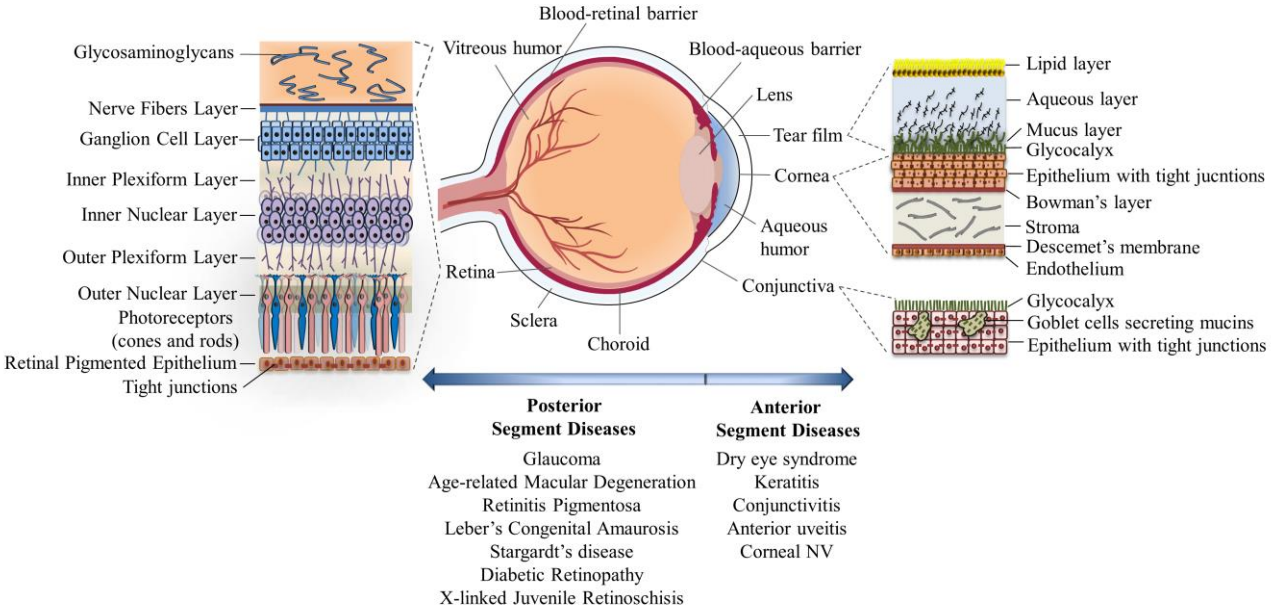


Fig. 1 Representation of the structure of human eye (in more detail the tear film, cornea, conjunctiva, vitreous humor and retina) and some examples of diseases affecting both anterior and posterior segments.

Both the anterior and posterior segments are also protected by blood-barriers, the blood-aqueous and the blood-retinal barriers, respectively. The blood-aqueous barrier contains the uveal endothelium and ciliary epithelium. This barrier restricts the access of compounds such as plasma albumin and hydrophilic drugs into the aqueous humor, but it is also responsible for the passage of nutrients essential for corneal function (del Amo and Urtti, 2008). The inner and outer blood-retinal barrier is formed by the retinal vessels' endothelial cells and the retinal pigment epithelium cells, respectively. In both parts of the blood-retinal barrier, the constituent cells are connected by tight junctions. This barrier plays a fundamental role in the regulation of nutrients flux and the restriction of drug diffusion into and out of the retina (Mannermaa et al., 2006).

There are several routes intended to reach either the anterior or posterior segments of the eye. Topical administration and subconjunctival injections are normally oriented towards treating the anterior segment whereas intravitreal (IVT) and subretinal injections are the most common methods used for the treatment of relevant diseases that affect the back of the eye.

In the next sections we will comparatively analyze the nanotechnology-based strategies that have been reported so far to deliver polynucleotides to both the anterior and posterior segments of the eye. We will highlight their potential for targeting specific tissues and thus, for the treatment of specific ocular diseases. We will conclude with a perspective of the challenges that need to be overcome for the clinical translation and industrial development of these nanomedicines.

2. Polynucleotides used for the treatment of ocular diseases

The polynucleotides that have been studied until now as potential treatments for ocular disorders are described below.

Plamid DNA (pDNA)

Plasmid DNA-therapeutics aims to express a specific therapeutic gene. Therefore, the plasmid needs to be internalized into the nucleus of the cell (which is still a challenge) where it will be transcripted into a messenger RNA (mRNA). Thereafter, the newly formed mRNA is transported into the cytoplasm where it is translated into the codified protein. The first nucleotide-based therapy reaching clinical trials was a pDNA construct proposed for the treatment of an immunodeficiency disease caused by an adenosine deaminase deficiency (Blaese et al., 1995). Since then, according to the clinical trials database (clinicaltrials.gov), several pDNA clinical trials have been conducted although only three of them have been oriented to the treatment of ocular diseases focusing on intraocular melanoma and allergic rhinoconjunctivitis.

Antisense oligonucleotides (AS-ODNs)

AS-ODNs are synthetic single-stranded RNA fragments (13 to 25 nucleotides) firstly described in 1978 (Stephenson and Zamecnik, 1978), that bind to complementary intracellular mRNA strands by base pairing, forming a short double helix and ultimately blocking its transcription into the undesirable protein. AS-ODNs can also modulate gene expression by enzymatic degradation of targeted mRNA by ribonuclease H (Walder and Walder, 1988). The activity of AS-ODNs is highly limited due to their poor intracellular uptake and poor stability in biological fluids (Opalinska and Gewirtz, 2002).

The only AS-ODNs-based drug (without the association to any type of carrier) approved by the FDA for an ocular condition was registered in 1998. This nucleic-acid based drug, fomivirsen, was marketed as Vitravene® for the treatment of cytomegalovirus (CMV)-induced retinitis in immunocompromised patients (Crooke, 1998). However, in 2004 Novartis Ophtalmics discontinued the product due to the significant decrease of Vitravene® sales as a consequence of the low number of patients infected with CMV. Other AS-ODNs are currently under clinical trials for the treatment of different ocular diseases (see Table 1). For instance, aganirsen (GS-101) has completed a phase III clinical trial for the topical treatment of corneal neovascularization and it is currently in phase II for the treatment of AMD, neovascular glaucoma, retinopathy of prematurity and diabetic macular edema.

Small interfering RNA (siRNA)

RNAi-based technology, namely siRNA is a promising alternative for treating eye diseases affecting both, the anterior and posterior segments of the eye. This is a double-stranded RNA (dsRNA) of 21-23 base pairs designed to specifically knockdown target genes (Elbashir et al., 2001). Unlike pDNA, this type of polynucleotide only needs to get into the cytoplasm of the cell where it is loaded into the RNA-induced silencing complex (RISC) (Hammond et al., 2000).

The first clinical trial using a siRNA (Cand5) was conducted in 2004 for the treatment of wet AMD and, since then, other clinical trials based on siRNA have been performed for ocular therapies such as Sirna-027 for choroidal neovascularization (CNV), PF-04523655 for diabetic retinopathy, SYL1001 for dry eye and SYL040012 and QPI-1007 for glaucoma (see Table 1). Moreover, several siRNA therapies are under preclinical development for corneal neovascularization (corneal NV), retinitis pigmentosa, diabetic retinopathy, and fibrotic eye disease, among others. Nonetheless, the majority of the undergoing siRNA studies target diseases affecting the retina.

As other polynucleotides, siRNA also suffers from poor stability in biological fluids and restricted capacity to enter cells. Different chemical modifications have been performed in the structure of polynucleotides especially to ameliorate their stability when in contact with biologic fluids thus, improving their bioavailability (Beigelman et al., 1995; Beverly et al., 2006; Chiu and Rana, 2003; Epstein and Kurz, 2007; Hall et al., 2004).

Aptamers

Aptamers are small molecules synthesized from DNA or RNA sequences that have the capacity to bind to specific proteins, as well as to nucleic acids and other compounds. Due to their unique three-dimensional structure they may act in a similar way as antibodies do but with the advantages of being non-immunogenic and highly stable molecules (Chandola et al., 2016). The only aptamer that has received marketing approval, in 2004, for ocular administration is Pegaptanib sodium (Macugen®), which is an RNA-based aptamer directed against vascular endothelial growth factor (VEGF) (Xu et al., 2008). There are other aptamers like E10030 also known as pegpleranib (Fovista®) and ARC1905 (Zimura®) that are under clinical trial for the treatment of AMD (Table 1).

Table 1. Products under clinical development for both the anterior and posterior segments of the eye.

Product	Indication	Target	Administration route	Developer	Status
pDNA					
CryJ2-DNA-LAMP	Allergic Rhinoconjunctivitis	JRC allergens	Intramuscular injection	Immunomic Therapeutics, Inc.	Completed Phase I
Mouse gp100	Intraocular melanoma	Gp 100	Intramuscular / epidermal jet injection	Memorial Sloan Kettering Cancer Center	Completed Phase I/II
siRNA					
QPI-1007	NAION	Caspase 2	IVT	Quark	Completed Phase I, Recruting Phase II/III
	APACG				Completed Phase II

PF-04523655	Wet AMD	RTP801	IVT	Quark/Pfizer	Completed Phase I and phase II
	DME				Terminated Phase II
SYL040012 (Bamosiran)	Glaucoma	β2 ADR	Eye drop	Sylentis	Completed Phase I and Phase II
SYL1001	Ocular pain associated to DES	TRPV1	Eye drop	Sylentis	Completed Phase I and Phase II
RXI-109	Subretinal fibrosis, Wet AMD	CTGF	IVT	Rxi	Recruting Phase I/II
AS-ODN					
GS-101 (aganirsen)	iCRVO Patients at Risk of Developing NVG	IRS-1	Eye drop	Gene Signal	Not yet recruting Phase II/III
ISTH0036	Glaucoma, undergoing trabeculectomy	TGF-β2	IVT	Isarna Therapeutics	Active Phase I
iCo-007	DME and DR	c-raf kinase	IVT	iCoTherapeutics	Completed Phase I
IONIS-GSK4-LRx	Undisclosed	Undisclosed	IVT	Ionis Pharmaceuticals	Active Phase I
Aptamer					
E10030 (Fovista®)	Wet AMD	PDGF-B	IVT	Ophthotech Corporation	Terminated Phase II and Phase III Active Phase III Active Phase I
ARC1905 (Zimura®)	IPCV, Dry and Wet AMD GA and MD	C5	IVT	Ophthotech Corporation	Completed Phase I Active Phase II

pDNA, plasmid DNA; siRNA, small interfering RNA; AS-ODN, antisense oligonucleotides; NAION, Non-arteritic anterior ischemic optic neuropathy; APACG, Primary angle closure glaucoma; AMD, Age-related macular degeneration; DME, Diabetic macular edema; DES, Dry eye syndrome; iCRVO, Ischemic central retinal vein occlusion; NVG, Neovascular glaucoma; DR, Diabetic retinopathy; IPCV, Idiopathic polypoidal choroidal vasculopathy; GA, Geographic atrophy; MD, macular degeneration; JCR, Japanese Red Cedar pollen; Gp 100, glycoprotein 100; RTP801, hypoxia-inducible factor 1-responsive gene; β2 ADR, β2-adrenergic receptor; TRPV1, transient receptor potential vanilloid 1; CTGF, connective tissue growth factor; IRS-1, insulin receptor substrate-1; TGF-β2, transforming growth factor β2; PDGF-B, platelet-derived growth factor; C5-complement component 5; IVT, intravitreal.

3. Synthetic nanocarriers for the delivery of polynucleotides to the eye

In general, the delivery of polynucleotides has been attempted using viral vectors (adenovirus (Ads), adeno-associated virus (AAVs), lentivirus, and retrovirus) and non-viral carriers (e.g. nanoparticles, liposomes, dendrimers, nanoemulsions, micelles, etc.). Currently, there are already some approved gene therapy products using viral vectors. These products include a recombinant Ads-p53 gene therapy for the treatment of head and neck squamous cell carcinoma (Gendicine®) (Pearson et al., 2004), an oncolytic virus that promotes cytotoxicity in cancer cells for nasopharyngeal cancer (Oncorine®), a retroviral vector loaded with a human cytocidal cyclin G1 construct for the treatment of solid tumors (Rexin-G®) (Gordon and Hall, 2010) and an AAV1 delivering a

human lipoprotein lipase (LPL) variant for the treatment of LPL deficiency (Glybera®) (Ylä-Herttuala, 2012). While these therapies have been approved in China (i.e. Gendicine®, Oncorine®), the Philippines (Rexin-G®) and Europe (Glybera®), major concerns associated to their immunogenic and mutagenic risks remain (Thomas et al., 2003). These concerns have motivated the search of synthetic delivery systems, which are safer and easier to produce in large scale.

Currently, there are a few ocular nanotechnology-based products on the market, that include a few over the counter products for the treatment of dry eye syndrome (DES), such as nanoemulsions (Lipimix™, Soothe XP®, Cationorm®), and liposomes (Lipomil®), as well as drug-containing nanomedicines, such as cyclosporin-A nanoemulsion (Restasis®), indicated for severe DES, a difluprednate nanoemulsion (Durezol®) indicated for the treatment of ocular inflammation, verteporfin liposomes (Visudyne®), a photodynamic therapy and pegaptanib (Macugen®), a PEGylated anti-VEGF, both approved for the treatment of AMD. Despite of this, the development of synthetic nanocarriers for the delivery of polynucleotides to the eye is still in an early stage. This early development is illustrated in Fig. 2, whereby the number of research articles describing non-viral carriers for ocular polynucleotide therapy has been scarce until the last decade.

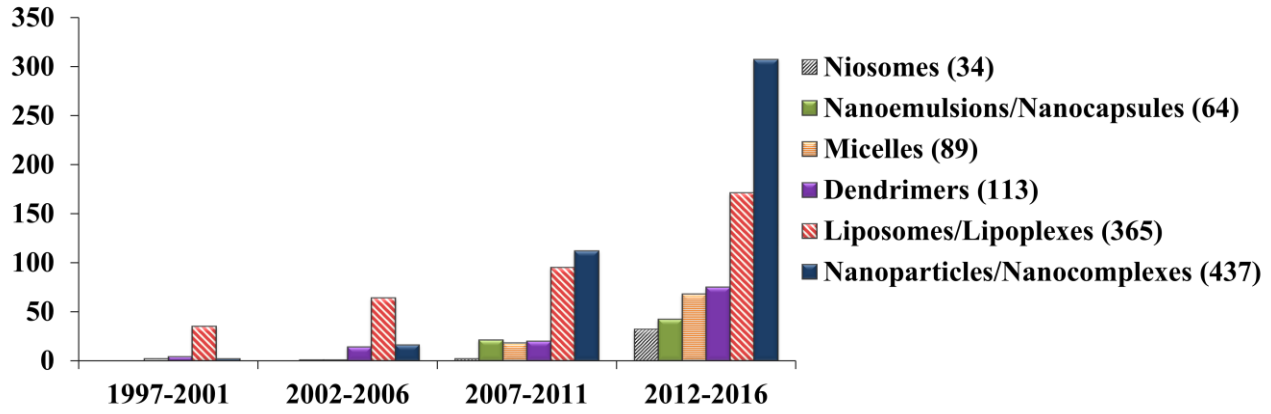


Fig. 2 Illustration of the evolution of the number of published research articles describing the use of non-viral carriers for ocular polynucleotides delivery from 1997 to 2016. Data obtained from Scopus. Search defined with the combination of keywords and or groups of keywords: [ocular, eye] and/or [pDNA, RNA, siRNA, antisense oligonucleotides, polynucleotides] and/or [nanoparticles, nanocomplexes, liposomes, lipoplexes, niosomes, micelles, nanoemulsions, nanocapsules, dendrimers]. Language: English.

An additional observation is that polymeric nanoparticles/nanocomplexes followed by liposomes have been the nanostructures that have received most of the attention up to now. Fig. 3 shows how most of the research so far has been concentrated on pDNA delivery although as described in the next section, the tendency is now being oriented towards RNA-based therapies.

Fig. 4 illustrates non-viral delivery systems currently under development for ocular polynucleotide/drug delivery. Overall, the composition and characteristics of the nanocarriers currently under investigation are discussed below.

Nanocomplexes and Nanoparticles

The design of these non-viral carriers for polynucleotide delivery has evolved over time going from simple nanocomplexes of cationic polymers, e.g. polyethyleneimine (PEI), and polynucleotides (dos Santos et al., 2006a; dos Santos et al., 2006b; Ketola et al., 2013; Kuo et al., 2005) to more defined nanoparticles. These nanoparticles have been developed using an array of biomaterials which include hydrophobic and amphiphilic polyesters, such as poly(lactic-co-glycolic acid) (PLGA) and PLGA-polyethylene glycol (PLA-PEG) (Csaba et al., 2005; Perez et al., 2001), which were investigated at first by our laboratory, as well as proteins such as gelatin (Xu et al., 2008), albumin (Arnedo et al., 2004), and cationic polymers, mainly poly-L-lysine (PLL) (Ketola et al., 2013; Männistö et al., 2002) and chitosan (Csaba et al., 2009; de la Fuente et al., 2008a; de la Fuente et al., 2008b).

Our group was among the pioneers in the development of nanoparticles for topical ocular drug delivery (Calvo et al., 1996; Calvo et al., 1994; Losa et al., 1991; Losa et al., 1993) and reported for the first time the potential of chitosan/hyaluronic acid nanoparticles for the delivery of polynucleotides, i.e. pDNA, to the eye (de la Fuente et al., 2008a; de la Fuente et al., 2008b). The inclusion of HA allowed a better retention and permeation through the rabbits' corneal epithelium, which resulted in a more efficient transfection of the epithelial cells (de la Fuente et al., 2008a; de la Fuente et al., 2008b). The combination of different characteristics of these systems, such as biocompatibility, mucoadhesion and targeting of CD44 receptors make them suitable carriers for polynucleotide delivery to the cornea and conjunctiva. In a different study, Urtti's group determined that the HA coating of DNA/PEI complexes decreases PEI's toxicity by shielding the positive charges and reducing non-specific interactions with cell membrane by the CD44 receptor targeting (Hornof et al., 2008).

With regard to the properties that influence the interaction of nanoparticles with the corneal epithelium, we have found that, in addition to the size (Calvo et al., 1996), the surface composition of the nanoparticles and their charge play an important role in their interaction with the corneal epithelium. For example, we observed that chitosan nanoparticles have the ability to interact with the ocular mucosa and be internalized by the corneal epithelial cells (de Campos et al., 2004). Other authors have explored the functionalization of particles with specific targeting ligands, such as transferrin and arginine-glycine-aspartic acid (RGD), which are expected to improve nanoparticle uptake by ocular cells (Chen et al., 2013; Singh et al., 2009).

Liposomes and lipoplexes

As an alternative to polymers, cationic lipids such as 3-β[N-(N',N' imethylaminoethane)- carbamoyl] cholesterol (DC-cholesterol) and 1,2-Dioleoyl-3- trimethylammonium propane (DOTAP) (Jääskeläinen et al., 2000; Lajunen et al., 2014; Matsuo et al., 1996; Rajala et al., 2014), have also been used to produce complexes with polynucleotides, named lipoplexes or liposomes. Cationic lipids, in addition to their capacity to complex polynucleotides, are supposed to help the liposomes interacting with the corneal epithelium (Jiang et al., 2012). Neutral lipids such as 1,2-dioleoyl-3- hosphatidylethanolamine (DOPE) have also been included in liposomal formulations as "helper" lipids since they can change the conformation of these systems to an inverted hexagonal organization thus facilitating their endocytosis and delivering the polynucleotide into the cytoplasm (Koltover

et al., 1998; Smisterová et al., 2001). The surface modification of liposomes with polymers has also been studied as a way to enhance their stability, e.g. through PEGylation (Bochot et al., 2002; Chen et al., 2013; Liu et al., 2011) and to facilitate their internalization and transfection efficiency through the use of peptide penetration enhancers (Mannermaa et al., 2005; Rajala et al., 2014).

Stimuli-responsive liposomes have also been developed. This is the case of the light-induced liposomal formulation containing verteporfin (Visudyne®), which has been marketed for the treatment of AMD. Due to the different laser applications in ophthalmology, and the time- and site-specific drug release from light-activated liposomes, these systems are of particular interest for the ocular field (Lajunen et al., 2016b). More specifically, indocyanine green-liposomes might be an attractive option for ocular drug delivery. Indocyanine green is an FDA approved imaging agent and the only one approved for clinical use under near infrered (NIR) light, which is less damaging that UV light. Moreover, a fast exposure of these light-activated liposomes to NIR light, led to a complete release of the loaded calcein and FITC-Dextran (Lajunen et al., 2016a).

Niosomes

These nanostructures are made of amphiphilic non-ionic surfactants such as Span® 60, Brij® 35, Brij® 78, Brij® 98 (Kaur et al., 2012; Saettone et al., 1996), which are known for their penetration enhancer capacity. Several niosome formulations have been developed for ocular drug delivery and some of them have shown promising results for ocular polynucleotide delivery (Ojeda et al., 2016; Puras et al., 2015; Puras et al., 2014). Still, as shown in Fig. 3, these carriers are the least common nanocarriers used for polynucleotide delivery to ocular tissues.

Micelles

Micelles comprise self-assembling diblock or multiblock amphiphilic molecules forming highly ordered monolayer structures. An example of a triblock copolymer micelle approved by the FDA for ophthalmic products is the micellar system formed by the copolymer poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) (Tong et al., 2007). This specific composition has been explored for the delivery of pDNA to different ocular tissues (Liaw et al., 2001; Tong et al., 2007).

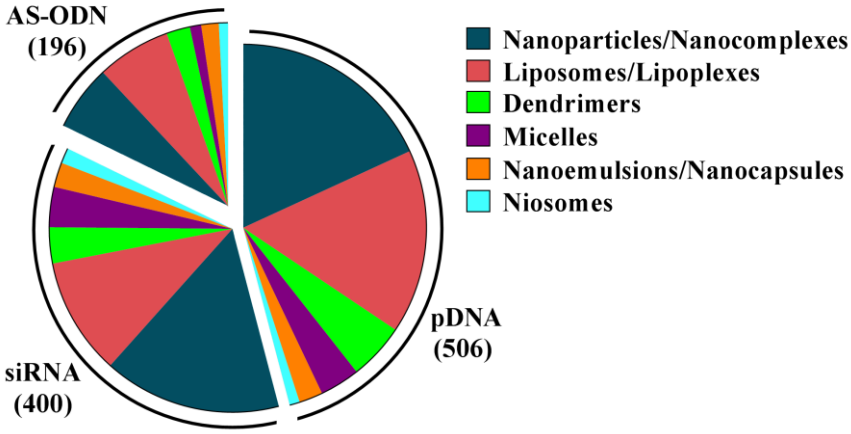


Fig. 3 Illustration of the number of published research articles describing the use of non-viral carriers for ocular pDNA, siRNA and AS-ODN delivery from 1997 to 2016. Data obtained from Scopus. Search defined with the combination of keywords and or groups of keywords: [ocular, eye] and/or [pDNA, siRNA, antisense oligonucleotides] and/or [nanoparticles, nanocomplexes, liposomes, lipoplexes, niosomes, micelles, nanoemulsions, nanocapsules, dendrimers]. Language: English.

Nanoemulsions and Nanocapsules

Nanoemulsions were evaluated in the early 90s by Benita's group for topical ocular drug delivery (Muchtar et al., 1992), and a few years later they were suggested for AS-ODN delivery (Teixeira et al., 1999). More recently, Benita's group evaluated cationic nanoemulsions which contain the surfactant DOTAP to enhance the carrier residence time on the ocular surface and efficiently deliver AS-ODNs to the retina (Hagigit et al., 2010; Hagigit et al., 2012).

Nanocapsules share common features with nanoemulsions and polymer nanoparticles, as they are formed by oily nanodroplets surrounded by a polymer coating. Our group pioneered the development of nanocapsules for topical ocular drug delivery (Calvo et al., 1997; Losa et al., 1993). Interestingly, working with PEGylated polyester nanocapsules we also observed that the PEG coating was critical in terms of preserving the stability of these nanocapsules in the ocular fluids and, this improved stability was translated into a greater transport of the nanocapsules across the corneal epithelium (de Campos et al., 2003). More recently, we have found that nanocapsules containing polyarginine and protamine arginine-rich polymer shells, have an improved ocular retention and can be used for corneal wound healing (Reimondez-Troitiño et al., 2016).

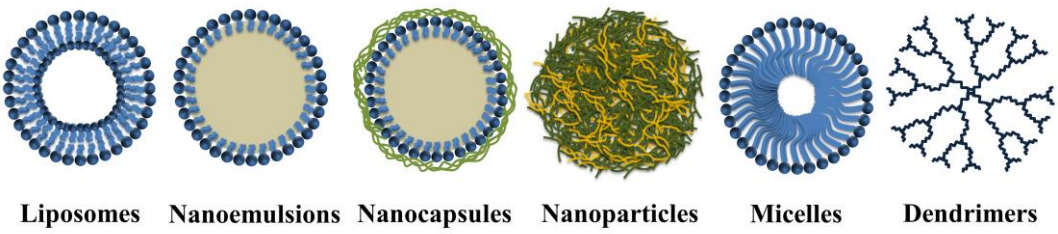


Fig. 4 Representation of the structure of different types of nanocarriers.

Solid lipid nanoparticles (SLN)

SLN are made of solid lipids such as Compritol®888 ATO, Precirol® ATO 5, Gelucire® 44/14 and stearylamine (del Pozo-Rodríguez et al., 2008; Li et al., 2008). These nanoparticles have been shown to facilitate drug penetration into the cornea (Cavalli et al., 2002; Li et al., 2008) and were used for the first time for ocular polynucleotide delivery in 2008 by del Pozo-Rodriguez (del Pozo-Rodríguez et al., 2008).

Dendrimers

Dendrimers are tree-like branched structures that consist of an inner core, repetitive branched units (i.e. different generations) and peripheral multivalent functional groups, which play a key role in the complexation with polynucleotides. The use of PAMAM (Chaum et al., 1999; Hudde et al., 1999) and PLL (Marano et al., 2004) based dendrimers for ocular polynucleotide delivery was first reported in 1999 and 2004, respectively. Since then, only a few reports have described the use of these nanocarriers for oligonucleotide (Marano et al., 2004; Wimmer et al., 2002) and pDNA (Hudde et al., 1999) ocular delivery and none for siRNA.

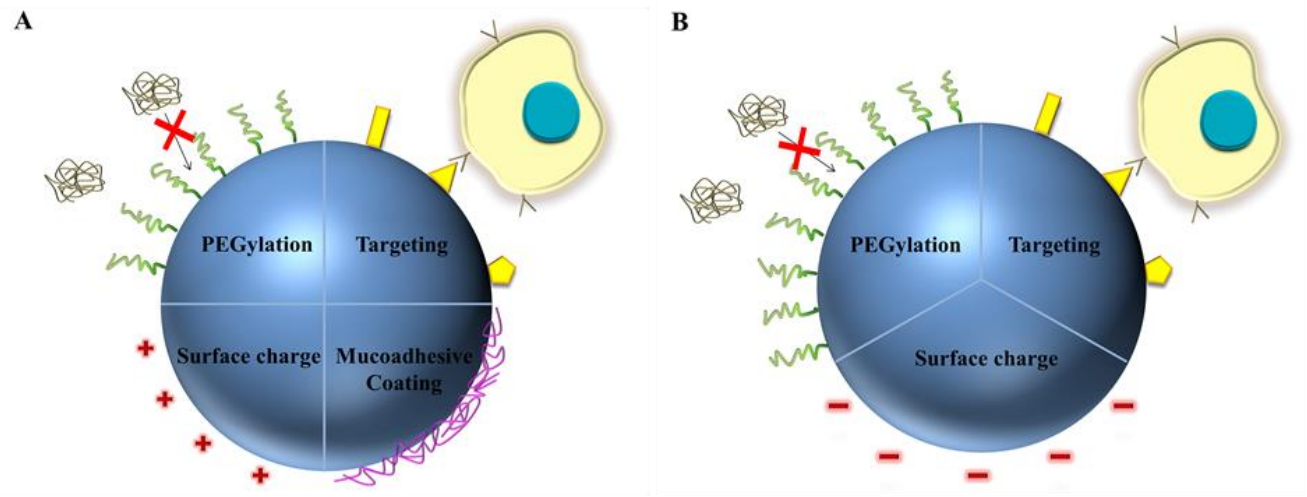


Fig. 5 Schematic representation of the main strategies used to develop nanocarriers aiming to treat different diseases affecting both anterior (A) and posterior eye segments (B). When targeting the anterior segment structures, the topically applied carriers usually present a positive surface charge and a mucoadhesive polymeric coating to increase the retention time in the ocular surface. They can also be PEGylated in order to be muco-penetrating and even include specific targeting moieties like arginine-glycine-aspartic acid sequences to target the desired tissue. When targeting the posterior eye segment, nanocarriers administered by intravitreal injection usually present a negative surface charge in order to avoid aggregation with the glycosaminoglycans present in the vitreous humor and a PEGylated surface to improve the diffusion through the vitreous. They might also present targeting moieties to target a specific tissue.

4. Site-specific nanocarriers-based polynucleotide delivery for the treatment of ocular pathologies

The main diseases affecting the anterior and posterior segments of the eye will be discussed in the next sections as well as the nanocarriers developed for polynucleotide delivery to both segments. Tables 2 and 3 summarize some of the nanocarriers used for the delivery of polynucleotides aiming at the treatment of the discussed diseases.

4.1. Nanomedicine approaches for the treatment of anterior segment ocular diseases

There are two main target tissues in the anterior segment of the eye, the cornea and the conjunctiva. The cornea represents a key challenge for many drugs due to its highly organized multilayer epithelium and the presence of tight junctions that limit the permeation of drugs and polynucleotides (Ruponen and Urtti, 2015). Several viral vectors and naked-polynucleotide formulations have reached the clinical development phase for the treatment of ocular disorders, although none of them have been marketed yet.

While the ocular delivery of polynucleotides has been achieved by physical means, such as electroporation (Blair‐Parks et al., 2002; Hao et al., 2009), iontophoresis (Berdugo et al., 2003; Hao et al., 2009), gene gun (Tanelian et al., 1997; Zhang et al., 2002) and sonophoresis (Yamashita et al., 2007), the use of nanocarriers offers specific advantages, such as (i) their nanometric size and components properties may allow their transport in the conjunctival and corneal epithelium (Amrite and Kompella, 2005), (ii) they may provide a sustained delivery of polynucleotides *in vivo* (Cohen et al., 2000; dos Santos et al., 2006a; Khan et al., 2004) and, (iii) they may target the cornea, the conjunctiva or both (de la Fuente et al., 2008a; de la Fuente et al., 2008b).

The most prevalent pharmacological ocular conditions in the anterior segment are DES, ocular inflammation (i.e. keratitis, allergic conjunctivitis, anterior uveitis), corneal wounds and, corneal NV.

Dry eye syndrome

DES is a multifactorial ocular pathology characterized by inflammation, pain and ocular discomfort due to insufficient tear secretion, excessive evaporation and alteration in the composition of the tear film (Pañeda et al., 2012). Current therapies for treating dry eye include drug-free artificial tears and nanosystems such as drug-free cationic nanoemulsions and cyclosporine A loaded nanoemulsions. As an alternative, polynucleotides have been proposed for the treatment of severe dry-eye associated to a deficiency of mucus glycoproteins, such as MUC5AC (Contreras-Ruiz et al., 2013; Konat Zorzi et al., 2011). For example, Contreras-Ruiz et al. (2013) developed cationized gelatin-based nanoparticles to deliver a plasmid encoding a modified MUC5AC protein (pMUC5AC). This nanoformulation was instilled to a dry eye mouse model and the result of the treatment was a reduction in ocular inflammation accompanied by an improved tear production (Contreras-Ruiz et al., 2013).

Keratitis, conjunctivitis, anterior uveitis

These are diseases related to inflammation in the cornea, conjunctiva and the anterior uvea, respectively. The most common treatment strategies for these types of infections are antimicrobial ophthalmic solutions in the form of eye drops containing different drugs (e.g. anti-histamines, non-steroidal anti-inflammatory drugs, antibiotics or corticosteroids). Polynucleotide-based therapies have also been considered as an alternative for

the treatment of severe infectious and inflammatory processes. For example, stromal keratitis and angiogenesis induced by herpes simplex virus-I (HSV) in mice have been reported to be significantly reduced by intravenous injection of cationic polyplexes of PEG-PEI-RGD and anti-VEGF siRNA (Kim et al., 2004a). In a different study, PEI-siRNA complexes targeting the HSV-1 infected-cell polypeptide 4 gene were evaluated on a mouse model of herpes simplex keratitis. Following topical administration, the said complexes were found to inhibit HSV-I replication *in vivo* for 96 h (Li et al., 2014).

Corneal neovascularization

Corneal NV is a pathological event that occurs associated with many ocular diseases that can cause blindness. Corneal NV means the formation of blood vessels within the transparent avascular tissue due to inflammation, infection and hypoxia, among other reasons. The available treatments include topical corticosteroids and non-steroidal anti-inflammatory eye drops and anti-VEGF-A compounds, such as bevacizumab, which were found to have limited clinical efficacy and negative side effects. Based on the critical role that angiogenesis plays in ocular neovascularization diseases, attempts have been made to attack these diseases using new anti-VEGF polynucleotide therapies (targeting VEGF or its receptors). PLGA nanoparticles loaded with a plasmid containing a small hairpin RNA (shRNA) expression cassette against VEGF-A (pSEC.shRNA.VEGF-A) were injected into the corneal stroma in a corneal NV mice model. The plasmid-loaded nanoparticles were found to be effective in reducing the corneal expression of VEGF-A (Qazi et al., 2012). In another study, human serum albumin (HSA) nanoparticles encapsulating a plasmid (pCMV.Flt23K) were also injected into the cornea of mice and tested for their efficacy against corneal NV (Jani et al., 2007). The results showed that HSA nanoparticles provided a 40% reduction corneal NV after 5 weeks of treatment. A faster response was observed upon subconjunctival injection in mice of PEGylated micelles containing the VEGFR1 plasmid (sflt-1). In this case, at seven days post-injection the corneal neovascularized area was reduced by 45% (Iriyama et al., 2011).

In summary, only a few studies, which are summarized in Table 2, have disclosed the efficacy of synthetic nanocarriers for the treatment of eye-surface diseases either following topical instillation or intra-cornea/conjunctival injection. Nonetheless, and despite their ability to improve the retention time of polynucleotides in the ocular surface and transfect tissues, i.e. cornea and conjunctiva, their efficiency is difficult to judge. In fact, the *in viv*o studies reported so far do not provide a comparison of these new polynucleotides therapies with the currently available treatments.

Table 2. Polynucleotide-loaded nanomedicines for the treatment of diseases affecting the anterior segment of the eye.

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Dry eye syndrome					
Gelatin nanoparticles	pMUC5AC	Eye drops	Dry eye mice model	Reduced ocular inflammation; improved tear production	(Contreras-Ruiz et al., 2013)
Herpes Simplex Keratitis					
PEG-PEI-RGD polyplexes	Anti- VEGF siRNA	Intravenous injection	HSK mice model	Reduced stromal keratitis and angiogenesis	(Kim et al., 2004b)
PEI complexes	ICP4-siRNA	Eye drops	HSK mice model	Inhibited HSV replication	(Li et al., 2014)
Corneal NV					
PLGA nanoparticles	pSEC.shRNA.-VEGF-A	Corneal intrastromal injection	Corneal NV mice model	Reduced VEGF-A expression	(Qazi et al., 2012)
HSA nanoparticles	pCMV.Flt23K	Corneal intrastromal injection	Corneal NV mice model	40% reduction of Corneal NV after 5 weeks	(Jani et al., 2007)
PEGylated micelles	psflt-1	Subconjunctival injection	Corneal NV mice model	45% reduction of Corneal NV after 7 days	(Iriyama et al., 2011)

PEG, polyethylene glycol; PEI, polyethyleneimine; RGD, arginine-glycine-aspartic acid; PLGA, poly(lactic-co-glycolic acid); HSA; human serum albumin, pMUC5AC, plasmid encoding the mucus glycoprotein MUC5AC; ICP4-siRNA, siRNA targeting the infected-cell polypeptide 4 gene of HSV-1; pSEC.shRNA.-VEGF-A, plasmid containing a small hairpin RNA expression cassette against vascular endothelial growth factor A; pCMV.Flt23K, plasmid encoding domains 2 and 3 of the Flt binding domain for VEGF; psflt-1, plasmid encoding soluble VEGF receptor 1; HSK, herpes simplex keratitis; Corneal NV, corneal neovascularization; HSV, herpes simplex virus.

4.2. Nanomedicine approaches for the treatment of back of the eye diseases

Diseases affecting the retina can potentially be treated with polynucleotides-based therapies. The retina is a photosensitive tissue composed of three main layers or cell types. The retinal pigmented epithelium is in the outermost layer followed by the photoreceptors (cones and rods), and the retinal ganglion cells in the innermost layer. Defects in these cell layers can lead to AMD (retinal pigmented epithelium and photoreceptors), retinitis pigmentosa, Leber's congenital amaurosis (LCA) (photoreceptors), glaucoma and optic neuropathy (retinal ganglion cells). According to the WHO, among the diseases affecting the posterior segment of the eye, glaucoma and AMD are the main causes for blindness.

Subretinal injection is the most effective way to deliver drugs to the photoreceptors and RPE layer of the retina. Additionally, drugs can be injected into the vitreous humor through IVT injection that is less invasive and allows for a broader and more uniform transduction of the retina. Despite their efficacy, these methods are invasive and not acceptable when there is the need for frequent intraocular administrations. Repeated injections may lead to undesired side effects like high risk of infections, cataract development, vitreous hemorrhage and even retinal detachment and endophthalmitis that can potentially cause vision loss. Besides the risks of repeated injections, the poor stability of polynucleotides in biological fluids and their short vitreal half-life urges the need of developing carriers able to protect and deliver them in a specific, efficient and sustained way.

As summarized in Table 3, several types of nanocarriers have been used to deliver polynucleotides to the back of the eye. Such systems have aimed to target different tissues (i.e. choroid, macula, and retina) or cell types (i.e. RGC, photoreceptors, and RPE) of this eye segment, expressing mutations responsible for diseases like AMD, glaucoma, CNV and X-linked juvenile retinoschisis (XLRS), among others. These potential nanomedicines have been injected into the subretinal space or the vitreous humor. In this sense, it has been reported that positively charged particles may aggregate upon interaction with components present in these compartments, i.e. HA and GAGs, and this aggregation would ultimately hamper their cellular uptake (Peeters et al., 2005; Pitkänen et al., 2003). However, the vitreous humor can also act as a reservoir where the carriers can gradually release the compound of interest to yield a sustained delivery of drugs to the retina (Bourges et al., 2003).

Glaucoma

Glaucoma is characterized by the progressive damage of the optic nerve leading to retinal ganglion cells death and permanent vision loss. According to the WHO, glaucoma is the second cause of vision loss worldwide after cataracts, being responsible for 8% of all blindness cases (Pascolini and Mariotti, 2011). Factors like local ischaemia-hypoxia, excessive stimulation of the glutamatergic system, alterations in glial cells, aberrant immunity and mainly high intraocular pressure (IOP) seem to contribute to glaucoma (Weinreb and Khaw, 2004).

The current standard treatment for glaucoma involves medication to lower IOP levels by means of either diminishing aqueous humor production (beta blockers) or improving its drainage (prostaglandin analogs). These treatments are chronic and they often suffer from limited patient compliance. In addition, the side effects associated to systemic absorption are no negligible. Therefore, there is a clear need for advanced treatments and delivery technologies.

In vivo studies of nanocarriers loaded with different drugs (e.g. timolol, brimonidine tartrate, pilocarpine) have shown promising results with increased bioavailability, prolonged retention time and sustained drug delivery in addition to minimizing systemic absorption of the associated drugs (Reimondez-Troitiño et al., 2015). Along the same line, a number of patents on ophthalmic nanoformulations such as nanoemulsions (Carli et al., 2013), and contact lenses delivering nanoemulsions (Chauhan and Gulsen, 2012), have been recently issued.

Therapies based on polynucleotides could improve the commercially available anti-glaucoma treatments. Trabecular meshwork, ciliary epithelium and muscle and ganglion cell layer are some examples of target tissues for polynucleotide-based glaucoma therapy. However, research in this line is in a very early stage and has mainly made use of naked polynucleotides or viral vectors administered topically or as IVT and subretinal injections.

The studies reported until now to contribute to the development of polynucleotide-based nanomedicines for the treatment of glaucoma have used model polynucleotides associated to lipid nanocarriers. For example, Matsuo and colleagues (Matsuo et al., 1996) studied different liposomes loaded with pDNA encoding β-Gal through topical instillation. *In vivo* data in rats showed gene β-Gal expression for up to a month in conjunctival, corneal and retinal ganglion cells after administration of N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate (TMAG) and DC-cholesterol liposomes. A different approach consisted of liposomes with a viral envelope-coating of inactivated hemagglutinating virus of Japan HVJ (Sendai virus) (Hangai et al., 1996; Hangai et al., 1998a, b), which was supposed to allow the fusion of the liposomes with the cell membrane and deliver the encapsulated nucleotide into the cytoplasm. These liposomes were loaded with LacZ pDNA and a high-mobility group 1 (HMG1) nonhistone nuclear protein, which guides the pDNA into the nucleus. The *in vivo* results following intravitreal and subretinal injections of these formulations to rat and mice presented LacZ expression in the photoreceptors for more than 30 days (Hangai et al., 1996). Moreover, these liposomes encapsulating FITC-labeled phosphorothioate oligonucleotides were injected into the anterior chamber of rats and monkeys. Fluorescence was detected in the trabecular meshwork of monkeys and in the iris and ciliary body of rats lasting as long as 7 and 14 days, respectively (Hangai et al., 1998b).

Johnson et al. (Johnson et al., 2008) synthesized what they called a "peptide for ocular delivery (POD), containing specific aminoacids ($GGG(ARKKAAKA)_4$), which theoretically endowed the peptide with cell penetrating properties, and used it for the delivery of pDNA encoding a red fluorescent protein (pCAGRFP) and siRNA to the eye. After topical instillation to mice, the pDNA-POD complexes were found to penetrate the corneal epithelium, sclera, choroid and even the optic nerve. The same nanocomplexes were also found to enter and deliver the associated siRNA in the GCL and RPE, following subretinal and IVT injection, respectively. A different formulation tested *in vivo* was the one combining pDNA with surfactant gemini and "helper" lipids DOPE and DOPE:1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DOPE:DPPC). After topical application, these nanoparticles were found in the limbus, iris and conjunctiva for 48 h and, following IVT injection they were localized within the nerve fiber and GCL of the retina (Alqawlaq et al., 2014).

Among the presented approaches only model polynucleotides and healthy animal models were used, thus no therapeutic effect or comparison with current treatments has been reported.

Age-related macular degeneration

AMD is the leading cause of irreversible blindness in people over 60 years old and it is predicted to affect about 196 million people worldwide by 2020 (Wong et al., 2014). This multifactorial disease leads to a progressive degeneration of photoreceptors in the central retina that can result in blindness. There are two forms of AMD, the "dry" and "wet" AMD. Wet AMD, also known as CNV, is caused by the promotion of blood vessels growth

mainly by the protein VEGF, which affects the central part of the retina, while dry AMD or nonexudative form is characterized by RPE cell death and consequent photoreceptor degeneration (Pañeda et al., 2012). Currently, AMD has no cure but there are some FDA approved treatments especially for the wet form like pegaptanib (Macugen®) an anti-VEGF aptamer, ranibizumab (Lucentis®) an antibody fragment, and aflibercept (Eylea®) a recombinant fusion protein, which is quickly becoming the gold standard treatment. Bevacizumab (Avastin®) is a monoclonal antibody which has also been used as an off-label treatment.

With the idea of exploring new treatments for AMD, different pDNAs have been associated to different polymers originating nanoparticles and nanocomplexes of various compositions. For example, HSA has been found to be able to condense DNA and protect it from degradation (Langer et al., 2003; Mo et al., 2007). A plasmid encoding the Cu and Zn superoxide dismutase gene 1 (SOD1), a gene whose deficiency is associated with CNV and RPE dysfunction, was complexed with HSA and injected intravitreally to mice. Unfortunately, *in vivo* results revealed that protein expression was only detected for up to 2 days after IVT injection (Mo et al., 2007).

In another study, PLGA nanoparticles were used to deliver a shRNA-expressing pDNA targeting hypoxia-inducible factor-1α (HIF-1α), which regulates the transcription of pro-angiogenic factors like VEGF (Zhang et al., 2010). The i*n vivo* results obtained following IVT injection to a laser-induced CNV rat model showed a decreased of VEGF expression and a reduction on the extent of NV.

A different strategy involving the intravenous administration of PLGA nanoparticles functionalized with an RGD peptide, a ligand of integrin receptors, has been evaluated in mice and also primates. The results indicated that the said nanoparticles loaded with the plasmid pFlt23k.NR were able to get into the retina lesion, promote the plasmid expression for up to 6 weeks and lower the CNV (Luo et al., 2013). In another study, the intravenous administration of anti-VEGF plasmid-loaded PLGA nanoparticles, functionalized with RGD and transferrin peptides, led to a high gene expression in RPE, and the subsequent inhibition of CNV (Singh et al., 2009). These positive results obtained following intravenous administration are surprising if we take into account the blood-retinal barrier that considerably limits access to the retina. In our understanding, a high dose will be required when using this modality of administration with the potential undesired off-target effects. Recently, Lajunen and co-workers (Lajunen et al., 2014) demonstrated that transferrin-decorated liposomes were able to reach the RPE by topical instillation in Sprague Dawley rats, thus avoiding the possible undesired side-effects associated with IVT and intravenous administrations.

PEGylated liposomes containing protamine sulfate (PS) and HA loaded with a siRNA targeting VEGF-R1 were also tested *in vivo* through IVT injection in a laser-induced mice model of CNV. This system was able to protect the loaded siRNA and decrease the CNV area (Liu et al., 2011).

Dendrimers have also been tested as polynucleotide carriers in CNV animal models and could be considered as potential strategies for AMD treatment (Marano et al., 2004; Wimmer et al., 2002). For example, Marano et al. (2004) developed different lipid-lysine dendrimers that led to a significant reduction in VEGF expression levels and were able to significantly inhibit CNV in a mice model. A long-term study using the laser-induced CNV

mice model revealed that a single IVT injection of dendrimers loaded with ODN-1 inhibited up to 95% the development of CNV in rats and the response lasted for up to six months. This system was able to penetrate through all the retinal cell layers up to the RPE (Marano et al., 2005).

Finally, the cationic nanoemulsions originally developed by Benita's group (Hagigit et al., 2010; Hagigit et al., 2012; Hagigit et al., 2008) and containing DOTAP were used to deliver a 17-mer partially phosphorothioated ODN directed to the VEGF KDR receptor. This cationic nanoemulsion was administered by topical instillation and IVT injection and the results showed that, as expected, only the injected nanoparticles were able to reach the inner nuclear layer (INL) of the retina maintaining therapeutic levels for about 72 h after injection. (Hagigit et al., 2010).

Retinitis pigmentosa

This disease affects one in 3,500 to 5,000 people worldwide and can be inherited as autosomal recessive (50-60%), autosomal dominant (30-40%) or X-linked (5-15%). RP can be the result of mutations in more than 60 genes being one of them rhodopsin, which accounts for 25% of autosomal dominant RP cases (Anasagasti et al., 2012).

Based on the PEG stabilizing properties, the PEG-substituted lysine CK30-PEG was used for the formation of complexes with pDNA molecules of interest for RP treatment (Liu et al., 2003; Ziady et al., 2003). The use of this carrier allowed for the efficient delivery of a plasmid containing *Rds* cDNA and a rod opsin (MOP) promoter, and its expression in photoreceptors of a RP mouse model preventing cone degeneration. Gene expression was detected at least one month post-treatment (Cai et al., 2010). More recently, Han et al. used these particles to compare the efficacy between a genomic DNA (gDNA), which included introns from the rhodopsin gene, and a rhodopsin complementary DNA (cDNA). The introduction of specific genomic sequences improved the rhodopsin expression levels, delaying photoreceptor cells death and improving functionality up to eight months in a rhodopsin knockout (RKO) mouse model (Han et al., 2015).

X-linked juvenile retinoschisis

XLRS affects one in 5,000 to 20,000 males who are diagnosed very early within their first years of life. The progression of this disease usually involves vitreous hemorrhage and retinal detachment that ultimately can lead to vision loss. Significant progress has been achieved in understanding the genetic and molecular mechanisms responsible for this disease. Currently, about 190 disease-causing mutations have been identified in the retinoschisis RS1 gene. The RS1 protein is secreted in the retina and it is responsible for maintaining the retina's integrity (Molday et al., 2012).

Gascón's group developed a hybrid nanostructure consisting of a dextran-protamine sulphate–pDNA complex (i.e. pCMS-EGFP or pCEP4-RS1) adsorbed onto the surface of solid lipid nanoparticles (Delgado et al., 2012). These plasmid-loaded particles were administered to rats through three different routes: IVT injection, subretinal injection and topical instillation. The results of these studies presented plasmid expression in the cornea after topical instillation, the nanocarriers were also able to transfect the RPE cells and photoreceptors by

subretinal injection while by IVT administration gene expression was mainly detected in the ganglion cell layer. In a subsequent study, the same nanoparticles and a different kind containing HA instead of dextran (HA-protamine-pDNA-SLN) were administered by IVT and subretinal injections to C57BL/6 wild type and Rs1h-deficient mice models. Both carriers were able to transfect different layers of the retina, following both administration methods. However, when subretinal injection was used a higher amount of RS1 expression was detected in the photoreceptors and the expression was maintained for up to two months after injection it in GCL and INL. In addition, both carriers were able to lead to a partial recovery of the retina that consisted of mainly in a decreased loss of photoreceptors and an amelioration of the organization of retina layers (Apaolaza et al., 2015).

Leber's congenital amaurosis

The prevalence of LCA is between 1 in 81,000 newborns (Stone, 2007). Fourteen mutated genes have already been identified and they are responsible for photoreceptor cell death at an early age causing blindness. About 6 % of LCA patients present mutations in RPE65 gene that encodes for all-trans-retinyl-ester hydrolase (den Hollander et al., 2008).

PEG-Lysine complexed to a pEPI-eGFP and phRPE65, containing a scaffold matrix attachment region (S/MAR) and a macular dystrophy 2 promoter (VMD2), were subretinally administered to wild-type mice and to an RPE65-deficient LCA mice model, respectively (Koirala et al., 2013) S/MAR inclusion was used considering its self-replication capacity residing inside cells for more than 100 generations (Piechaczek et al., 1999) and increase gene expression (Kim et al., 2004b; Klehr et al., 1991). The treatment led to higher DNA expression levels that lasted at least two and a half years in the wild-type mice and despite the fact that only about 20% of the RPE cells expressed the gene, it still led to improvements in RPE65-deficient LCA disease that were noticed up to six months (Koirala et al., 2013).

More recently, a different strategy combining DOTAP/DOPE/cholesterol liposomes with protamine-plasmid complexes, nuclear localization signaling and cell-penetrating transactivator of transcription (TAT) peptides was developed. These liposomes were administered by subretinal injection in a RPE65-associated blind mice model and the results showed the capacity of these nanocarriers to maintain gene expression for at least three months. Furthermore, *in vivo* results revealed that the treatment led to blindness correction (Rajala et al., 2014).

Stargardt's disease

This autosomal recessive disease has no cure yet and it is the most common inherited juvenile macular degeneration affecting one in 8,000-10,000 people worldwide. Stargardt's disease causes problems to adapt to darkness and a progressive and irreversible loss of central vision. The disease is caused by mutations in the ABCA4 gene that encodes a protein of the ABC lipid transporter family (Allikmets et al., 1997). Hundreds of disease-causing mutations have been identified in this gene that can lead to Stardardt's or to cone-rod dystrophy and several forms of autosomal recessive RP (Cremers et al., 1998). To the extent of our knowledge, only PEG-Lysine complexed to pDNA have been assessed for this disease and gene expression was detected for about eight months improving mice's recovery of dark adaptation (Han et al., 2012).

Diabetic retinopathy

This ocular pathology is the most frequent complication of diabetes mellitus, a disease that according to the WHO affects more than 422 million individuals around the world. Diabetic retinopathy which affects more than 93 million people results from vascular abnormalities causing glial dysfunction and death of retinal neurons, and ultimately, blindness (Yau et al., 2012). Monoclonal antibody-based therapies, such as Eylea® and Lucentis® are some of the FDA approved treatments for this disease. As an alternative, different siRNA therapies, such as those targeting the connective tissue growth factor (CTGF) (Winkler et al., 2012), VEGF (Jiang et al., 2009), fibronectin, collagen and laminin (Oshitari et al., 2005), HIF-1α (Jiang et al., 2009), or hypoxia-inducible gene RTP801 (Nguyen et al., 2012) have been proposed. However, these siRNAs have not been used in association with nanocarriers so far.

Different approaches making use of anti-angiogenic microRNAs (Mitra et al., 2016) and plasminogen fragments (Park et al., 2009) associated to PEG-Lysine have also been explored. These nanocomplexes were intravitreally injected in a mouse model for late-onset diabetic retinopathy. A single administration reduced the expression of VEGFR-2, suppressing angiogenesis for at least three months after treatment (Mitra et al., 2016). In another study, PLGA nanoparticles loaded with a plasminogen fragment Kringle 5 (K5) plasmid were administered by IVT injection in oxygen-induced retinopathy (OIR) and streptozotocin-induced diabetic mice models. Similarly, a single injection led to a significant reduction of retinal vascular leakage and attenuated VEGF over-expression and retinal NV for at least one month (Park et al., 2009).

Despite the ability of synthetic nanocarriers for ocular polynucleotide delivery and all the potential advantages and the promising results observed in animal models, their delivery efficiency is still low. On the other hand, the majority of the clinical trials using polynucleotides for ocular diseases have used naked polynucleotide treatments and about 20% have combined them with viral vectors, especially AAVs,. These clinical trials have been oriented to the treatment of macular degeneration, LCA and Leber´s hereditary optic atrophy. Despite of this, it should be recognized that major advances in ocular polynucleotide-based therapies have already been achieved and that this field is now opening opportunities for future developmental programs. The main approaches used to improve non-viral carriers performance in the treatment of the previously discussed diseases affecting both anterior and posterior segments are summarized in Fig. 5 A and B, respectively.

Table 3. Polynucleotide-loaded nanocarriers for the treatment of conditions affecting the posterior segment of the eye.

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Glaucoma					
TMAG-DC-Cholesterol, liposomes	pCMV-β-Gal	Eye drops	Healthy rat	β-Gal expression in conjunctiva, cornea and RGC for 1 month	(Matsuo et al., 1996)
HVJ-AVE liposomes	pCMV-β-Gal	IVT and subretinal injections	Healthy rat	β-Gal expression in neural retina for 1 month	(Hangai et al., 1996)
HVJ-AVE liposomes	Phosphorothioated oligonucleotides	Injection into the anterior chamber	Healthy rat and monkeys	Fluorescence in trabecular meshwork for 7 (monkeys) and 14 days (rat)	(Hangai et al., 1998b)
Gemini nanoparticles	pCMV-tdTomato	Eye drops and IVT injection	Healthy mice	pDNA detected in retina's nerve fiber and GCL 48h after IVT injection	(Alqawlaq et al., 2014)
Age-related macular degeneration					
HSA nanoparticles	pSOD	IVT injection	Healthy mice	Protein expression detected 2 days after injection but not after 7 days	(Mo et al., 2007)
PLGA nanoparticles	pshHIF-1α	IVT injection	CNV rat model	Decreased VEGF expression and reduced CNV for 1 month	(Zhang et al., 2010)
RGD-functionalized PLGA nanoparticles	Flt23k plasmid	Intravenous injection	Primate and mice CNV models	Lowered CNV; improved 40% mice's vision	(Luo et al., 2013)
RGD/transferrin functionalized PLGA nanoparticles	Anti-VEGF intraceptor plasmid	Intravenous injection	CNV mice model	47-73% reduced CNV area, 2 weeks after treatment	(Singh et al., 2009)
Lipid-lysine dendrimers	Anti-VEGF oligonucleotide	IVT injection	CNV mice model	Inhibited by 95% the development of CNV for 4-6 months	(Marano et al., 2005)
PS/HA PEGylated lyposomes	siRNA targeting VEGF-R1	IVT injection	CNV mice model	Decreased CNV area	(Liu et al., 2011)
Cationic nanoemulsions (oleylamine, DOTAP, AOA)	AS-ODN against VEGF KDR	Eye drops, IVT injection	Healthy rabbits	Injected nanoemulsions reached the retina's INL	(Hagigit et al., 2010)
Retinitis pigmentosa					
CK30PEG nanoparticles	gDNA	Subretinal injection	RKO mice model	Improved rhodopsin levels and improved photoreceptors functionality for 8 months	(Han et al., 2015)

CK30PEG nanoparticles	MOP-NMP vector carrying *Rds* gene	Subretinal injection	Rod-dominant RP mice model	Prevention of cone degeneration	(Cai et al., 2010)
X-linked juvenile retinoschisis					
Dextran/PS SLN	pCMS-EGFP pCEP4-RS1	Eye drops Subretinal and IVT injections	Healthy mice	Transfection of different ocular tissues	(Delgado et al., 2012)
Dextran/PS SLN HA/PS SLN	pCAG-GFP-CMV-RS1	Subretinal and IVT injections	Healthy mice Rs1h-deficient mice model	Partial recovery of retina for 2 months	(Apaolaza et al., 2015)
Leber's Congenital Amaurosis					
CK30PEG nanoparticles	pEPI-EGFP pEPI-hRPE65 containing S/MAR and VMD2 promoter	Subretinal injection	Healthy mice RPE65-deficient mice model	Gene expression lasted for 2.5 years in the healthy mice Improved the phenotype of RPE65-deficient mice	(Koirala et al., 2013)
DOTAP/DOPE/ cholesterol/PS liposomes containing NLS and TAT	pcDNA3 pGFP chicken Rpe65 cDNA	Subretinal injection	RPE65-deficient mice model	Gene expression in RPE for 3 months Blindness correction	(Rajala et al., 2014)
Stargardt's disease					
CK30PEG nanoparticles	pEPI-CMV-EGFP with ABCA4 cDNA and MOP-ABCA4	Subretinal injection	ABCA deficient mice model	Gene expression in the retina for 8 months Improved recovery of dark adaptation	(Han et al., 2012)
Diabetic retinopathy					
CK30PEG nanoparticles	pCAG-miR200-b-IRES-eGFP	IVT injection	Ins2^{Akita} mice	Angiogenesis marked suppression	(Mitra et al., 2016)
PLGA nanoparticles	pK5	IVT injection	OIR mice model Diabetic mice model	Reduced retinal NV for 1 month	(Park et al., 2009)

TMAG, N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate; DC-cholesterol, 3-β[N-(N',N' imethylaminoethane)-carbamoyl] colesterol; HJV-AVE, inactivated hemagglutinating virus of Japan-artificial viral envelope; HSA, human serum albumin; PLGA, poly(lactic-co-glycolic acid); RGD, arginine-glycine-aspartic acid; PS, protamine sulfate; HA, hyaluronic acid; SLN, solid lipid nanoparticles; DOTAP, 1,2-Dioleoyl-3- trimethylammonium propane; AOA, arginine octadecyl amide; DOPE, 1,2-dioleoyl-3- hosphatidylethanolamine; NLS, peptide of nuclear localization signaling; TAT, cell-penetrating transactivator of transcription; pCMV-β-Gal, plasmid containing cytomegalovirus pomoter and enconding β-galactosidase gene; pSOD, plasmid encoding superoxide dismutase gene; pshHIF-1α, plasmid containing a small hairpin RNA targeting the hypoxia-inducible factor 1α; AS-ODN, antisense oligonucleotides; VEGF, vascular endothelial growth factor; gDNA, genomic DNA; pEPI-EGFP, plasmid encoding a green fluorescent protein; S/MAR, scaffold matrix attachment region; VMD2, vitelliform macular dystrophy 2 promoter; pK5, Kringle 5 plasmid; pCAG-GFP_CMV-RS1, plasmid encoding both GFP and retinoschisin; IVT, intravitreal; CNV, choroidal neovascularization; RKO, rhodopsin knockout; OIR, oxygen-induced retinopathy; GCL, ganglion cell layer; RGC, retinal ganglion cells; GCL, ganglion cell layer; INL, inner nuclear layer; RPE, retinal pigmented epithelium

5. Translational aspects of ocular delivery of polynucleotides

Despite the multiple reports centered on the benefits of nanotechnology for treating ophthalmic conditions it is surprising that only a small number of these products have obtained marketing authorization. As indicated in section 3, besides a variety of emulsions and liposomes, which are over-the-counter products for the treatment of DES, there are nanoemulsions containing specific drugs, such as cyclosporine A (Restasis®, Lipomil®) and difluprednate (Durezol®) for the treatment of ocular inflammation, and liposomes containing verteporfin (Visudyne®) and the PEGylated anti-VEGF aptamer, pegaptanib (Macugen®), both for the treatment of AMD. The majority of these products are emulsions composed of well-known oils (castor oil or medium-chain triglycerides oil), emulsifiers (polysorbate 80, pemulen, poloxamer 188, tyloxapol or cetalkonium chloride) and excipients to maintain tonicity and pH. However, the application of more sophisticated technologies for the development of oligonucleotide-based nanomedicines is associated to significant challenges as highlighted below.

The term nanomedicine includes an ample range of products with enormous variations in size, shape, materials and other characteristics. This heterogeneity makes consensus definitions and classifications of these products difficult to establish from the regulatory standpoint, thus complicating the generation of appropriate regulatory guidance. Both, the FDA and the EMA, however, consider that the development of a product that falls within the definition of nanotechnology may require special attention in terms of toxicity and safety.

Market authorization of pharmaceuticals requires a full physical and chemical characterization of the drug. The increasing complexity of nanomedicines indicates that characterization has most likely to be tailored for the specific product in development. The initial phases of product development may require a thorough characterization to select the specific tests that will subsequently be used to release batches and test the quality of the final product. Therefore, as with other medicinal products, it is important to identify the critical quality attributes (CQAs) that maintain a direct relationship with products quality, efficacy and toxicity; these CQAs should be the basis for the product's quality control throughout its lifecycle. CQAs for ophthalmic nanomedicines may include aspects related to nanotechnology such as particle size, size distribution, surface charge, hydrophilicity; but also attributes related to pharmaceuticals, such as purity, stability, sterility, and manufacture controls. Among the ophthalmic products approved so far, only the cationic nanoemulsion containing cyclosporine A specifically lists CQA related to nanotechnology: particle size and zeta potential. This is important as it is well known that size has a direct impact on the biodistribution and clearance of the nanomedicine. For instance, subconjunctival administration of fluorescent polystyrene nanoparticles has shown that 20 nm nanoparticles exhibit a rapid clearance from ocular and periocular tissues while 200 nm nanoparticles made of the same material are retained in the eye for a period of two months (Amrite and Kompella, 2005). It should also be considered that the nanomaterial administered to the eye can aggregate, thereby influencing the *in vivo* behavior of the nanomedicines.

On the other hand, although small scale manufacturing of different types of nanomedicines in research laboratories is fairly common, with the exception of nanoemulsions, the experience in large-scale manufacturing is still limited. Large-scale manufacturing requires standardization and manufacturing under Good

Manufacturing Practices (GMP). Factors affected by the scale-up include changes in particle size, drug loading, quality and quantity of impurities, structural alterations, among others. In particular, for oligonucleotides changes in manufacturing processes may lead to decreased stability or even degradation of the product, as a consequence, a very well controlled small-scale process may turn out to be unreliable or non-reproducible at a larger scale if the process has not been efficiently controlled during the scale-up. For multi-step processes it is usually useful to establish mid-process controls that can inform of the quality of the intermediate products in order to better control the process as a whole.

An additional specific requirement of ocular pharmaceuticals is sterility. Many materials used to produce nanomedicines are susceptible to routine sterilization techniques such as gamma irradiations or autoclaving. For small or malleable particles double filtration may be an option for sterilizing the final product but for bigger rigid particles aseptic manufacturing may be the only option.

Many nanocarriers are specifically designed to improve delivery of the active ingredient, thus it is expected that biodistribution of the nanomedicine and its unformulated counterpart should be significantly different. In fact, for ocular administration, it is crucial to determine whether the formulation changes the ability of the drug to enter systemic circulation. Many approaches to develop nanomedicines for eye conditions seek precisely this aim; to reduce the amount of compound that enters systemic circulation reducing the potential systemic side or off-target effects of the drug. One of the main challenges of pharmacokinetic (PK) studies of nanomedicines is to address the fate of the free drug following administration of a nanomedicine. For efficacy assessment the amount of free drug reaching the intended site should be measured. On the other hand, the distribution of the complete nanomedicine and its fate following delivery should be studied in order to address eventual toxicities associated to the nanocarrier. For larger molecules, such as oligonucleotides specific methods used to assess the free drug should be used to quantify the amount of drug reaching the target site. Subtle variations in the characteristics of nanomedicines may result in altered patterns of biodistribution; therefore it is generally expected that PK profiles of nanomedicines have greater variability than PK profiles obtained with unformulated drugs. In addition, for ocular pharmaceuticals administered in eye drops the percentage of entrapment, which is the amount of drug in one eye drop, is difficult to control further increasing the variability of the PK profiles. These inherent characteristics of nanomedicines for ophthalmic use should be taken into account when designing PK studies to avoid unnecessary duplication of experimental work. The surface characteristics of the nanomedicine have a strong impact on the absorption and distribution of the drug and can be modified by surface coatings in order to obtain particles with the desired characteristics.

It is generally accepted that specific types of nanomedicines may raise concerns in terms of toxicity, but the general battery for assessing toxicity of drugs in a preclinical setting should be able to identify these toxic effects and their potential relation to dose. Knowledge about the differences in biodistribution between the unformulated drug and the nanomedicine are helpful to pinpoint possible target tissues for toxicity. Also, additional tests may be needed if components of the nanomedicine are known to have dose-limiting toxicities or if any of the components are not naturally degraded and excreted. Particular attention should be made on potential immunotoxic effects. These toxicities are not necessarily deduced from the unformulated drug as they

may arise from specific interaction of the drug with other components of the nanocarrier or by interactions of the nanomedicine with proteins present in biological fluids. The physical characteristics of the nanomedicines may also increase their potential to interact with biological components. In the particular case of the eye, interaction with melanin may alter the PK profile of the drug in the eye generating locally high concentration of the drug upon release from melanin (Agrahari et al., 2016). Formulations reaching the bloodstream may also potentially interact with components of the complement system or coagulation inducing side effects (Yousefi et al., 2014). The assessment of these toxicities is not necessarily straightforward as animal models are not necessarily good predictors of human immune systems. In these cases, *in vitro* tests with human cells can be used to complement animal studies.

The production of nanosystems usually requires the use of surfactants. Surfactants lower the surface tension between the nanosystem and the dispersion liquid acting as stabilizing agents. Usually there is a correlation between the amount of surfactant used and the size of the nanoparticles, with low concentration of surfactant generally yielding smaller particles. Unfortunately, surfactants may cause ocular irritation when administered at high concentrations and need to be removed from the formulation or used in concentrations that are compatible with the ocular surface. For ophthalmic products this approaches are combined with the aim of reducing the amount of surfactants to concentrations that are tolerable by the eye without removing them completely as they usually act as penetration enhancers facilitating the entrance of compounds (Leonardi et al., 2014).

Clinical translation of nanomedicines for ophthalmic conditions has different requirements depending on the indication and route of administration. Topical ocular eye drops are the preferred option for treating the anterior segment of the eye whereas nanodrugs for the back of the eye are usually developed to release drug over an extended period of time to reduce the frequency of administration. Nanomedicines that include oligonucleotides should protect them against degradation by nucleases. Human biological fluids contain higher concentration of nuclease activity than most animal models, therefore stability studies of the nanomedicine need to be complemented to assess the stability of the drug in humans (Martínez et al., 2014). In addition, ophthalmic nanomedicines need to comply with the general requirements for drugs administered by the ocular route. For topical forms parameters such as sterility, osmolality, antimicrobial agents, buffering, viscosity, pH, particulate matter and compatibility with packaging have to be considered.

Finally, it should be mentioned that development of complex delivery systems for ophthalmic drugs is highly costly. As previously mentioned, the processes for developing nanomedicines may require additional data not generally required for small molecules. This, added to the insufficient regulatory framework and the potential complexity of the scale-up processes may further increase the associated costs of developing nanomedicines. As such, it seems difficult for the industry to design developmental plans where the size of the ophthalmic market outweighs the efforts and financial expenses associated to the development.

Currently there are several advanced preclinical programs developing nanomedicines with small drugs for different ocular conditions. Most of these drugs are already commercialized and are being reformulated in order to improve their efficacy as well as to find new indications for them. Overall, the more advanced technologies

include the production of nanoemulsions and liposomes. As these compounds reach the market it is expected that nanoformulations are also incorporated into the pipelines of more complex molecules such as oligonucleotides or peptides.

6. Conclusions and future perspectives

Although therapies involving polynucleotides for treating ocular diseases are in an early development stage, preliminary human clinical trials begin to show promising results. Currently, there are two FDA approved nucleic acid-based drugs for eye conditions: Vitravene®, an AS-ODN for cytomegalovirus-induced retinitis treatment in immunocompromised patients, and Macugen® an aptamer designed to treat wet AMD. Curiously, this aptamer is PEGylated and, as such, maybe considered as a nanomedicine. In principle polynucleotide-based drugs do not comply with the best physicochemical properties to be used as effective drugs, however, their chemical modification and the development of easy to produce nanocarriers offer a great window of opportunity for the exploitation of these new therapies. Currently, several ocular diseases of the anterior and posterior segment of the eye such as glaucoma, AMD, ocular pain associated to dry eye and CNV are under gene-based clinical trials evaluation. Development of effective future ocular treatments will be a combination of understanding the diseases genetic basis as well as improving and developing long-term and nontoxic ocular drug delivery systems for both segments of the eye. It is believed that AS-ODN and RNA-based therapeutics (specially siRNA-based as it is a potent inhibitor of protein expression) will continue further development reaching the market in a reasonable time frame being the major task to achieve their nanosystems delivery along with investigating alternative routes of administration.

If nanotechnology is successfully combined with polynucleotides, their delivery, the greatest hurdle holding these drugs from the clinic, could be overcome. The following years will tell if these combined approaches can be used in the treatment of severe ocular diseases that nowadays relay on painful, inconvenient and inefficacious treatments. Much effort will have to be put into place for the efficient delivery of the genetic material to targeted cells/tissues being one of the most challenging tasks to be accomplished by multidisciplinary research teams composed of researchers, clinicians and pharmaceutical companies.

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