NANO AND MICROTECHNOLOGIES FOR OPHTHALMIC ADMINISTRATION. AN OVERVIEW.

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ABSTRACT

Ocular drug delivery is one of the most challenging fields of pharmaceutical research. They are generally employed to overcome the static (different layers of cornea, sclera, and retina including blood aqueous and blood-retinal barriers) and dynamic barriers (choroidal and conjunctival blood flow, lymphatic clearance, and tear dilution) of the eye. Ophthalmic formulations must be sterile, and the biomaterials used in the preparation of pharmaceutical systems completely compatible and extremely well tolerated by ocular tissues. The location of the target tissue in the eye will determine the route of administration. Ophthalmic administration systems are intended for topical, intraocular and periocular administration. In this review we describe the main pharmaceutical nano- and microsystems currently under study to administrate drugs in the eye, covering microparticles, nanoparticles, liposomes, microemulsions, niosomes and dendrimers. We have performed the corresponding revision of the published scientific literature always emphasizing the technological aspects. The review discusses also the biomaterials used in the preparation of the nano and microsystems of ophthalmic drug delivery, fabrication techniques, therapeutic significances, and future possibilities in the field.

KEYWORDS: Microparticles, nanoparticles, liposomes, microemulsions, niosomes, dendrimers, intraocular administration, periocular administration, ophthalmic topical administration, biomaterials.

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ABBREVIATIONS

ACV	Acyclovir
ACA	Alkyl cyanoacrylate
ACZ	Acetazolamide
AIDS	Acquired immune deficiency syndrome
AmpB	Amphotericine B
ARMD	Age-realted macular edema
AS-ODNs	Antisense-oligodesoxinucleotide
AUC	Area under the curve
BSA	Bovine serum albumin
BSS	Balanced saline solution
C24	Poly-24-oxyethylene cholesteryl ether
CH	Sodium cholate
Cht	Cholesterol
Cmax	Maximal concentration
CMC	carboxymethyl cellulose
CMV	Cytomegalovirus
CNV	Choroidal neovascularization
CSN	Chitosan
CyS	Cyclosporines
Cy A	Cyclosporine A
DDBA18	Dioctadecyl-dimethyl-ammonium bromide
DMPC	1,2-dimyristoyl-sn-glycerol-3 phosphocholine
DMPE	Di-myristoyl-phosphatidyl-ethanolamine
DNA	Deoxyribonucleic acid
DOPS	2-dioleoyl-sn-glycerol-3-phospho-L-serine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DP	Dicetylphosphate
DPPC	Dipalmitoyl phosphatidylcholine
DPPS	Dipalmitoylphosphatidylserine
DPTs	Dendritic polyguanidilyated translocators
DR	Diabetic retinopathy
DSEP	Polyethylene glycol-distearoyl ethyl phosphocoline
DSPC	Distearoyl phosphatidylcholine
DSPE	1,2-Distearoyl-sn-glycero-3 phosphatidylethanolamine
Dx	Dexamethasone
EE	Encapsulation efficiency
EGFP	Green fluorescent protein
EMA	European medicines agency
FDA	Food and Drug Administration
GCV	Ganciclovir
GDNF	Glial-cell-line-derived neurothropic factor
GSH	Gluthatione
HA	Hyaluronic acid
HEMA	2-hydroxyethyl methacrylate

HET-CAM	Hen's egg test-chorioallantoic membrane
HPMC	Hydroxypropyl methyl cellulose
HSV-1	Herpes simplex virus type 1
HUVECs	Human umbilical vein endothelial cells
IgG	Immunoglobulin G
IOBA-NHC	Instituto de Oftalmobiología Aplicada- Normal Human Conjunctiva
IOP	Intracoular pressure
K5	Plasminogen kringle
LC	Liquid crystalline
LCT	Lecithin
LPS	lipopolysaccharide
LUVs	Large unillamelar vesicles
ME	Microemulsion
MLV	Multillamelar vesicle
MPEG-2000-DSPE	1,2-Distearoyl-sn-glycero-3 phosphatidylethanolamine-n-((maleneimide/polyethyleneglycol)-2000)
MP	Microparticle
NHC	Normal human conjunctiva
NP	Nanoparticle
NTX	Naltrexone hydrochloride
O/O	Oil-in-oil
O/W	Oil-in-water
OND	Oligonucleotide
PACA	Poly(alkyl cyanoacrylate)
PAMAM	Polyamidoamine
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PCL	Polycaprolactone
PEG	Poly(ethylene gycol)
PEI	Polyethylenimine
PGE2	Prostaglandine E2
PLA	Polylactic acid
PGA	Polyglycolic acid
PLGA	Poly(lactic-co-glycolic) acid
PMA	Polymethacrylic acid
PPI	Polyethylenimide
PS	Phosphatidilserine
PS-OND	Phosphorothioate oligonucleotide
PVA	Polyvinil alcohol
PVR	Proliferative vitreoretinopathy
RA	Retinoic acid
REV	Reverse phase evaporation
RGC	Retinal ganglion cells
Rh	Rhodamine
RNA	Ribonucleic acid
RPE	Retinal pigmented epithelium
SA	Stearylamine

SLA	sulfacetamide sodium
SLPN	Solid lipid nanoparticles
SUV	Small unillamelar vesicle
TA	Triamcinolone acetonide
TBUT	Tear break-up time
TGF-B2	Transforming growth factor beta 2
TRD	Tractional retinal detachment
VEGF	Vascular endothelial growth factor
VIP	Vasointestinal active peptide
W/O/W	Water-in-oil-in-water

1.- INTRODUCCION

1.1. Nano- and microsystems for ophthalmic administration

Ocular disorders treatment can benefit from the use of ophthalmic formulations based on nano- and microsystems. In the case of diseases affecting the ocular surface or if the drug has to reach the anterior segment of the eye, the topical administration is the preferred route. The conventional ophthalmic preparations used in this case (eye drops) show as great inconvenient the short residence time of formulations on the ocular surface, due to drainage of solution, tear turnover, its dilution by lacrimation and the low corneal permeability of most of the drugs. All these limitations cause poor ocular bioavailability and it is estimated that approximately only the 5% of instilled drug would reach the target site [1].

The use of pharmaceutical systems able to increase the retention time of drugs on the ocular surface, and in some cases to enhance the corneal transport, are intended to increase the drug bioavailability after topical administration, reducing the number of applications per day and increasing the patients' compliance. Colloidal systems as liposomes, nanoparticles, niosomes and microemulsions have been developed to increase ocular drug bioavailability. Most of them are also useful for delivering the drug in the target site. Most recently, dendrimers have emerged in the ophthalmic field to augment the poor bioavailability of drugs applied on the surface of the eye exploring their ability to interact with the mucins of the ocular surface. The specific structure of these biopolymers makes them also useful for targeting. Furthermore, they are one of the most promising non-viral transfection agents explored in gene therapy.

Regarding the pathologies affecting the posterior segment of the eye, the objective is to deliver the drug in the vicinity of the target tissue. For these pathologies the use of topical administration results ineffective and systemically administered drugs cannot

achieve therapeutic drug concentrations in the posterior segment of the eye due to the blood-aqueous and blood-retinal barriers. The more suitable route is then the intraocular injection of the active substance, dissolved or suspended in a vehicle, or its periocular injection if the drug can cross the different tissues to reach the back of the eye. The main lack-point of these administrations is the need of repeated injections to achieve therapeutic drug levels for prolonged periods of time, which is generally necessary to treat the chronic pathologies affecting the posterior segment. In that sense intraocular and periocular implants and microparticulate delivery systems may address the risk of side effects associated with repeated injections as they are able to release the drug for long periods of time. At this level, nanosystems are useful tools to protect the active substance of the external environment and to target the drug to ocular tissues.

Ocular drug delivery is one of the most challenging fields of pharmaceutical research. It is not only necessary to overcome the static (different layers of cornea, sclera, and retina including blood aqueous and blood-retinal barriers) and dynamic barriers (choroidal and conjunctival blood flow, lymphatic clearance, and tear dilution) protecting the eye, but they must also fulfil more restricted specifications than the devices designed for other administration routes. The drug delivery systems prepared must be sterile and the biomaterials used in the preparation of the systems must be completely compatible and extremely well tolerated by ocular tissues.

In this review we describe the main nano- and microsystems under study to administrate drugs in the eye, covering microparticles, nanoparticles, liposomes, microemulsions, niosomes and dendrimers (figure 1). We have performed the corresponding revision of the published scientific literature published during the last decades always emphasizing the technological aspects. The review discusses also the biomaterials used in the

preparation of nano- and microsystems for ophthalmic administration, fabrication techniques, therapeutic significances and future possibilities in the field.

1.1.1. Microparticles

Microparticles (MPs) are solid drug carriers with diameter of 1 to 1000 µm, capable to provide sustained and controlled release of the loaded active agents, while the non-released substance is protected from degradation and physiological clearance. A large variety of active compounds have been encapsulated in microparticles for ophthalmic administration, mainly for intraocular drug delivery. The main advantage of these formulations is that they can release the drug over the time with one single administration, having the same effect than multiple injections. These therapeutic systems are usually prepared with a polymer or mixture of polymers and can include one or more active substances. By physical structure, microparticles are classified in microcapsules and microspheres. Microcapsules are constituted by a drug core, which is surrounded by a polymer layer (reservoir structure). Conversely, in the microspheres the drug is dispersed through the polymeric network (matrix structure) [2].

The main advantage of microspheres over conventional systems for ocular drug delivery is that they are able to increase the efficiency of drug delivery by improving the release profile and also reduce drug toxicity. Furthermore they can be injected as suspension using conventional needles (27-34G) so no surgery is needed. Recent studies have demonstrated that microparticles tend to aggregate once injected in the vitreous cavity, behaving as an *in situ* forming implant [3]. The size of microparticles seems to be advantageous also for periocular administration. After injection, microparticles are retained in the administration site proximities where they can progressively release the

loaded actives, differently from smaller particles (nanoparticles) that are mainly cleared by the systemic and lymphatic system [4].

1.1.2. Nanoparticles

Nanoparticles (NPs) are defined as solid, submicron-sized drug carriers with diameters ranging from 1 to 1000 nm [5]. Similarly to microparticles, depending on their structure, NPs can be subdivided into nanospheres and nanocapsules. Nanospheres are constituted of a dense solid polymeric network with a matrix type structure, in which the drug can be either adsorbed on the particle surface or encapsulated within the particle. Nanocapsules are small reservoirs consisting of an inner liquid core, where the active substance is usually dissolved, surrounded by a polymeric membrane. In nanocapsules the active substance can also be adsorbed on the capsule surface.

Solid lipid nanoparticles (SLNPs) were developed as an alternative to colloidal drug delivery systems [6] (lipid emulsions, liposomes and polymeric NPs). Their structure consists of a solid lipid core and an amphiphilic surfactant as an outer shell. The presence of these compounds allows the incorporation of lipophilic and hydrophilic active ingredients [7].

Although the potential of big nanoparticles to sustain the delivery of drugs has been shown, microparticles give better results due to the substantial difference of size. However, the smaller size of nanoparticles makes them suitable to be taken up by cells, for example retinal pigmented epithelium cells (RPE). This fact reveals the utility of these systems to treat retinal disorders. Nanoparticles seem to be more useful for targeting specific molecules of genetic material into the cells than for sustaining the delivery of drugs [8-10].

Of special interest is the use of bioadhesive NPs to administrate drugs on the ocular surface. Their size does not induce patients' discomfort and allows them an intimate contact with ocular mucins, increasing the retention time of the loaded compounds and also improving their corneal penetration [11,12].

1.1.3. Liposomes

Liposomes are spherical vesicles composed of an aqueous core enclosed by concentric phospholipid bilayers. They are spontaneously formed when phospholipids, which have an amphiphilic nature, are dispersed in aqueous medium [13]. The size of liposomes is in the range from 10 nm to 10 µm. According to their size and the number of lipid bilayers, liposomes are classified into small unillamelar vesicles (SUVs), large unillamelar vesicles (LUVs) and multillamelar vesicles (MLVs) if more than one bilayer is present (Table 1) [14,15].

The effectiveness of liposomes as drug delivery systems depends on many factors such as the type of lipid employed, size and surface charge of liposomes, characteristics of the therapeutic agent and the properties of the definitive liposomal formulation developed [16-19]. Liposomes present the advantage of encapsulating both lipophilic and hydrophilic molecules. Hydrophilic drugs are entrapped in the aqueous core while lipophilic compounds are incorporated in the lipid bilayers [16].

Liposomes are considered as promising nanocarriers for ocular administration because they are biocompatible, biodegradable and relatively non-toxic. They have received considerable attention as ocular drug delivery systems owing to their ability to increase the drug residence time on the ocular surface and hence its corneal penetration, especially the positively charged liposomes, able to totally cover the ocular surface once instilled [20]. Related to intraocular route, liposomes have demonstrated to protect and

increase the half-life of fragile active substances avoiding repetitive administrations to achieve therapeutic levels in the site of action [21,22]. In the case of intravitreal administration, liposomes have demonstrated to be useful in relatively long-term release of lipidic pro-drugs [23-25]. Liposomes have been already used in the ophthalmic clinical practice[26].

Positively-charged (cationic) liposomes have high efficiency for entrapping DNA or RNA, due to electrostatic interactions [27-29]. These complexes formed by DNA and positively charged liposomes are called lipoplexes and they are used frequently as gen carriers to treat ocular pathologies [30,31].

1.1.4. Microemulsions and niosomes

Microemulsions (MEs) are pharmaceutical systems composed of an oily phase, an aqueous phase and a combination of surfactant and co-surfactant at appropriate ratios to stabilize the system. ME are an attractive alternative to conventional topical formulations since they are thermodynamically stable systems, can be easily prepared and sterilized, and present the advantage to incorporate water-soluble and lipophilic drugs. Moreover, the surfactants can act as penetration enhancers to increase the drug permeability across the cornea. In addition, MEs usually are low viscosity systems with a Newtonian behavior, low surface tension values and transparent appearance because the nano-size drop (<150 nm) of the dispersed phase. As a result, ME allow an adequate mixture with tears and also guarantee a good spreading onto the ocular surface. Thus, ME can be administered as eye drops and results in a high patient's compliance [32-37].

Niosomes are formed from the self-assembly of non-ionic amphiphilic compounds in aqueous media resulting in bilayer vesicles of size 10-0.5 µm**.** These relatively new systems are interesting for ocular drug delivery. They have some of the advantages of

liposomes, because of their ability to entrap both hydrophilic and lipophilic actives either in an aqueous layer or in vesicular membrane, but with the improvement of a better chemical stability. Niosomes also possess some similarities with microemulsions, because of their surfactant composition, able to increase the drug permeability through the corneal tissue [38]. Various studies have demonstrated their ability to increase the bioavailability of antihypertensive and opioid antagonist drugs, among others, after instillation [39-41], due to permeation enhancement behaviour. Other possible reasons are the better spreading ability of niosomes on the lipophilic corneal surface and increased viscosity compared with drug solutions [42].

1.1.5. Dendrimers

Dendrimers are new polymers with a high potential in drug delivery [43-46]. The term ‘dendrimer’ arises from the Greek *dendron*, meaning ‘tree’, and *meros* meaning ‘part’. It graphically describes the structure of this class of synthetic macromolecules that have highly branched, three-dimensional features that resemble the architecture of a tree [43]. Despite their large molecular size (5,000-500,000 g/mol), dendrimers are structurally well-defined and have low polydispersity. A typical dendrimer consists of three main structural components: (i) a multifunctional central core, (ii) branched units and (iii) surface groups [47,48]. The branched units are organised in layers called ‘‘generations’’, and represent the repeating monomer unit of these macromolecules [43]. Generally the number of generations is represented as a digit preceded by the capital letter “G” (i.e. G2: generation 2). On a molecular level the dendritic branching results in semi-globular to globular structures of a few nanometres of diameter with a high density of functionalities on the surface [49]. These pendant groups can be easily chemically modified. This fact can be used to functionalize the dendrimer with targeting

ligands able to direct it to the desired tissue [47,50-52].

Dendrimers fit with the definition of "nanocarrier'', term used to describe "the hybrid multifunctional systems with sizes typically ranging between 1-200 nm which may deliver the bioactive agent at the targeted site with improved therapeutic activity over the free form of bioactive agent [53]". Although the use of dendrimers in drug delivery is yet less developed than most of the previously described drug carrier systems, as it will be shown in this review, they present high potential for ocular drug delivery and have demonstrated to be useful in different ophthalmic administration routes.

1.2. Biomaterials used in the preparation of nano and microsystems for ocular drug delivery

1.2.1. Microparticles and nanoparticles

Depending on the nature of the polymer (erodible or biodegradable and non erodible or non biodegradable) micro and nanoparticles can disappear or remain in the site of action after releasing the drug. Among the biodegradable polymers employed to prepare micro and/or nanoparticles the most used have been polyesters (lactide and glycolide copolymers, polycaprolactones, poly(β-hydroxybutyrates)), polyamides (including natural polymers such as collagen, gelatin, and albumin, or semisynthetic pseudo-poly(amino acids) polymers such as poly(N-palmitoyl hydroxyproline ester)), polyurethanes, polyphosphazenes, polyorthoesters, polyanhydrides and poly(alkyl cyanoacrylates) [54,55]. In this review we will spotlight in some of the most relevant biomaterials.

Some of the first attempts to administrate NPS on the ocular surface were carried out with poly(alkyl cyanoacrylate) (PACA) nanosystems in the 80's [56]. PACAs are hydrophobic bioerodible polymers commonly used as tissue adhesive. They are

biocompatible polymers that degrades *in vivo* by hydrolysis of ester bonds of the alkyl side chain of the polymer, leading to water soluble degradation products, alkylalcohol and poly(cyanoacrylic) acid [57]. Currently, these polymers have been replaced in the development of particulate systems for ocular drug delivery.

Chitosan (CSN) has been widely employed to prepare nanoparticles for ocular drug delivery. It is a cationic high molecular weight heteropolysaccharide composed mainly of β-(1,4)-2-deoxy-2-amino-D-glucopyranose units and partially of β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose. Free amino groups of chitosan can undergo protonation thus, making it soluble in aqueous acid solution (pH < 6.5), which avoids the use of hazardous organic solvents while handing it. Because of favorable biological properties such as biodegradability, biocompatibility and non-toxicity, chitosan has attracted great attention in pharmaceutical and biomedical fields [58,59]. Its cationic nature makes it an interesting candidate to DNA electrostatic complexation [60]. Furthermore, chitosan has bioadhesive nature, by electrostatic interactions with the negatively charged at physiologic pH of the mucins sialic acid groups [61,62]. This interaction with mucins has been used by several authors to improve the bioavailability of drugs after topical instillation by increasing the ocular surface retention time of drugs encapsulated in chitosan NPs. CSN can be used to prepare nanoparticles alone [63-66] or in combination with other compounds such as alginate [67], lecithin [68], etc. Some interesting combinations of chitosan with other polymers such as hyaluronic acid have demonstrated to be also useful in gene transfection at corneal level [69,70]. CSN is also an interesting biomaterial to coat NPs, liposomes or niosomes, improving their mucoadhesiveness on the ocular surface and the drug corneal permeation [41,64,71].

The most widely employed polymer to prepare biodegradable nano and microspheres are the lactic and glycolic acid polymers and their derivatives: poly(lactic) (PLA), poly(glycolic) (PGA) and their copolymers poly(lactic-co-glycolic) acid (PLGA). These polymers are already approved for human purposes by the FDA and EMA. A biodegradable implant (0.45 x 6 mm) prepared with PLGA and loaded with dexamethasone (Ozurdex®) has been recently commercialized to treat persistent macular edema associated with diabetes, retinal vein occlusions, uveitis and post-cataract surgery [72]. PLA, PGA and PLGA are well tolerated *in vivo* and accepted for parenteral administration. Their biodegradation products, lactic and glycolic acids, are also biocompatible and easily eliminated from the body thorough Kreb's cycle [73]. The rate of degradation of PLGA systems depends on: (1) the copolymer molecular weight, length of lactic and glycolic blocks and ratio of lactic and glycolic acids (2) structure and morphology, such as crystallinity and shape of the sample, (3) the way the materials are processed, (4) the particle size (total surface area) (5) the type and amount of drug contained in the formulation and (6) the environment in which the system is placed [74-79]. Modifying these factors, the degradation rate can be varied from days to months. For example, 50:50 lactide/glycolide copolymers have an *in vivo* half-life of degradation of several weeks, whereas 65:35, 75:25 and 85:15 lactide/glycolide copolymers have progressively longer degradation half-life [55].

Poly(ε-caprolactone) (PCL) is a material approved by the FDA for human use also widely used in the preparation of nano and microspheres. It is an aliphatic polyester able to undergo bulk erosion by random hydrolytic chain cleavage in physiological conditions in the human body. The degradation of PCLs may require from 2 to 4 years, which is especially interesting for the preparation of long term implantable devices.

However, the degradation process can be modulated with the use of additives able to enhance the hydrolytic reaction of degradation[55].

In the case of SLNPs, the lipids employed are usually triglycerides, partial glycerides, fatty acids, steroids or waxes. All types of emulsifiers can be used but, depending on the lipid selected, the efficiency of the combination to prevent particle agglomeration has to be adapted.

1.2.2 Liposomes

Liposomal membranes are normally composed by a mixture of phospholipids of natural or synthetic origin. Among the natural phospholipids, those from egg or soybean lecithin (phosphatidylcholine (PC), phosphatidylserine, glycerosphosphocoline, etc) are principally employed. Concerning the synthetic phospholipids, the most used are phosphatidylcholines derivatives (dipalmitoylphosphatidylcholine (DPPC), 1,2-dimyristoyl- sn-glycero-3-phosphocholine (DMPC), etc.) [80], phosphatidylethanolamines (dimyristoylphosphatidylethanolamine (DMPE), etc) [81], phosphatydilserines (2-Dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), etc), phosphatidic acids (2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA), etc) and PEGylated phospholipids (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (MPEG-2000-DSPE), etc) [82]. To obtain positively-charged lipid vesicles cationic lipids as dioleoyl-3-trimethyleammonium propane chloride (DOTAP) or stearylamine (SA) can be included in the bilayer. To prepare negatively-charged liposomes anionic compounds as dicetylphosphate (DP) are employed [20].

Lipid vesicles also commonly include additives as cholesterol (Cht) and antioxidants as α-tocopherol or ascorbic acid. Cht improves the mechanical properties of bilayers,

provides rigidity to the liposomal membranes and avoids the lost of encapsulated drugs. Antioxidants, are also commonly incorporated to protect the phospholipid from oxidation [83,84]. These compounds avoid lipid peroxidation and can also have therapeutic effect. Furthermore, sphingosine, glycoplipids or other amphiphilic compounds such as polyethylene glycol (PEG) can be added to modify intrinsic properties of liposomes [85,86].

1.2.3. Microemulsions and niosomes

The amount and type of surfactants and co-surfactants, required to achieve the pseudo ternary-phase diagram corresponding to the ME region, are a critical point to elaborate MEs for ophthalmic administration, as ocular tolerance must be assured [87]. The most frequently used are non-ionic surfactants as poloxypropylene/polyoxyethylene block copolymers (poloxamers), polysorbates, polyethylene glycol and tyloxapol. Amphiphilic surfactants as lecithin are also often employed. Hwever, ionic surfactants are actually toxic to the ocular surface so their use in ocular formulations is very restricted. The co-surfactant provides higher fluidity of the interfacial film and a higher mean drop size which ensure a homogeneous repartition of ME compounds among droplets [88]. The most used co-surfactants are glycols, amines of short chains and low molecular weight alcohols (except hexanol and pentanol due to their high toxicity).

The choice of the oily phase depends mainly on the drug solubility. Usually, polar oils and triglycerides with medium or long chains are preferred because of their ability to form micelles in the inner aqueous phase and solubilize lipophilic drugs. The oily phase selected has to be well tolerated in the eye. Vegetable oils (soja oil, castor oil) triglycerides, Miglyol 812® (caprylic/capric acid triglyceride), isopropyl myristate, fatty acids as oleic acid and esters of saccharose are the most used [32,89,90]. The aqueous

phase must contain additives as electrolytes, antibacterial compounds and isotonic agents. It has to be mentioned that these components of the aqueous phase can dramatically influence the ME formation and its stability, so extensive formulations studies are required [32].

In case of niosomes, non-ionic surfactant (polysorbate, sorbitan esters, polyoxyethylenes, etc.) are used. Cholesterol is usually included in a 1:1 molar ratio (non-ionic surfactant: cholesterol) in most formulations to stabilize the bilayer [38]. Similarly to liposomes, several molecules can be also included in the formulation to obtain negatively and positively charged vesicles [40].

1.2.4. Dendrimers

There are two general synthetic approaches used to generate dendrimers: (i) divergent, and (ii) convergent. The appropriate route depends mainly on the kind of monomer employed and the target polymer structure [43]. In the divergent approach the dendrimer is synthesised from the core as the starting point and built up layer by layer (generation by generation) [91]. The alternative convergent approach [92] starts from the surface and ends up at the core, where the dendrimer segments (dendrons) are coupled together [47]. Other synthetic strategies such as the “lego” or the “clic” chemistry are also being explored to obtain dendritic structures [48,93]. It is worthy to mention that the synthesis of dendrimers is not only limited to almost spherical structures, but also to the synthesis of hybrid systems, such as dendritic branched polymers, grafted-dendritic structures, cyclodextrin-dendrimers hybrids, etc. [94,95]. Although there is wide literature concerning the synthesis of dendrimers and their use in different fields (biosensors, catalysis, etc.) [96], only a few families of dendritic structures are currently being evaluated for their use in biomedicine, due to

biocompatibility issues [97]. The most promising dendrimers currently under study for medical purposes are the polyamidoamine dendrimers (PAMAM) and the dendrimers derived from polyethylenimine, also named PPI, among others [46,49,98,99].

1.3. Methods of preparation of nano- and microsystems for ocular drug delivery.

Although there are a wide variety of methods of preparation of nano- and microsystems for drug delivery, in this review we will only focus on the methods mostly used to prepare systems destined to ocular administration.

1.3.1. Microparticles

Many microencapsulation procedures are modifications of the following basic techniques: solvent extraction/evaporation, aggregation by pH adjustment or heat, coacervation (phase separation) and spray-drying [100].

Among them, for example, spray-drying has been used to encapsulate poor aqueous soluble drugs as triamcinolone acetonide (TA) and ciprofloxacin to treat ocular diseases [101]. This technique is relatively simple, although it is not useful for highly temperature-sensitive drugs. Furthermore, the resultant microspheres are not always able to efficiently control the drug release due to its highly porous structure.

Coacervation is another method classically employed to microencapsulate drugs. In this technique the use of organic solvents and coacervating agents are required. These compounds can remain in the microparticles once prepared, producing ocular toxicity. The use of supercritical gases as phase separating agents has been recently introduced to avoid potentially harmful residues in the microspheres [101].

The technique for microspheres formation destined to ocular delivery most reported is the solvent extraction/evaporation method (evaporation of a solvent from an emulsion). This technique requires a dissolution or dispersion of the active substance in a first

solvent containing the matrix-forming polymer (inner phase). After that, the emulsification of this polymer solution in a second continuous phase immiscible with the inner phase is performed. Then, the extraction of the dispersed phase solvent is carried out by evaporation at room conditions or under vacuum. Finally, the immature microspheres are harvested and freeze-dried or desiccated. Lyophilization is preferred because the stability of the final product is increased. Depending on the drug solubility, oil-in-water (O/W) or oil-in-oil (O/O) emulsions can be prepared. In case of polypeptides, proteins and other biotechnological products techniques based on the water-in-oil-in-water (W/O/W) emulsion, in which the active is first dissolved in the inner aqueous phase (W1), are generally chosen [102]. However, knowing the instability of such macromolecules in the organic-aqueous interfaces formed [103,104], some alternatives are currently under study to avoid such problem, such us the inclusion of albumin or PEG as stabilizers [105], or the use of the macromolecules in its solid state by the formation of a solid-in-oil-in-water emulsion (S/O/W) [106,107].

1.3.2. Nanoparticles

There are several methods to prepare NPs that can be classified by whether (a) the formulation requires a polymerization reaction, (b) the formulation is achieved directly from a macromolecule or preformed polymer, (c) the system is formed by a desolvation and denaturation process of natural proteins or (d) NPs are formed by spray-drying [5]. Other methods include (e) NPs prepared by the emulsification/solvent evaporation method or (f) by nanoprecipitation.

Depending on the type of carrier, the materials used and the intended application, some methods are chosen over others. For example, to prepare PACA NPs the preferred methods are the emulsion polymerization method and the interfacial polymerization

technique. In the first case nanospheres (size between 50 and 300 nm) stabilized with a stabilizing agent (poloxamer, dextran, etc.) are produced, while in the case of interfacial polymerization oil-containing nanocapsules are obtained [57].

Other methods applicable to a wide range of polymers are based in macromolecules denaturation (mainly natural proteins such as albumin or gelatin). The denaturation takes place typically by increment of temperature [108]. For termosensitive drugs, this denaturation can be produced through changes in pH, charge or addition of desolvating agents (such as ethanol or concentrated inorganic salt solutions) [109].

An interesting method for NPs preparation using hydrophilic polymers such as chitosan is the ionotropic gelification. This technique is based on the cross-linking method using ionic counterions like tripolyphosphate, dextran sulphate, cyclodextrins or alginate as cross-linking agents [63,67,110], instead of toxic conventional cross-linking agents such as glutaraldehyde. This method has gained much attention because it is simple, non-toxic and involves a mixture of aqueous phases at room temperature avoiding organic solvents [111]. In the case of chitosan NPs, they retain their cationic nature, required for its bioadhesiveness and permeating properties [112].

Other methods available to prepare NPs for ocular administration are the emulsion-solvent evaporation method [113-115], spray-drying [116] (both previously described) and the nanoprecipitation method. To obtain particles in the nano-range using the emulsion method, the droplets formed during the emulsification step must be very small. To this, several methods as high shear homogenization, ultrasound or high pressure homogenization can be used [6]. In the nanoprecipitation method the polymer is dissolved in an organic solvent and then is added drop-wise to an aqueous phase that is stirred until the organic phase has been evaporated [117].

1.3.3. Liposomes

Some of the most widespread used procedures for liposomes elaboration are based on the thin-film hydration method (Bangham technique), sonication, extrusion, reverse phase evaporation (REV), freeze-thaw method, French pressure, ethanol injection or detergent dialysis [13,118-121]. When necessary, the preferred liposomal formulation can be elaborated by combining more than one strategy.

The thin-film hydration technique consists on the solubilization of the lipid components in an organic solvent and its posterior slow removal by evaporation in a vacuum evaporator. A thin film of dry lipids is formed on the inner surface of the evaporator flask. Next, lipid film is hydrated with an aqueous solution and liposomes are formed spontaneously [13]. After that, and to obtain an homogeneous vesicles size, sonication and/or extrusion by polycarbonate membranes can be carried out. An schematic representation of this procedure is presented in figure 2.

The freeze-thaw method is an extension of the lipid film hydration method. Once formed, liposomes are vortexed with an aqueous solution until the lipid film is completely suspended and MLV are frozen, vortexed again and extruded. In this process SUVs are ruptured and defused to form LUVs. During this procedure the solute is allowed to equilibrate between the aqueous core and outside the liposomes. As many freeze/thaw/extrusion cycles as necessary are performed. This method is widely used to protein entrapment [122].

Liposomes can be also performed by sonication of a lipid mass dispersed in the aqueous solution (large scale manufacture liposome) followed, if necessary, by extrusion processes [123].

The REV method is based on a co-solvated lipid solution that is dispersed in the aqueous solution with the drug. The lipid mixture is first dissolved in an organic solvent

that is evaporated under reduced pressure. After that, the formed lipid film is re-dissolved in a different organic phase (diethyl-ether or isopropyl-ether), in which the lipid vesicles will be formed after the addition of the aqueous solution. The organic solvent is again removed under pressure to obtain a semisolid-gel. The resulting liposomes are called “REV liposomes”. LUV and oligollamelar vesicles are performed with this method directed to the encapsulation of large macromolecules [15].

1.3.5. Microemulsions

ME are prepared by relatively simple, inexpensive processes that do not require of a complex technology. Basically, there are two main procedures for MEs preparation: (1) Autoemulsification process, where MEs are elaborated by spontaneous formation of the droplets when constituents are incorporated by gentle mixing and (2) process based on supply of energy by using a high-pressure homogenizer to obtain the droplet size desired after mixing the emulsion components.

Niosomes intended for ophthalmic administration can be prepared using all methods already mentioned for liposomes, being the film hydration technique [38] and REV method [40] the most employed.

1.3.6. Dendrimers

The unique structure of dendrimers, with units branching from a core with void spaces within and between the branches, and an abundance of surface functional groups, makes them useful for complexing or entrapping various therapeutic agents. Hydrophobic drugs are typically located inside the structure, in the dendrimer core, through hydrophobic interaction, in a “host-guest” system [47,48,124,125]. In fact, PAMAM dendrimers can be used to increase the solubility of hydrophobic drugs such as

ibuprofen, 5-aminosalicylic acid or diclofenac [126-128]. Hydrophilic drugs are generally located on the dendrimer surface, thanks to electrostatic interactions (complex) [52,129,130] or covalent links (conjugates) [131-135]. In all cases drugs are continuously released from the systems for hours [127-131]. A specific case of dendritic complex is the combination of cationic dendrimers and oligonucleotides (OND). The complex is called dendriplexes [43,48,136], by analogy with similar complexes formed by liposomes and DNA called lipoplexes. The dendrimer-OND interaction is due to the characteristic high density of positive charged on the dendrimer surface [48,99,136,137]. Dendriplexes are able to protect the nucleic acid from degradation and enhance the transfection in comparison to naked OND. They are considered one of the most promising non-viral vectors currently in development for gene therapy [93,125,136,138].

1.4. Routes of administration of nano and microsystems for ocular drug delivery

As mentioned before, the location of the target tissue in the eye will determine the route of drug administration. As the eye is designed to avoid the entrance of exogenous substances, the drug must overcome the different protecting barriers to achieve the target tissue. In this section we will briefly describe the characteristics of each administration route underlining the main advantages and disadvantages. The different administration routes described in the text have been compiled in figure 3.

1.4.1. Topical administration

As previously commented, although the ocular topical administration of drugs offers obvious advantages such as easily administration and no need of qualified healthy staff, it undergoes important limitations. The main drawback is the low bioavailability of

drugs due to rapid clearance from the ocular surface and limited permeability through the cornea. Furthermore, topically administered drugs cannot reach the back of the eye in effective concentrations so its utility is restricted to the treatment of diseases affecting the ocular surface and when the drug has to reach the anterior segment of the eye.

The technological strategies used to increase the bioavailability of active substances after ocular instillation are based on the increment of their retention time on the ocular surface, mainly by interaction with ocular mucins, and on the corneal permeability enhancement. In that scenario, the use of solid microsystems is quite limited, due to their size. On the contrary, mucoadhesive nanosystems (nanoparticles, liposomes, niosomes, dendrimers, etc.), able to attach to the ocular surface and microemulsions offer, as it will be presented in this review, a very promising approach.

1.4.2. Intraocular administration

Intraocular administration involves the deliver of a relatively high dose of drug inside the eye with few or no systemic side effects. It includes several administration routes covering the anterior (intracameral administration) and the posterior segment (intravitreal administration, subretinal administration, etc.).

Although “intracameral” can be literarily used to define “injection of drug in the ocular globe”, it is exclusively used to describe the injection of formulations into the anterior chamber of the eye. This route is generally used during cataract surgery.

The intravitreal injection of drugs is the most efficient route to treat disorders in the back of the eye. This administration route presents clear advantages in comparison to topical and systemic administration. Furthermore, this local administration assures a very low prevalence of systemic side effects. However it is accompanied of important disadvantages. The first one is that it is an invasive route so the injection is performed

directly in the ocular cavity. This provokes rupture of delicate tissue structures and also compromises the immunity privilege of the eye, which can cause infections. Furthermore, as direct deposit of the drug is performed, retinal toxicity can be produced due to the initial high concentrations achieved. Secondly, for most of the active substances, the clearance from the vitreous is very rapid, with elimination half-lives of few hours in most of the cases. This involves the need of frequent repeated injections, which in turns, increases the risks associated to intravitreal injection, such as endophthalmitis, damage to lens or retinal detachment among others.

Chronic ocular disorders can be most efficiently treated by maintaining the drug concentrations within the therapeutic window at the target tissue in the eye and reducing the frequency of administrations. To reach this goal microsystems are useful tools currently under development. As it has been already mentioned, the main advantage of these systems is that they can control the release of the active substance for weeks, even months, reducing the frequency of injections and maintaining sustained adequate concentrations in the vitreous. In the case of nanosystems, their utility is more focused on the protection of the active substances of the external environment and their targeting to damaged ocular tissues. This strategy is of especial relevance in the case of active bioengineered compounds (peptides, proteins, nucleic acid etc.).

Alterations in the outer retina can be also treated by subretinal injections. The risk of this administration is evident and it is currently tested in preclinical studies. Cai et al, for example, have evaluated the subretinal administration of DNA-peptide NPs to treat the photoreceptors degeneration [139]

1.4.3. Periocular administration

Periocular injections are a less invasive alternative, potentially safer than intraocular injections, that offers an interesting approach for retinal drug delivery. Periocular refers to the region surrounding the eye and is a general term that includes peribulbar, posterior juxtascleral, retrobulbar, sub-tenon and subconjunctival routes. Depending on the injection, a drug administered by this route can access the target sites in the posterior segment through the sclera, the choroid and/or aqueous humor and vitreous. Drugs can reach intraocular tissues after periocular administration, even if they are macromolecules or low-molecular-weight drugs. However, they can be rapidly eliminated from the administration site, via the lymphatic flow in the conjunctiva and episclera, and/or the blood flow of the conjunctiva and choroid.

Although the periocular administration of drugs has been used primarily to be targeted to the anterior segment (anti-inflammatory agents and anesthetics by subconjunctival injection) [140], the interest of this route is now also directed to the posterior segment and, specifically, to retinal delivery as an alternative to intravitreal injections [141-143]. While intravitreal injections have shown to be more effective to treat retinal ganglion cells and inner retinal interneurons disorders, it has been demonstrated that periocular injections are more effective for delivery to the outer retina (photoreceptors and RPE) [144].

1.5. Specific requirements for ocular drug delivery

1.5.1. Sterility

A mayor concern in the administration of ophthalmic formulations is to avoid microbial contamination. For this reason, a properly sterilization and maintenance of the systems is always required prior to and/or during their use. A final sterilization is preferred over the preparation of the formulations under aseptic conditions. Among the available

sterilization methods, sterilization by membrane filtration, ethylene oxide, gamma irradiation and autoclaving are included.

A number of drawbacks limit the utility of these methods in ophthalmic drug delivery. These limitations are usually related to the stability of the materials (drug and/or polymer) or the production of toxic residues during the process. Some of the biomaterials commonly used to prepare micro and nanosystems for ophthalmic administration, such as PLGA or phospholipids, are sensitive to the conventional sterilization methods usually employed (heat and ethylene chloride).

In the case of polymers, gamma irradiation is the preferred option. It has a high capacity for penetration (the dose required to assure sterilization of a pharmaceutical product is 25 kGy) [145]. However, gamma-irradiation of bioresorbable polyesters, such as PLA or PLGA, induces dose-dependent chain scission as well as molecular weight reduction, affecting the properties of the final product [146]. The degradation rate of polymeric biomaterials due to gamma-irradiation has been linked to radical formation [147]. This problem seems to be diminished by using low temperatures during the exposure time of the different systems to gamma-radiation and has been already reported for sterilization of microspheres [145]. In the case of PLGA microparticles, several studies have demonstrated that gamma irradiation under low temperature did not change the essential characteristics of formulations; drug release rate, morphology, particle size and particle size distribution, etc. [148,149].

Nanoparticulate systems could also be potentially sterilized by other methods such as sterilization by membrane filtration, or autoclaving. Sterilization by filtration does not affect materials' properties (polymer or drug) since it is based on physical removal of microorganisms through 0.22 µm membrane filters. However, it can only be used with NPs which have a narrow size distribution and a mean particle size below 200 nm to

avoid membrane clogging, and it is not suitable when the drug is adsorbed at the NPs surface or when the suspensions are too viscous. Heat sterilization by autoclaving is a highly effective technique but involves high temperatures (120ºC) that may produce degradation or decomposition of the material (polymer and/or drug). Although this technique is not suitable with most systems, solid lipid NPs can be sterilized by autoclaving. It has been shown that after autoclaving, SLNPs almost maintain the spherical shape without any significant increase in the size of the NP distribution [7].

Concerning liposomes, sterilization presents serious difficulties that compromise the use of liposomes as drug delivery systems. Sterilization by heating can degrade liposomes as they are thermolabile and lipids can be hydrolyzed with high temperatures (autoclaving at 121ºC or by gamma irradiation). Therefore, the most indicated sterilization method is based on filtration by polycarbonate membranes of 0.22 μm pore size, nevertheless if liposomes of 0.22 μm diameter of less are not the desired end point this filtration step cannot be used to sterilize the liposomes products. In addition, phase transition temperature determines filtration process, and liposomes passing through 0.22 μm can modify their size. Hence, in these cases, working under aseptic conditions from the beginning of the elaboration process combined with sterilizing filtration (if possible) is the selected alternative [85].

So, the deficient and chemical stability of lipid vesicles and the loss of stability during heating limit the sterilization methods to sterile filtration and aseptic working conditions. Nevertheless, lyophilisation is one of the most promising methods to keep liposomes stable during long term storage[150]. As we previously commented, liposomes are susceptible to chemical and physical instabilities as fusion/aggregation of lipid membranes, precipitation phenomena, drug loss, lipid oxidation, phospholipid hydrolysis, etc. Lyophilisation or freeze-drying technique offers the opportunity to

remove water from liposomal systems resulting in a solid porous cake which must be reconstituted with aqueous solutions before use. After freeze-drying, the porous cake can be sterilized by gamma irradiation without serious damage for the liposomal system preventing hydroxyl radicals formation by water exposition to gamma irradiation. Hence, freeze-drying enhance liposomal stability and protects lipid vesicles from alterations during gamma irradiation process.

As usual, lyophilisation technique requires a good defined protocol [151-153] and in addition, presents some disadvantages: Lipid bilayer of phospholipids can result damaged by crystal formation during freezing process and, water removal during sublimation step can lead to a disruption of the lipid bilayer. As consequence, aggregation/fusion phenomena can occur during the hydration step and liposomal system is modified. Nevertheless, there are strategies directed to protect liposomes from water removal alterations. Composition of lipid bilayer is one of the factors to take into account, the addition of cholesterol and vitamin E avoids crystal formation during water removal. Nowadays, cryoprotectors are frequently used with good results to overcome problems derived from freeze-drying. Saccahrides as sucrose, lactose, dextran, trehalose and other molecules as lysine protect phospholipids from water removal modifications since sugars can replace the water molecules as sublimation occurs and thereby bilayer conformation remains stable. A vitrification model has also been proposed for saccahrides, especially for trehalose, which also protects phospholipid during freeze-drying process by a vitreous layer formation around the lipid membrane[154]. The ratio sugar/phospholipids should be appropriate for freeze-drying protocols in order to protect liposomes from lyophilisation process maintaining physical and chemical properties and stability after rehydration of the anhydrous cake[152]. In some cases, polymers as hyaluronic acid can act as cryoprotectants, so the use of bioadhesive

polymers is an important technological resource for formulations destined to ophthalmic route[155]. As an example, Ahmad et al. developed mitoxantrone liposomes (DOPC, cholesterol, cardiolipin, alphatocopheryl succinate) by ethanol injection technique. As lyoprotectant, sucrose or trehalose was employed at different concentrations. There were no differences in size pre-lyo and post-lyophilisation formulas when sucrose/lipid ratio 7.5/1 was used. The lyophilized formula resulted stable for 13 months after storage at 2-8ºC and liposomes reconstituted were physically and chemically stable for 8 hours at room temperature (20–25ºC)[156].

After lyophilisation of liposomes, sterilization by gamma irradiation can be performed at a dose of 25 KGy following European Pharmacopea guidelines[157]. Physical and chemical studies before and after lyophilisation and sterilization processes are required to check if alterations in the liposomal systems appear. Perrie et al. developed a combined technique based on freeze-drying and posterior gamma sterilization applied to liposomal vaccines containing a protein able to responses against *Mycobacterium tuberculosis*. Cationic liposomes, composed by dimethyldioctadecylammonium bromide, 1.25 mg/ml; and α,α'-trehalose 6,6-dibehenate, 250 μg/ml, carried out by solvent evaporation method. Cryoprotectants as sucrose, trehalose and lysine were tested to obtain the optimal ratio for stability and sterility posterior studies. The freeze-drying protocol consisted of pre-freezing liposomal formulation at -70ºC for 30 minutes and followed by drying in two steps: -50ºC for 48h and at -30ºC to 20ºC for 6 h. After that, gamma sterilization at a dose of 25 KGy (room temperature) was carried out. Lysine, sucrose and trehalose /liposomal formula in a ratio 4/1, 8/1 and 10/1 respectively were the most effective concentrations to show lyoprotectant effects. Mean volume diameter size, zeta potential, dynamic viscosity, pH and residual moisture content, among others, were measured prior to and post sterilization. Results show no

differences for each parameter analyzed after gamma irradiation, except pH which values were not influenced by sterilization but for cryoprotectant choice [158].

As conclusion, lyophilisation and posterior gamma irradiation can result appropriate methods to enhance stability and sterility of liposomal formulations, nevertheless, cryoprotectants choice, freeze-drying protocols and gamma irradiation doses have to be evaluated carefully.

Microemulsions sterilization processes are relatively simple and easy to carry out. Sterilization by autoclaving or sterilizing filtration are good alternatives for these systems [159]. No extended studies about niosomes sterilization are available, however, the mentioned sterilization for ME, except filtration, that similarly to liposomes, could modify the vesicles size, might be also applied for niosomes. In the case of dendrimers, thanks to their small particle size (tipically lower than 10 nm) they should be sterilized by sterilizing filtration through 0.22 µm membrane. However, to our best knowledge, no extensive studies have been performed in this sense.

1.5.2. Tolerance

Tolerance studies for ophthalmic formulations can be performed *in-vitro* and *in vivo. In-vitro* tolerance involves viability assays in cell lines. There are different cell lines to perform these studies. Immortalized corneal and conjunctival cell lines are employed to evaluate tolerance of topical formulations[160-165]. Immortalized ARPE cell lines and primary cultures of retinal cells[166-169] are employed for formulations destined to intravitreal administration. Other immortalized cell lines as macrophages are useful to test *in vitro* tolerance as they are very sensitive.

In vivo ocular tolerance of drug delivery systems depends on their composition and site of administration.

I*n vivo* tolerance of topical formulations has been examined by several authors. For example, Contreras-Ruiz et al. evaluated the alterations of the tear film physiology and ocular surface tissues after the instillation of hyaluronic acid-chitosan NPs in rabbit eyes[170]. 30 µl of a NPs suspension (0.5 mg NPs/ml of buffer) were instilled every 30 minutes for a period of 6 hours. Clinical signs observed 24 hours later were compared with those obtained just before the instillation. No signs of ocular discomfort or irritation after exposure were shown and ocular surface tissues and tear film physiology remained unchanged. Nagarwal et al. evaluated the tolerance of 5-fluorouracil loaded chitosan NPs applied topically in rabbit eyes. In this work authors used the Draize test to check swelling and discharge of the conjunctiva and corneal integrity. No signs of ocular inflammation, tissue alteration, conjunctiva congestion, swelling or discharge were reported[171].

Liposomes are supposed to result no toxic for the ocular surface as the tear film contains a similar composition (aqueous phase and lipids components), however, cationic and anionic liposomes may induce some ocular toxicity produced by the surface charge [111,172]. Stearylamine is known to destabilize lysosome membranes and subsequent hydrolases release results in cellular damage. SA-liposomes also produce ocular surface toxicity as function of SA concentration [173,174]. Taking these studies into account, Schaeffer et al. demonstrated the adequate tolerance of a indoxole liposomal formulation composed by PC:SA:Cht in a molar ratio 7:2:1. Authors administered the liposomes preparation in male Sprague-Dawley white rats twice daily for 8 days and evaluated changes of corneal cells. The histological evaluation of rat corneas did not show ocular toxicity as stroma hyalinization [175].

Alonso et al. designed a system combining liposomes and chitosan nanoparticles to apply as eye drops. Liposomes were prepared according to the solvent evaporation method followed by extrusion at phase transition temperature until desired liposome size close to 400 nm was obtained. Three different liposomes compositions were prepared: L1 consisted of di stearoyl phosphatidylcholine:di palmitoyl phosphatidylserine:cholesterol (DSPC :DPPS:Cht (6:0.1:3 molar ratio), L2 consisted of DPPC:Cht (6:4 molar ratio) and L3 was composed of DSPC:Cht (6:4 molar ratio). After that, the liposomes-chitosan nanoparticles were performed by freeze-drying process. The size of the complexes ranged from 750 to 400 nm and the surface zeta potential was between +6 and +15 mV. These complexes resulted well tolerated by the ocular surface as they did not present toxicity when incubates with IOBA-NHC cells. Besides, *in vivo* studies on New Zealand rabbits demonstrated that these nanosystems were non-irritating and compatible with ocular surface [71].

Concerning ME, Benita et al., for example, developed a positively-charged emulsion with phospholipids, poloxamer 188 and SA which employed in an acute ocular tolerance assay in rabbit eyes. Administration of a drop hourly of the cationic microemulsion was well tolerated with no damage on the ocular surface during the 5 days of the study [176]. However the toxicity of surfactants and co-surfactants may compromise ocular tolerance so deep studies are necessary for each individual ME formulation [89].

The scarcely number of niosome tolerance studies performed, *in vitro* and *in vivo*, showed no signs of toxicity. Abdelbary and El-gendy (2008) prepared niosomes using three different non-ionic surfactants: polysorbate 60 or polysorbate 80 or polyoxyethylene lauryl ether, always in combination with Cht and PD. Authors tested the presence of any redness, inflammation or increased tear production upon the

application of each niosomal preparation to the eye of albino rabbits. Observations were performed at 1, 24 and 48 hours after instillation using the contralateral eye as control. No signs of toxicity of damaged tissues were observed [38]. More recently, Abdelkader and collaborators (2012) performed a complete study conjunctival and corneal toxicity of niosomes combining different in vitro tests: The hen's egg test-chorioallantoic membrane (HET-CAM), bovine corneal opacity and permeability (BCOP) test and histological examination of excised corneas [177]. They tested niosomes prepared with and loaded with Cht, DP, Sorbitan monostearate (Span 60), poly-24-oxyethylene cholesteryl ether (C24) sodium cholate (CH) and also tested each isolated components. Only one of the tested ingredients, CH, showed moderate irritation, however such an effect was diminished when incorporated into niosomes.

It is generally accepted that the cationic dendrimers are less tolerated than the anionic ones. Authors referred this lower tolerance to the high density of positive groups on the dendrimer surface that can disrupt and alter in some extent the biological membranes [178,179]. However, some authors have administered PAMAM dendrimers on the ocular surface showing a similar toxicity behavior than linear polymers. Vandamme and Brobeck [180] tested the tolerance of 2% of PAMAM (4G) solution with different chemical groups on the surface (amino, carboxyl and hydroxyl) groups on albino rabbits. The clinical observations and ocular reactions (redness, conjunctival chemosis, discharge, iris and corneal lesions), evaluated 1, 24, 48, 72, 96 h and 7 days after application, revealed that the aqueous polymer solutions of the dendrimers tested did not cause any ocular irritation and did not induce a watering reflex. However, Lopez and co-workers (2009) observed that cationic PAMAM (G3 & G5) dendrimers resulted relatively toxic for the corneal epithelium at concentrations 1.3-12.5 μg/ml. These authors demonstrated that the coupling of the dendrimers with PEG chains importantly

reduced the toxicity of the systems [181].

PLA and PLGA are the most studied polymers for intraocular administration. Regarding micro and nanoparticles prepared with PLA and PLGA, they have been mainly administered by intravitreal and periocular route. In the tolerance studies performed in animal models (rabbits) and humans it has been observed that once injected in the vitreous, PLGA microparticles suffer aggregation [3,182]. Preliminary investigation in 25 human eyes showed that this tendency of the PLGA microspheres to aggregate and condensate at the site of the injection have a positive effect, not only because they act as *in situ* implant, diminishing the surface area and probably prolonging the drug release time, but also because this aggregation leaves free visual axis, so patient's vision is not compromised [3]. Once in the vitreous PLA and PLGA begin to hydrolyze, disappearing in several weeks [3,182]. In studies carried out by Moritera et al., the elimination of these polymers from the vitreous seems to accelerate in eyes underwent vitrectomy in rabbits [183]. Although no histologically retinal nor choroidal damage were reported after intravitreal injection of PLGA microspheres by Koobehi and co-workers [184], a mild localized foreign body reaction was denoted by Veloso et al., [185], similar to the ones reported for sutures and disappeared 2-4 weeks after administration [182]. Authors observed that this foreign body reaction, associated with PLGA in the eye gradually decreased with time, was similar to that described for microspheres intramuscularly injected in rabbits [186]. In the case of PLGA NPs, Gupta et al analyzed the ocular tolerability of sparfloxacin loaded PLGA NPs with the hen's egg chorioallantoic membrane test (HET-CAM) [187]. For the procedure, 0.5 ml of the formulation were instilled directly onto a 2x2 cm window made in the shell, allowing the formulation to be in contact with the chorioallantoic membrane for 5 minutes. Then, vascular damage was assessed. Results indicated that sparfloxacin NPs were nonirritant

and well tolerated. Zang et al administered 100 µl of a dexamethasone (Dx) loaded PLGA NPs suspension (equivalent to 2 mg of Dx) with a 30G needle into the vitreous cavity in rabbit eyes [188]. NPs allowed a sustained release of Dx for at least 50 days in the tissues and, after a certain time, they gradually sedimented to the areas next to the retina. Clinical observation, including fundus observation and photography, IOP measurement and B-scan ocular ultrasonography were used to determine possible abnormalities that could be attributed to the intravitreal sustained-release of Dx from NPs, but authors did not find any evidence of this relation.

Barbosa-Alfaro et al., have recently evaluated the tolerance of PLGA microparticles after sub-tenon injection. Five mg of dexamethasone (Dx) microspheres (165.6±3.6 µgDx/mgMP) (828 µg Dx) were administered by sub-tenon injection in rabbits' eyes. Neither adverse clinical nor histological signs were observed six weeks after periocular administration [189]. Only a moderated conjunctival congestion was observed in both eyes (treated and untreated) probably due to the sub-tenon injection itself [190]. Other authors either found any inflammation sing, including redness and edema after a single subconjuntival injection of 5 mg PLGA-microspheres loaded with celecoxib in rats [149].

Considering intraocular particles prepared with other biopolymers, Merodio et al evaluated the retinal toxic response induced by ganciclovir (GCV) loaded bovine serum albumin NPs in Wistar rats' eyes. 5 µl of a NPs suspension (200 mg NPs/ml equivalent to 5 mg GCV/ml), were administered with an intravitreal injection using a 29G needle. The effects of NPs administration were analyzed 1 week and 2 weeks post-injections. Albumin NPs in contact with the vitreous did not show evidence of inflammatory reaction in the retinal tissue, alterations of the surrounding ocular tissues or any autoimmune response able to stimulate photoreceptor degeneration[191].

In vivo tolerance of intravitreous poly-Ɛ-caprolactone implants have been already performed in pigmented rabbits. In this study a device loaded with dexamethasone was inserted into the vitreous of the animals. Clinical and histologic observation demonstrated that the system resulted well tolerated in the animals[192].

The nature of the biopolymer is critical in terms of intraocular response. Rincon et al studied the tolerance of microparticles prepared from an elastin-like-family biopolymer (poly (valine-proline-alanine-valine-guanine; VPAVG). The thermoresponsive polymer did not induce any cytotoxicity on macrophages up to 60mg/mL. No inflammatory response was observed after subcutaneous injection in the hind-paw of rats. However, 45% of retinal detachment was observed in the animals receiving intraocular injection of poly (VPAVG) microparticles (2,5mg)[15,193].

Periocular and intraocular liposomes administration also require tolerance studies Especially intravitreal injection can induce blurring vision, decrease of visual acuity and can lead to the apparition of opacities and cloudiness limiting their use for intravitreal purpose [194,195] [196]. Tremblay and co-workers intravitreally injected in 40 eyes of twenty pigmented rabbits amphotericine B (AmpB)-liposomal formulation (lipid composition PC:Cht:tocopherol succinate in a molar ratio 5:3:1 and AmpB concentrations of 1, 5, 10 or 20 ng). Control preparations, also injected, consisted on empty liposomes, PBS and free AmpB free. A part from the presence of vitreous bodies in most of the eyes injected, authors did not detect retinal damage in the group treated with Amp-Liposomes or empty liposomes. However, animal group received AmpB solution presented retinal necrosis or atrophy in 5 of the 16 eyes treated due to the intrinsic toxicity of the drug[197]. Freeman et al. developed a liposomal formulation composed by dioleyl phosphatidyl choline (DOPC), dimyristoyl glycerol (DMG), triolein and Cht entrapping a GCV derivative. Liposomes tolerance was studied after

intravitreal injection of drug loaded and unloaded liposomes showing no retinal damage for any of the preparation tested [198].

Hereafter the review will be focused on the description of research works published in the last decades that describe the high potential of microparticles, nanoparticles, liposomes, microemulsions, niosomes and dendrimers for ocular drug delivery. Some relevant studies in the field of gene therapy and transfection have been also commented in the corresponding sections. As summary and guidance for readers, the basic information of each study has been assembled in Table 2.

2. MICROPARTICLES FOR OPHTHALMIC ADMINISTRATION

Several preclinical studies have demonstrated the efficacy of microparticles releasing encapsulated molecules in a controlled manner from weeks to months depending on the polymer characteristics. These systems can be administrated by different ophthalmic routes (topic, intraocular and periocular) depending on the target tissue to which they are intended.

2.1. Microparticles for topical administration

The utility of microparticles for topical therapies of ocular diseases are limited by (a) the short residence time of the microparticles in the ocular surface and (b) the particles size, that should not exceed 10 µm to avoid scratching feeling [199,200]. For the above reasons the topical administration of microparticles is not very common, and only some authors have explored the possibility of using polymeric biodegradable microparticles to administrate drugs on the ocular surface.

Recently, Addo et al. developed a topic microparticulate formulation of tetracaine that

produced an anesthetic action during common surgical procedures on the ocular. Microparticles (mean particle size of ~4 µm) were prepared by spray-drying method using a bovine serum albumin–chitosan (BSA-CSN) solution. This formulation presented a minimum cytotoxicity and optimum human corneal epithelial cells (HCET-1) cellular uptake (detectable 15 min after instillation and maintained for at least 24 hours). *In vivo* studies in rabbits showed that two drops of tetracaine microparticles (0.5%) suspended in saline solution increased the duration of anesthetic action of the drug up to 4-fold in comparison with the standard marketed solution while maintained the onset of action statistically similar to a commercial 0.5% tetracaine hydrochloride ophthalmic solution (Alcon Pharmaceuticals) [201].

As previously mentioned, one of the main reasons hindering the use of topical microspheres is its short contact time on the ocular surface. Some authors have developed bioadhesive microspheres to increase the drug bioavailability by prolonging the contact time of the formulation with the ocular surface. In this sense Sensoy and co-workers studied the efficacy of topic treatment with bioadhesive microspheres loaded with sulfacetamide sodium (SLA) for the treatment of keratitis in rabbits. Microspheres were prepared by spray-drying method using natural (pectin), semi-synthetic (hydroxypropyl methyl cellulose -HPMC-) and synthetic (polycarbophil) mucoadhesive polymers combined at different ratios. Authors found that SLA-loaded polycarbophil microspheres (size 1.87µm) formulation had the highest production yield (62.7%), exhibited the lowest initial burst release within the first 5 min (15.25%) and prolonged the release rate of SLA (71% of the drug was released after 9 h), resulting the most suitable for ocular application. The *in vivo* studies carried out in rabbit eyes with keratitis showed that the eyes treated with polycarbophil microspheres suspended in light mineral oil completely eradicated the bacteria from eyes after 6 days of

administration twice a day of the microparticles formulation [200].

Choy et al., [202,203] used PEG as a mucoadhesion promoter. These authors administered nile red-labeled PLGA-PEG microparticles (diameter lower than 10 μm) on the ocular surface of rabbits by instillation of the microparticles suspensions, or by the administration of mannitol minitablets containing the microparticles, in the cul-de-sac. Authors observed that while the aqueous suspension induces tear production rapid and drainage, the minitables formed a microenvironment of relatively high viscosity due to mannitol dissolution increasing the microparticles retention time on the ocular surface up to 1 hour, according to fluorescence images.

2.2. Microparticles for intraocular administration

Intravitreal microparticles allow the release of the encapsulated drug bypassing the blood–ocular barrier. The main advantage of microparticles is that they can be injected as a suspension using conventional needles. The microspheres are suspended in a physiological vehicle, typically phosphate buffer solution (PBS) or balanced salt solution (BSS). In some cases more viscous vehicles; such as hyaluronic acid (HA) or HPMC solutions to improve the suspension's injectability [185].

The use of microparticles as drug carriers to treat posterior segment diseases by intravitreal injection has been of great interest in the last decades. Many authors have tested this strategy to treat diseases such as proliferative vitreoretinopathy (PVR), age-related macular degeneration (ARMD), diabetic retinopathy (DR), uveitis, macular edema or citomegalovirus (CMV) retinitis and most recently optic nerve diseases such as glaucoma and retinitis pigmentosa.

Moritera and co-workers [183,204] prepared PLA microspheres loaded with adryamicin. Suspensions of microspheres (mean particles size 50 μm) were injected in

the vitreous of normal rabbit eyes and in a rabbit model of PVR. Authors observed that the retinal detachment was decreased from 50% to 10% in PVR rabbits after 4 weeks of the administration of 10 µg of the encapsulated drug. Authors found a significant decrease in the retinal toxicity of the single injection in comparison with the administration of the same amount of drug in solution, with no histological abnormalities, nor electrophysiological changes in the eye. Another interesting work was the study carried out by Giordano and co-workers [205] for the intravitreous administration of retinoic acid (RA) loaded in PLGA microparticles, also for the treatment of PVR in a rabbit model. The microparticles prepared *in vitro* released the drug for 40 days. *In vivo* studies showed that the incidence of tractional retinal detachment (TRD) resulted effectively reduced two months after a single injection of RA-loaded microspheres (110 µg RA) in comparison to blank microspheres.

The intravitreal administration of antivirals drugs as acyclovir (ACV) and ganciclovir (GCV) using microparticles has attracted the attention of several research groups. For example, Conte et al. [206] developed a controlled release formulation from different PLA and PLGA polymers loaded with ACV using the spray-drying technique. The formulations were tested as an alternative to intravenous or intravitreous administration of the drug in solution in the treatment of acute retinal necrosis caused by virus injections. After *in vivo* injection of PLA microparticles (25 µm diameter) into rabbit eyes, drug levels were detected in the vitreous for fourteen days after administration. In an interesting study carried out by Veloso et al. [185] was tested the antiviral effect of GCV released from PLGA. GCV-loaded microspheres (size 300-500 µm) containing 864.04 µg of the drug prepared from PLGA 50:50, were injected in infected rabbits' eyes. In treated eyes, vitritis, retinitis and optic neuritis decreased during the 14 days of the study, in contrast to control eyes. Immunofluorescence of virus antigens in retinal

tissues was not shown in treated eyes. Histopathology analysis showed minimal focal disruption of the retinal architecture in eyes injected with GCV-loaded microspheres, while some was observed in eyes injected with blank microspheres. No adverse tissue reaction was clinically or histopathologically observed in the eyes injected with ganciclovir-loaded microspheres after 8 weeks. Authors concluded that these formulations should be an alternative to the frequent injections of GCV solution required to maintain intraocular therapeutic drug levels. In another study of microparticles loaded with GCV, Duvvuri and co-workers injected PLGA microspheres loaded with GCV dispersed in a thermogelling PLGA-PEG-PLGA solution in rabbits' eyes. In this work, microspheres were prepared by the solvent evaporation method using PLGA polymers with different lactic:glycolic ratios (50:50 and 65:35). After intravitreal injection of microparticle formulation (196 μg of GCV), authors showed that drug levels in the vitreous were higher and maintained for prolonged time after the GCV-microparticles injection (approximately 0.8 μg/ml for 14 days) in comparison with drug solution (above 0.8 µg/ml for 54 hours) [207].

Intravitreal administration of microparticles in the treatment of pathologies as intraocular immune disorders, uveitis and other intraocular inflammation has also been extensively investigated. PLGA 75:25 microparticles of approximately 50 µm loaded with Cyclosporine (CyS) were tested for the treatment of uveitis and other intraocular immune disorders. The particles maintained therapeutic concentrations for at least 65 days in the disease-related tissues such as the choroid-retina and iris-cilliary body after injection into the vitreous body of healthy rabbits. The mean residence time of the active substance was increased around 10 times compared to CyS solution [208]. Barcia et al. [209] developed microspheres (53µm) for the sustained delivery of dexamethasone destined to prevent intraocular inflammation. Microspheres were prepared using PLGA

50:50 containing 1410 μg of dexamethasone and were injected in an animal model of uveitis provoked by lipopolysaccharide (LPS) injection. Authors showed that the intraocular inflammation was significantly reduced in animals receiving the Dx-loaded microspheres compared to control (blank microspheres). In order to simulate secondary uveitis a second injection of LPS was performed 30 days after microparticles administration. No inflammation was observed in the animals treated with dexamethasone loaded PLGA microspheres after this second LPS injection.

It is interesting to remark that some studies performed with microspheres loaded with anti-inflammatory drugs have been already performed in humans. For example, Cardillo and co-workers [3] reported human studies of the intravitreal injection of TA-loaded PLGA microspheres. Nine volunteer patients suffering diffuse macular edema were treated with the TA-microspheres. The efficacy of the formulation was compared to conventional TA injections. Interestingly, the eyes treated with TA microspheres showed marked decrease of retinal thickness as well as improved visual acuity for 12 months.

Currently, the increasing development of bioengineered products (peptide, proteins nucleic acids, etc.) potentially useful in the treatment of diseases in the posterior segment of the eye makes necessary the development of microparticulate systems able to administrate such molecules in a effective manner. This fact would open the door to new therapies resulting especially hopeful for the diseases currently without effective treatment. In that sense, for example, Andrieu-Soler et al. [102] developed PLGA microspheres loaded with a proteic neurotrophic factor (glial-cell-line-derived neurothropic factor; GDNF) prepared according to the W/O/W emulsion technique. The microparticles were able to control the protein release (10 ng/d) for three months according to *in vitro* studies and resulted efficient preventing the retinal degeneration in

a rd1 mouse model for 17 days. Thereafter, a glaucoma animal model (rats), based on IOP elevation (episcleral injection of hypertonic NaCl solution) was also used to test the efficacy of the PLGA microencapsulation of GDNF. With this objective, in a first work, Jiang et al [210] tested microparticles prepared by the W/O/W emulsion technique. The *in vitro* release, previously evaluated was of 35.4 ng/mg over 71 days [211]. Nine weeks after intravitreal injection of GDNF loaded microspheres in the animal models, a significant increase of the survival of retinal ganglion cells (RGCs) and their axons was demonstrated. Checa-Casalengua et al. [106] developed PLGA microparticles (20-40 μm) loaded with an antioxidant (Vitamin E) and GDNF by using a microencapsulation method derived from the S/O/W emulsion technique. In this method the biotechnological product was suspended in the oil as a first step of the microspheres formation, maintaining the stability of the protein during and after the procedure. The microspheres prepared according to this methodology were able to release the protein in its bioactive form for more than 130 days. Afterwards, authors observed that a single injection of 0.025 mg of microspheres (0.64 ng GDNF) in the same animal model of glaucoma previously described (IOP elevation) were enough to promoted RCG survival of 72.7% eleven weeks after injection, in contrast to 36.6%, 30.6%, and 29.0% of RGC survival observed in eyes treated with GDNF in solution, vitamin E and blank microspheres respectively.

2.3. Microparticles for periocular administration

As mentioned earlier, the periocular administration arises as an interesting alternative route to avoid some of the side effects related with intravitreal injections. Microparticles administered by this route should be maintained in the periocular space for a long time in order to provide sustained drug levels and enhanced drug absorption into the

intraocular tissues. In this sense, Amrite and. Kompella [212] studied the effect of the particle size of the disposition of non-biodegradable nanoparticles (20 and 200 nm) and microparticles (2 µm) after their subconjunctival injection in rats. The authors observed that only the microparticles (2 µm) and nanoparticles (200 nm) were retained at the site of administration for at least 60 days because nanoparticles of smaller size were rapidly cleared by the systemic and lymphatic circulation [4].

The efficacy of microparticles in delivering drugs to the posterior ocular tissue via periocular routes have been demonstrated in several preclinical studies. Many interesting works are related with the periocular administration of microparticles loaded with anti-inflammatory agents. For example, Kompella and co-workers prepared PLA-budesonide microparticles (3.60 ± 0.01 µm) and nanoparticles (345 ± 2 nm) using the solvent evaporation method from an O/W emulsion. PLA-budesonide microparticles (75µg of budesonide), nanoparticles (50 µg of budesonide) and budesonide solution (75 µg) were subconjunctivally administered in rats. The ocular tissues (retina, vitreous, lens and cornea) levels were compared at different times. On day 1, drug levels in the solution and nanoparticles groups were higher in all tissues evaluated, when compared with the group receiving microparticles. On the other hand, on days 7 and 14, drug levels in the microparticles group were higher in comparison with the solution and nanoparticles groups. Thus, subconjunctivally administration biodegradable microparticles were found to be useful in sustained retinal drug delivery [213]. The potential usefulness of subconjunctival injection of celecoxib-microparticles in treating diabetes-induced retinal abnormalities was studied by Ayalasomayajula and Kompella. Celecoxib-microparticles (3.9 ± 0.6 µm) were prepared by the solvent evaporation method using PLGA 85:15. Microparticles (containing 75 µg of celecoxib) were administrated by subconjunctival injection in a rat model of diabetes-induced retinal

oxidative stress. Results showed that microparticles were able to release the drug in a sustained way during 14 days in different tissues (retina, vitreous, lens and cornea). Furthermore, the diabetes –induces biochemical markers thiobarbituric acid reactive substances and 4-hydroxynonenal were significantly reduced. Microparticles also slightly inhibited the gluthatione (GSH) depletion and the increase in GSSG/GSH [214]. In other work, sterilized celecoxib-microparticles (dose 750 µg of celecoxib) were administrated by subconjunctival injection in a streptozotocin diabetic rat model. At 2 months after administration of celecoxib-microparticles, the drug levels could be quantified in sclera-choroid, retina, vitreous and cornea. Furthermore, the authors showed that a single injection of the microparticulate system was able to delay the development or progression of the early pathophysiological changes in the retina as a result of diabetes. These findings were demonstrated by the celecoxib-microparticles which significantly reduced the diabetes-induced elevations of prostaglandin E_2 (PGE_2) secretion, VEGF (vascular endothelial growth factor) protein, the vitreous-plasma protein ratio and blood-retinal barrier leakage without causing any damage to the retina [149].

Kimura et al. evaluated the effects of PLA microspheres loaded of adriamycin, a potent antimetabolic agent, to prevent post surgical fibrosis after glaucoma filtering surgery in rabbits. PLA-microparticles of adriamycin were prepared by the solvent evaporation method from an oil in oil emulsion (O/O). Adriamycin-microparticles (doses, 100 µg or 200 µg) were subconjunctivally injected. Results showed that the intraocular pressure (IOP) in the eye treated with adriamycin microparticles (100 µg) was significantly lower than the IOP in control eyes from 7-12 days. This effect was more prominent in the eyes treated with adriamycin microparticles (200 µg), where the IOP was lower than control from 6-16 days. The duration of patent filtering bleb in the eyes treated with the

Adriamycin- loaded microparticles was longer compared with the control eyes [215]. Saishin *et al.* evaluated the subconjunctival injection of sterilized microspheres system to release PKC412, a kinase inhibitor, in a porcine model of laser-induced choroidal neovascularization (CNV). PKC412-microspheres were prepared from an O/W emulsion using PLGA/glucose as biodegradable polymer. Subconjunctival injection of PKC412-microparticles (25% and 50% of PKC412, approximately) caused significant suppression of the CNV development at rupture sites in Bruch's membrane in pigs. In these experiments authors also evaluated PKC412 levels in different ocular tissues, showing that twenty days after injection of 50% PKC412 microparticles, drug levels were detectable in vitreous, retina and choroid [190].

Microspheres loaded with bioengineered products have also been administered by the periocular route. Gomes do Santos et al (2006) prepared nanosized complexes of antisense TGF-β2 phosphorothioate oligonucleotides (PS-ODN) with and without polyethylenimine (PEI) encapsulated in PLGA microspheres ("trojan" microspheres) using a solvent evaporation method from a double emulsion (W/O/W). A volume of 100 µl of a suspension of 4-5 mg of microparticles (550 ng PS-ODN) in sodium hyaluronate 1.35% were administered by subconjunctival injection in a rabbit experimental model of filtering glaucoma surgery. Results showed a significant increase in the intracellular penetration of ODN in conjunctival cells and demonstrated a 100% bleb survival. The subconjunctival injection prevented post-surgical fibrosis following trabeculectomy for 42 days. [216]. Other example is the use of PLGA microspheres loaded with anti-vascular endothelial growth factor (anti-VEGF) RNA aptamer to reduce the formation of new blood vessels in the eye [217]. The microparticles were able to deliver the aptamer in a sustained manner (average rate of 2 µg/d). The released aptamer retained its bioactivity, according to its antiangiogenic effect in human umbilical vein

endothelial cells (HUVECs). After *in vitro* permeation study it was observed that microparticles were able to release the aptamer through the sclera for a period of 6 days. Not only subconjunctival, but other periocular routes have been also evaluated. Interesting results have been obtained by administering drug-loaded microparticles in the sub-tenon virtual space. In our research group, Barbosa-Alfaro and co-workers [189] prepared Dx-loaded PLGA microspheres (Figure 4) for periocular administration that allowed the Dx *in vitro* released for 9 weeks. Five mg of Dx-loaded microspheres (828 µg Dx) were administered by sub-tenon injection in rabbits' eyes. Two weeks after injections Dx reached peak concentrations of 579.0 ng/g, 1749.6 ng/g and 69.2 ng/ml in choroid-RPE, retina and vitreous respectively.

Sub-tenon injection has been even evaluated in humans. In a relevant work carried out by Panganelli and co-workers, it was reported that the sub-tenon injection of a combination of microspheres loaded with ciprofloxacin hydrochloride (2.0 mg) with a solution of 25 mg of TA were able to prevent the ocular inflammation and infection after cataract surgery. Ciprofloxacin microspheres (mean size 1.07±0.35 µm) were prepared with a spray-drying using PLGA 50:50. Authors compared the combined treatment (ciprofloxacin microspheres and TA) with topical administration of prednisolone (1%) and ciprofloxacin (3%) eye drops during 28 days. Both treatments were evaluated in terms of efficacy (anterior chamber cell and flare, conjunctival erythema, ciliary flush or symptoms of ocular inflammation) and safety (intraocular pressure, biomicroscopy and ophthalmoscopic findings). Results demonstrated that a single periocular injection had same therapeutic response and ocular tolerance as continuous administration of eye drops in controlling inflammations after cataract surgery [101].

3. NANOPARTICLES FOR OPHTHALMIC ADMINISTRATION

The ability of nanoparticles to control the release of the loaded drug is very limited, due to its high surface area. However, the attractiveness of nanoparticles for ocular drug delivery is focused on two main aspects. The first one is their capacity to intimately interact with tissues, due to its reduced size. This characteristic makes nanoparticles excellent candidates to increase the retention time of the loaded drugs on the ocular surface. Similarly, nanoparticles can also develop intimate contact and can be phagocyted by other ocular tissues such as RPE cells, which results very suitable as targeting systems. Secondly, when nanoparticles are designed for other ophthalmic administration routes (periocular, intraocular), they resulted optimal systems to protect labile molecules against the external environment, increasing their half-life.

3.1. Nanoparticles for topical administration

As previously explained, the penetration of drugs through the cornea to the anterior segment structures present some limitations that could be partially solved with the use of nanoparticulate systems, since they offer the possibility to enhance the delivery and transport of drugs across these tissues. One of the first studies with NPs administered topically was made 25 years ago. In that study, PACA NPs loaded with progesterone were prepared and applied to rabbit eyes, and the time profile of the drug in several ocular tissues was assessed [56]. The emulsion polymerization technique was chosen by the authors to prepare these NP, which gave an encapsulation efficiency of 99%. The study was designed to compare progesterone loaded NPs with a solution of drug without polymer (used as control). However, the release of the drug from NPs was not as effective as expected. They observed interactions between drug and polymer that made progesterone less available for penetration on the precorneal area.

Nowadays it is possible to find studies with promising results with materials like PLA, PLGA, CNS or albumin.

PLGA NPs were prepared by the nanoprecipitation technique to encapsulate flurbiprofen as antiinfammatory agent in ocular surgery[218]. NPs' size ranged between 232 and 277 nm and gave an encapsulation efficiency of nearly 95%. According to the authors, formulations did not show toxicity on the tissues and demonstrated to be more effective than sodium arachidonate (anti-inflammatory reference drug) after topical instillation in the rabbit eye.

CNS is one the most promising polymers in the development of NPs for topical drug delivery. De Campos et al studied CNS NPs, prepared by the ionic gelation technique loaded with the hydrophobic peptide Cyclosporine A (CyA). The topical administration of this peptide has been used in a variety of ocular surface phenomena as dry eye syndrome, vernal conjunctivitis and corneal allograft rejection. CyA presents very low aqueous solubility, and it has been the object of numerous work looking to novel trends for topical administration [219]. These authors determined whether the ophthalmic administration of CyA could be improved by interactions of NPs with corneal and/or conjunctival tissues. A CyA suspension in chitosan was used as control. The mean particle size of NPs was around 300 nm and the drug loading values were around 9%. Animals treated with CyA-NPs had higher drug levels in cornea and conjunctiva than those treated with control, with a maximum at 2 hours post-administration. Therapeutic levels were found at least during 24 hours post-administration in conjunctiva, and 48 hours in cornea. Brimonidine tartrate, an alpha-2 agonist ocular hypotensive drug with neuroprotective action, was also encapsulated in CNS NPs prepared by the ionic gelation technique [220]. The *in vitro* release study showed sustained release over 4 hours in PBS (pH 7.4) and the *in vivo* experiments showed a significant sustained effect

of NPs in comparison with conventional brimonidine eye drops. A mixture of CNS and Carbopol® was used to elaborate NPs loaded with the miotic agent pilocarpine [221]. The incorporation of Carbopol® also contributed to the mucoadhesive properties of the formulation. *In vitro* release tests were compared with those obtained with a pilocarpine solution, a gel and liposomes. In all cases, the best slow-release profile of pilocarpine occurred with nanoparticles. Moreover, in the *in vivo* miotic study, NPs formulations showed the most significant long-lasting decrease in the pupil diameters of the rabbits. Other mucoadhesive agent, hyaluronic acid, was also combine to CNS to produce bioadhesive NPs intended for the administration of two hypotensive agents, dorzolamide and timolol [222]. After *in vivo* administration of these formulations in albino rabbits it was demonstrated that the combination of both bioadhesive polymers resulted very efficient at reducing IOP in comparison with a solution of the drugs at the same concentration.

Other approaches have also been done with ACV pro-drugs encapsulated in PLGA NPs (160-190 nm) dispersed in a thermosensitive gel (PLGA-PEG-PLGA). The formulation remained liquid at room temperature and formed a gel at eye temperature after topical administration. This strategy allowed the formation of a depot in the eye surface that prevented the fast drainage of NPs, the interaction of pro-drugs with tear proteins, tear dilution of pro-drugs and pro-drug metabolism. The *in vitro* pro-drug release from NPs in buffer (40 mg in 1 ml PBS) exhibited a biphasic pattern with an initial burst release. This initial release was reduced when NPs were suspended in the thermosensitive vehicle. In the thermosensitive gel, NPs also showed a longer release in comparison with control (NPs without thermosensitive gel; 6 days versus 3 days) [223].

Cationic nanoparticles have been also tested as transfection agents. For example, Konat et al. described the transfection of a new plasmid designed to encode the human gel-

forming mucin MUC5AC, whose expression is usually decreased in ocular surface alterations such as the dry eye syndrome. The plasmid was entrapped in hybrid cationized gelatin NPs produced by the ionic gelation technique (diameter smaller than 150 nm; zeta potential between +20 and +30 mV; plasmid association higher than 90%) [224]. Transfection studies were made in a conjunctival and a corneal epithelial cell lines grown at a final confluence of 75%. Cells were incubated with the NPs for 3 hours (5 µg of plasmid/well). As controls, authors used naked plasmid associated with a commercial transfected agent (JetPEI-RGD). The formulations produced an efficient transfection of MUC5AC in both cells lines, however only the conjunctival cell line was able to express MUC5AC after NPs transfection. These results were corroborated after *in vivo* administration in rabbits, where it was observed that the treatment with NPs significantly increased the MUC5AC expression in the conjunctiva.

SLNPs' efficacy as topical drug delivery systems has also been studied recently with different active substances. Baicalin-loaded SLNPs[225], an anti-inflammatory and analgesic agent, were prepared following the emulsification/ultrasonication method (average diameter of 91.42 ± 1.02 nm and encapsulation efficiency around 63%). In the permeation studies made in excised rabbit corneas, results demonstrated that the system was able to enhance the corneal penetration of the drug in comparison with a baicalin solution used as control. The *in vivo* evaluation of tobramycin-loaded SLNPs (100 nm) administered on the corneal surface in rabbit eyes, showed a significantly higher bioavailability of the drug in the aqueous humor in comparison with an equal dose of a tobramycin solution [226]. The ocular delivery of acyclovir was also enhanced by incorporating it into SLNPs (400-777 nm), having a faster permeation through excised rabbit cornea [227]. Additionally, SLNPs have been loaded with methazolamide, an antiglaucoma active agent, showing a higher therapeutic efficacy and more prolonged

effect than a drug solution and a commercial formulation[228]. Another anti-glaucoma drug, timolol maleate, was encapsulated in SLNPs (160 nm) showing similar results [229]. Similarly, the *in vivo* efficacy of CyA was enhanced with its incorporation in these systems[230]. Furthermore, non-viral vectors based on SLNPs have also been studied in gene therapy for the treatment of ocular diseases. Delgado al co-workers tested the transfection efficacy of SLNP formulations in rat eyes by monitoring the expression of enhanced fluorescent protein (EGFP) after intravitreal, subretinal and topical administration. Depending on the administration route, the expression of EGFP was detected in different types of cells, even after topical application of the system [231].

3.2. Nanoparticles for intraocular administration

As it was already commented, NPs have been evaluated after intravitreal and subretinal administration.

The encapsulation of AG1478, an epidermal growth factor receptor and tyrosine kinase inhibitor, which is able to promote optic nerve regeneration, was evaluated by Robinson et al recently. Intravitreal injections (5 µl) of AG1478 loaded-PLGA NPs (359 ± 54 nm; 50 mg MPs/ml) were evaluated in a rat optic nerve crush injury model. Four weeks after the injection, authors were able to detect NPs in the vitreous and could find fiber growth through the injury site [232]. In other study, the molecule tetra-iodothyroacetic acid, able to block angiogenic action over VEGF and erythropoietin, was encapsulated in PLGA NPs. The effect of the intravitreal injection of this system (75 ng) was evaluated in mice. While the administration of the drug alone had no statistically significant effect on retinal neovascularization compared with the vehicle, the administration of a single injection of loaded-NPs significantly reduced retinal neovascularization by 30.9% three

days after injection [233]. The effect of nanoparticle-mediated gene delivery of angiogenic inhibitors on retinal inflammation, vascular leakage and neovascularization, has also been studied in a diabetic retinopathy rat model. An expression plasmid of plasminogen kringle (K5), a natural angiogenic inhibitor, was encapsulated in PLGA:CNS NPs (260 ± 30 nm, plasmid loading of 11 ± 1.4 µg/ml) by the emulsion-evaporation technique. K5-NPs were injected into the vitreous (8.8 µg/eye) and results showed that, for at least four weeks, K5-NPs attenuated vascular endothelial growth factor and reduced leukostasis and vascular leakage in the rat model used [234].

Advances have been already performed in the subretinal administration of NPs. Farjo et al. determined the efficiency of compacted DNA NPs to transfer a plasmid which encoded a green fluorescent protein (EGFP). NPs (4 mg/ml of DNA in saline solution) were administered by subretinal injection (1 µl) in mice. 2 days post-injection the examination of the tissues showed a fluorescent signal in the inner plexiform layer and in retinal ganglion cells. Substantial EGFP fluorescence was also detected in the lens, cornea and trabecular meshwork with no evidence of cellular infiltration or inflammation [235]. Hayashi et al (2009) determined the workability of subretinal delivery of goat immunoglobulin G (IgG) electrostatically adsorbed onto gold NPs in a model of retinal degeneration in rabbits. Results showed that, one month after the injection, IgG-loaded NPs could be still detected and located in the outer segments of the retina [236]. Other authors studied the therapeutic efficacy and safety of compacted DNA-NPs gene delivery into the subretinal space of a juvenile mouse model of retinitis pigmentosa. Rod function was measured by electroretinography showing a modest but statistically significant improvement compared with controls after injections [237]. Cai et al determined the utility of small NPs (diameter < 8 nm) composed of a therapeutic gen in a mouse model of retinitis pigmentosa with a phenotype of slow retinal

degeneration (rds). In this work, NPs were compacted by a peptide with a high transfectivity activity (CK30PEG, a PEG substituted 30-mer lysine peptide). Authors injected 0.3 µl of a suspension of NPs in saline solution (3.06 µg/ml) into the subretinal space in the superior temporal quadrant of the animals. As controls, saline solution and uncompacted plasmid DNA carrying the same therapeutic transgene, were used. The efficacy of the system was evaluated in terms of its ability to rescue the disease phenotype in the rds model, measuring the levels of several photoreceptor-specific proteins typically reduced in this animal model. Subretinal delivery of NPs transfected nearly all of the photoreceptor population and did not produce irreversible defects in retinal function. Interestingly, NPs were able to generate a cone function restoration to almost normal levels and a rod function improvement[139].

3.3. Nanoparticles for periocular administration

The effectiveness of NPs by periocular administration for sustained transcleral drug delivery to the retina is highly limited by their size. As mentioned previously, it seems like only larger NPs (200 nm and above) are appropriate carriers, due to the fact that smaller NPs (20 nm or less) are washed very fast by the systemic or lymphatic circulation [238]. The clearance that takes place with larger NPs is mainly produced by retention and degradation at the site of administration, until NPs reach a size that is small enough for entry into the systemic or lymphatic circulation[239].

Efficacy studies of NPs administered via periocular injection have been published. The effect of subconjunctivally administered budesonide NPs has been evaluated in Sprague-Sawley rats[240]. Budesonide loaded NPs were prepared with polylactide polymer by a solvent evaporation technique (345 nm, encapsulation efficiency of 65%) and were injected into the subconjuntival space with a 27G needle (50 µg

NPs/injection). A budesonide solution was used as control. Drug levels in retina and other tissues were determined at 1, 3, 7 and 14 days post-injection. At the end of day 7, budesonide concentration in cornea and vitreous was higher in the NP-treated group than in animals that received the budesonide control solution. On day 14, drug levels were below limits for both solution and budesonide NPs groups in all the tissues analyzed.

4. LIPOSOMES FOR OPHTHALMIC ADMINISTRATION

Liposomes are a very interesting strategy to increase the bioavailability of drugs in ophthalmology. They exhibit excellent ocular tolerance and can be used to entrap both hydrophilic and hydrophobic drugs with high efficiency, being also useful in gene therapy. They can be easily coated with different polymers conferring additional characteristics to the systems. However liposomes present limitations concerning sterilization and stability, which partially restrain their use.

4.1. Liposomes for topical administration

Liposome preparations to treat anterior segment disorders are designed to increase the drug residence time and enhance transcorneal permeation. As already mentioned, in the preparation of liposome-based formulation the lipid selection plays a fundamental role. Cationic liposomes, which are composed by molecules with positive charge as dioleoyl-3-trimethyleammonium propane chloride (DOTAP) or stearylamine (SA) [172,241], seem to interact with the negatively charged mucins present at the corneal surface [242]. Several authors noticed that positively-charged liposomes produced an increase in residence time and, subsequently an increase in the drug bioavailability. For example, Schaeffer et al. prepared positively charged liposomes composed by PC, Cht and SA in

a 7:2:1 molar ratio by thin-film hydration method to study the uptake by the cornea and the transcorneal drug flux. Authors observed that these positively-charged liposomes suffered statistically higher corneal uptake than neutral and anionic liposomes. In fact, penicillin G entrapped-positively unillamelar liposomes enhanced transcorneal flux 4-fold in comparison with an equivalent concentration of free drug [175]. Law et al. also tested anionic and cationic liposomes to administrate ACV on the ocular surface. Positively-charged liposomes were constituted by PC from fresh egg yolk, Cht and SA and negatively charged liposomes were composed by PC, Cht and dicetylphosphate (DP) (1.6:1:0.15 molar ratio for both charged liposomal formulations). The drug-lipid hydration method was used to form ACV-liposomes that were then dispersed in a buffer solution (pH=7.4). In comparison with free ACV, cationic and anionic liposomes enhanced the bioavailability of the therapeutic agent as consequence of their intimate contact with the ocular surface that promoted an increase on the formulation residence time. Cationic liposomes presented higher ACV concentrations in the cornea than the anionic vesicles due to their electrostatic interaction with ocular mucins that allowed the formation of a completely coated layer on the ocular surface increasing the contact time. On its turn, although negatively charged liposomes were flexibly deposited on the corneal surface they were rapidly drained [20,243]. According to these findings, Saettone et al. compared a positively charged liposomal formulation (PC, Cht and SA) prepared by thin film hydration method following extrusion with a currently marketed ACV ointment. Liposomes (size 370.9±5.6 nm and ACV-loading 1.20 mg/ml) increased the drug concentration in the aqueous humor of New Zealand rabbits when compared with the ointment at the same ACV concentration [244]. These promising results promoted studies related to ocular pharmacokinetics and the delivery of active substance entrapped in liposomes to the vitreous humor by topical application. Tu et al.

prepared negatively charged GCV-loaded liposomes by the REV method (lipid components, PC:Cht:Na deoxycolate 12:1.7:1, wt:wt) . Liposomes were suspended in 5% (wt:vol) sacharose solution obtaining (GCV final concentration of 1 mg/ml) and filtered by 0.22µm pore membranes. Liposomes resulted in a size of 200 nm, zeta potential was clearly negative (-52.5±6.8 mV) and encapsulation efficiency was close to 52%. Although GCV liposomes and GCV solution presented similar precorneal clearance, probably due to the absence of any viscous agent, *in vitro* transcorneal studies of the GCV liposomes demonstrated a 3.9-fold higher permeability than that of GCV solution. GCV liposomes and GCV solution presented an aqueous humor concentration-time profiles described by 2-compartmental pharmacokinetics with first order transcorneal penetration and both dosage forms (liposomes and solution) allow GCV detection in aqueous humor, lens and vitreous. Liposomal preparation increased drug concentration by 2- to 10-fold in all these tissues (100-500 ng/g for GCV liposomes compared with 30-300 ng/g for GCV solution) [245].

Novel strategies directed to increase the retention time of the liposomal formulation at the site of action are focused on their combination with bioadhesive polymers. Although bioadhesive polymers can produce blurring vision at the moment of instillation [246], recently works show that when liposomes are suspended in hydrogels matrices, they increase the viscosity and residence time of the formulation, because of their adhesive nature, and can also prolong the drug release rate. [14].

Budai et al. performed multillamelar vesicles encapsulating ciprofloxacin (CPX) by thin-lipid hydration method without posterior extrusion. Liposomes, composed by lecithin (LCT) or DPPC (extrusion was not performed), were suspended in different bioadhesive polymer solutions: a poly(vinyl alcohol) (PVA) solution 0.14% or polymethacrylic acid (PMA) 0.1% solution and were compared with PVA (0.14-15%)

and PMA (0,1-5%) CPX-containing gels. Release studies in PBS (pH 7.2) showed about 60% and 80% of total CPX released after 300 min for LCT liposomes-PVA 0.14% and liposomes-PMA 0.1% combination respectively. Liposomes-PVA 0.14% increased release half-time from 72 min, values obtained for hydrogels prepared at the same concentration but without liposomes, to 212 min for LCT vesicles and 644 min for DPPC vesicles. Authors observed that the polymers-liposomal formulations behave like concentrated (3-10%) hydrogels without liposomes. In addition, these authors noticed that lipid composition also determine drug profile release. The higher release rates observed for lecithin formulations, in comparison to DPPC formulations would be explained by the higher membrane fluidity and permeability (lower main phase transition temperature) offered by the saturated and unsaturated lipids composing LCT, in comparison to the more rigid DPPC. Authors pointed out that the presence of drugs dissolved in the lipid bilayer could modify this behaviour[247]. Hosny et al. also developed CPX-liposomes constituted by soy LCT and Cht at different molar ratios by REV technique [118]. The maximum encapsulation efficiency (EE) (73.04%±3.06%) was obtained for liposomes LCT:Cht 5:3 (molar ratio). Furthermore, the inclusion of SA in the molar ratio 5:3:1 (LCT:Cht:SA) resulted in a EE of 82.01%±0.52%. In order to get a prolonged release effect of the active substance, liposomes were coated with a high viscosity polymer, Carbopol® 940 [248-250] at 1.5%. This combination promoted an increment in the time needed to release the 75% of the dose from 2h for non-charged liposomes to 6 hours for cationic liposomes and 10 hours for Carbopol® coated liposomes. Moreover an increment in CPX transcorneal permeation of 5-folds and 3-folds for coated and uncoated liposomes respectively were observed in comparison to drug solution [251]. Similar results were obtained by these authors when CPX was substituted by gatifloxacin [252]. Additionally, oxoflacin (0.3%) was also incorporated

in REV liposomes containing DPPC:Cht:SA (4:3:1 molar ratio) dispersed in a thermosensitive gel composed by chitosan and β-glycerophosphate salt (1.8% w/v and 3.8% w/v respectively). The presence of this hydrogel increased the amount of drug permeated through the cornea in 7-fold in comparison to the drug aqueous solution at the same concentration. The good results obtained were partially attributed to the capacity of chitosan to interact with ocular mucins and also to open tight junctions of the epithelial cells promoting the transcorneal flux [253].

Not only nanoparticles but also liposomes have been evaluated as potential strategy to increase the bioavailability of CyA. Milani et al. developed liposomes incorporating CyA for preventing graft rejection. Large unilamelar vesicles composed of PC and phosphatidylserine (PS) in a 7:3 molar ratio were prepared by dialysis of mixed lipid-detergent micelles method [254]. Grafted animals divided into different groups received empty liposomes, CyA oily eye drops (olive oil) or CyA-liposomes five times a day, daily for 10 days. After treatment, a 77% rate of graft survival was obtained with the CyA-liposomes group while the rate was 37% for non-treated group and 45% for the olive oil CyA group [219,255]. Another technological strategy to increase CyA bioavailability is to include liposomes loaded with CyA in a collagen shields to achieve higher concentrations of the therapeutic agent in cornea, sclera, aqueous humor and vitreous [256]. This study showed that animals received CyA-liposomes dispersed in collagen shields had CyA concentration levels in aqueous humor higher than animals received CyA-liposomes or CyA eye drops.

Liposomes have been also investigated for the topical delivery of intraocular pressure (IOP) lowering agents. Acetazolamide (ACZ) and pilocarpine HCl [257] are some examples of the exceptional behaviour of topical liposome based formulations for glaucoma treatment. El-Gazayerly et al. prepared REV liposomes composed by

PC:Cht:SA and PC:Cht:DP both in a 7:2:1 molar ratio containing ACZ. Unilamellar liposomes with a particle size in the range of 300-500 nm were obtained. In this study positively-charged liposomes had higher encapsulation efficiency (49.5±91.95%), compared to the negatively-charged liposomes (29.27 ±2.16%). Moreover, cationic liposomes remained longer on the ocular surface, promoting a more pronounced and sustained reduction in IOP New Zealand rabbits [258].

4.2. Liposomes for intraocular administration

Current trends for liposomes directed to posterior segment are focused on targeting and protecting the active substance increasing the half-life and improving the therapeutic effect. Liposomes encapsulating molecules for intravitreal delivery can control and relatively prolong the release of the active substance in the site of action. Besides, liposomes protect fragile molecules, can improve drug stability in biologic fluids and can reduce retinal toxicity [259]. However, problems related to stability in biological fluids and storage which limited drug capacity, and difficulties in sterilization make the development of liposomal formulations difficult to perform.

Several studies have demonstrated that drug-liposomes complexes diminish drug toxicity because of a limited amount of drug is in contact with tissues. According to this fact, some drugs characterized by its high toxicity in the site of action have been formulated as liposomal form. Amphotericin B (AmpB) is a clear example [260,261]. Barza et al. showed that AmpB liposomal formulation (SUVs) composed by PC:Cht:tocopherol succinate (5:3:1 molar ratio) produced less toxic effects than a AmpB marketed solution after a single intravitreal injection in healthy pigmented rabbits. According to that results, lesions as focal retina damage, retinal atrophy or

necrosis, typically associated to AmpB solution were not present in animals that received AmpB-liposomes [197].

A novel formulation based on a pro-drug of GCV, 1-O-hexadecylpropanediol-3-phospho-ganciclovir (HDP-P-GCV), entrapped into liposomes was developed by Freeman et al. to CMV infection treatment. Liposomes were prepared by REV with a complex formula: HDP-P-GCV:DOPC:DOPG:Cht at a molar ratio of 12:48:10:30. Pro-drug final concentration to intravitreal injection ranged from 0.2 mM to 2 mM. A single intravitreal injection of HDP-V-GCV-liposomes (0.2 mM) was able to protect the treated eyes against CMV retinitis for 4-6 weeks in the CMV retinitis animal model developed by the researchers. Authors claimed that this novel formulation could resulted very promising for retinitis treatment by intraocular injection [262].

There are several works employing liposomes to encapsulate DNA or RNA for gene therapy which are administered by intravitreal route [29].

Some works have evaluated the efficacy of lipoplexes to retinal diseases treatment [30,31]. The first liposomal formulation for intravitreal injection marketed was Vitraven™ (Formivisen, Isis Pharmaceuticals, Carlsbad, CA) (approved by the FDA in 1998). Vitraven™ is composed by liposomes encapsulating an antisense oligonucleotide to the treatment of Citomegalovirus retinitis in AIDS patients [263,264].

In another work, liposomes containing DOTMA **(***N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride):DOPE (Dioleoylphosphatidylethanolamine) in a molar ratio 1:1 and DOTMA:Cht (1:1 molar ratio) were prepared according to the thin layer method by Sasaki et al. After sonication and extrusion, to obtain homogeneous sizes for lipid vesicles, liposomes were incubated with the plasmid DNA (pDNA) combining in a molar ratio1:2 (pDNA:cationic liposome charge ratio). pDNA:DOTMA:Cht showed the

highest transfection efficiency in the ocular tissue compared to pDNA alone and other lipid composition formulations prepared [265].

After intravitreal injection, lipoplexes must overcome two important barriers. The first one is the high viscosity of the vitreous that not only avoid the lipoplexes diffusion to reach the site of action (neuronal retina or RPE) but also induce lipoplexes aggregation. Secondly, the presence of the polyanionic glycoaminoglycans in the vitreous can displace the DNA from the complex. Improvements on lipoplexes formulation are nowadays under study to minimize these problems, for example by using pegylated lipoplexes. As example, Peeters et al. developed pegylated lipoplexes composed by DOTAP:DOPE (1:1 molar ratio) and DSPE-PEG from 0 to 16.7% by thin lipid hydration method. Liposomes size range from 233±12 nm to 106±5 nm decreasing size when increasing PEG and zeta potential ranged from 39±1 mV to 0±1 mV decreasing charge when increasing PEG. This study concludes that a concentration as 16.7% of DSPE-PEG in lipoplexes can avoid aggregation phenomena in the vitreous promoting transport of lipoplexes to the RPE [266]. Bochot et al. (2002) also developed pegylated liposomes for the intravitreal delivery of an oligonucleotide model, pdT16. The liposomes, composed of PC:Cht:PEG-DSEP (polyethylene glycol-distearoyl ethylphosphocholine) 64:30:6 molar ratio, were prepared by organic solvent evaporation method following cascade extrusion. The liposomal suspension was incubated with the oligonucleotide and 10 cycles of freeze-thaw were carried out to obtain the liposomal formulation containing pdT16 (250 µM) with a vesicle size of 150 nm and an encapsulation efficiency of 17.7%± 6 4.7%. Intravitreal injection of liposomes loaded with oligonucleotide were able to protect the active substance against degradation and, besides, liposomes prolonged the pdT16 release into the vitreous, retina and choroid [267]. Lajavardi et al. (2009) also used pegylated liposomes to

improved intravitreal delivery of a proteic substance, VIP (vasointestinal peptide) for the treatment of uveitis inflammation. Labelled Rhodamine liposomes composed by PC:PG:Cht:PEG-DSPE:PE-Rh (final molar ratio 47:10:35:5:3) (final lipid concentration 50 mM) were prepared by thin-layer method followed by extrusion and VIP was encapsulated by freeze-thaw method (150 µM) which lead in liposomes sizes in the range from 300 nm to 600 nm with a negative zeta potential. These mentioned liposomes, were able to protect protein from degradation in the vitreous, being dispersed in a hyaluronic acid matrix (1.2%) in order to maintain a sustained release of VIP [268]. Based on previous studies developed by Bochot et al., Camelo et al. determined the intraocular and biodistribution of Rhodamine liposomes (Rh-Lip) empty and loaded with Vasointestinal peptide (VIP) (VIP-Rh-Lip) 24h following intravitreal injection into healthy rats and, besides, authors evaluated the cells and organs implicated in the internalization of both liposomal preparations (unloaded and VIP loaded). Rh-Lip was prepared by thin layer method (composition and final molar ratio were PC:PG:Chol:PEG-DSPE:PE-Rh and 47:10:35:5:3 respectively). VIP was entrapped in Rh-Lip by freeze-thaw method (final VIP concentration in VIP-Rh-Lip was 0.55 mg/ml). After intravitreal injection of both liposomal formulations, liposomes stayed mainly in the posterior segment of the eye and some liposomes were internalized by retinal Müller glial cells. After VPI-Rd-Lip intravitreal injection, VIP was detected in macrophages and T cells indicating that VIP-liposomal formulation may be involved in regulating immune response in the eye [269].

4.3. Liposomes for periocular administration

Liposomes loaded with antigens had been proposed as periocular vaccines. Previous studies have demonstrated the main role of periocular vaccination for preventing HSV

infection in rabbits[270-273]. According to these results, Cortesi et al. developed positively-charged liposomes composed by PC:Cht:$DDBA_{18}$ (dioctadecyl-dimethyl-ammonium bromide), in a 8:8:1 molar ratio, and incorporated a recombinant secreted form HSV-1 glycoprotein B(gB1s) with a protective immunity activity and two peptides mimicking gB1s, DTK1 and DTK2, with additional antiherpetic activity. Liposomes were prepared by thin-lipid evaporation method and sonicated to obtain a homogeneous size. The final liposomal formulation was left maturate overnight to increases encapsulation efficiency. Liposomes presented a size close to 300 nm and a zeta potential about 23 mV. For *in vivo* evaluation, New Zealand rabbits were vaccinated by periocular administration (3 vaccinates for 3 weeks) with liposomes entrapping DTKs, liposomes including gB1s and empty liposomes. Afterwards, animals were infected with pathogenic HSV-1 variant and clinical eye signs and symptoms (conjunctivitis, iritis, epithelial keratitis, and corneal clouding) were evaluated on day 3,5,7,10 and 14 post-infection. 6 of 9 animals treated with liposomal formulation containing both peptides and gB1s, survived compared to 0 survivals in animals vaccinated with empty liposomes. It was demonstrated that the liposomal formulations prepared increased the survival of HSV infected animals [246]

5. MICROEMULSIONS AND NIOSOMES FOR OPHTHALMIC ADMINISTRATION

5.1. Microemulsions for topical administration

ME allow drug delivery and drug permeation across the ocular surface. Several authors have developed microemulsions for topical administration to solubilize drugs and to increase its ophthalmic bioavailability. Depending on the charge and the viscosity of the ME, the ocular contact time of the formulation can be increased.

CyA is an example of a drug incorporated in a ME. Restasis™ (Allergan, Inc., Irvine, California) is the first ME commercialized containing CyA 0.05% as ophthalmic formulation. Restasis contains polysorbate 80 and carbomer 1342 at alkali pH to stabilize the CyA -loaded anionic lipid emulsion. Several studies support a safety and favorable profile for CyA 0.05% in ophthalmic emulsion (marketed formulation) for ocular diseases as severe dry eye and meibomian gland dysfunction [274,275].

Fialho et al. formulated dexamethasone in a ME elaborated by titration method in which dexamethasone was dispersed in the oily phase, composed by isopropylmiristate. After that, surfactant (Gremophor EL, a castor oil derivative) and water were added and mixed to form an O/W emulsion. Then, the co-surfactant, propylenglycol, was added and mixed until a transparent system (microemulsion) was found. This ME presented suitable properties for topical administration in terms of pH, surface tension and viscosity. The formulation resulted stable for a three months period of time. As expected, the ocular irritation test (a modified Drazie test) showed that this ME did not produce significant alterations. The most interesting data are related to the bioavailability of the drug. 10 minutes after dexamethasone-microemulsion instillation, drug concentrations were found in the aqueous humor which also were detectable 480 minutes after topical administration, compared with a commercialized dexamethasone ophthalmic suspension which was not detectable in the aqueous humor 360 min after instillation [276]. In this case the surfactants and co-surfactants act as penetration enhancers promoting the drug to cross the corneal barrier and the viscosity of the external phase (40.27 ±0.98 mPa·s) increased ME retention time onto the ocular surface.

The surface charge of ME has been studied in order to increase the retention time onto the ocular surface and, subsequently, to achieve therapeutic drug levels in the anterior

and posterior segment of the eye. For example, Klang and co-workers (2000) formulated indomethacin in a positive-charged and in a negative-charged ME, and compared them with an indomethacin solution. Indomethacin ME components were: poloxamer and glycerol as non-ionic surfactant, the oily phase was composed by Lipoid 80, a medium lipid chains composition, and α-tocopherol as antioxidant. To obtain positive and negative microemulsion were employed a cationic surfactant, stearylamine and an anionic lipid, deoxycholic acid, respectively. Indomethacin 0.1% was dissolved in the oily phase, and both phases (aqueous and oily) were heated separately to 70ºC, then heated together to 85ºC, and mixed using a high-shear Polytron® mixer. After cooling, emulsion was homogenized, filtrated by 0.45 µm and sterilization process by autoclave took place. The mean droplet size were 110 ± 37 nm and 164 ± 63 nm for positive and negative microemulsions respectively with no changes after sterilization process. Besides, indomethacin was chemically stable over a 24 months storage period. Positively charged emulsion enhanced drug corneal penetration achieving higher concentrations of indomethacin in aqueous humor and sclera-retina compared to solution and negatively-charged ME. In addition, positively-charged ME was well tolerated by animals with no irritation or inflammation after eight consecutive instillations hourly. The electrostatic effect between the positively-charged emulsion drops and negatively-charged cornea may be related with this high indomethacin bioavailability [277]. In this case, drug bioavailability increase in both, cationic and anionic formulations, explained by ME colloidal properties. Since the control formulation does not produce surface eye alterations so there is not an enhanced penetration effect and vehicle viscosity is not high enough to increase retention time (2 cps). Nevertheless, the positively charged ME is able to interact with anionic epithelial

corneal surface increasing indomethacin bioavailability compared to both anionic ME and ophthalmic indomethacin suspension.

Alany et al. evaluated the behaviour of phase-transition ME after instillation and posterior dilution with natural tears. Authors elaborated ME phase-transition systems with different viscosities incorporating a hydrophilic model drug, pilocarpine hydrochloride. ME phase-transition were comprised by two non-ionic surfactants, sorbitan mono laurate (Crill 1) and polyoxyethylene sorbitan mono-oleate (Crillet 4), the oily phase was ethyl oleate (Cromadol EO) and the aqueous phase was composed by water at different ratios. Water content affects the viscosity of each formulation, the higher the content of the aqueous phase, the higher the viscosity of the ophthalmic preparation. So, depending on the water concentration different systems were selected: w/o ME 5%, w/o ME 10%, liquid crystalline structure (LC) 26%, o/w coarse emulsion (EM) 85% and 100% solution (w/w). The oil:surfactant mixture weight ratios were kept constant with pilocarpine hydrochloride being incorporated at 1% (w/w). Finally, therapeutic efficacy was analyzed in terms of miotic response in male New Zealand rabbits. All tested preparations showed adequate surface tension to spread homogeneously over the cornea (34±1.6 dyn/cm). Regarding to rheological behaviour, LC structure resulted pseudoplastic (with an apparent viscosity of 5487 mPa·s) while the rest of formulations exhibit a Newtonian flow 112 and 167 mPa·s for 5% and 10% ME respectively). Pilocarpine *in vitro* release studies, performed in Franz diffusion cells, resulted dependent on the viscosity, and consequently on water content, so ME 5% exhibited the faster release followed by ME 10% and finally LC. Regarding the miotic response, the colloidal formulation showed higher therapeutic efficacy in comparison with the solution, employed as control. The phase change in ME produces a viscosity increment and a flow behaviour change (from Newtonian to pseudoplastic).

Consequently, a magnification in ocular residence time and an increased drug bioavailability was observed [37,278].

The use of bioadhesive polymers in the aqueous phase of the ME demonstrated to enhance the drug bioavailability. In that sense, Chauhan et al. developed timolol ME in 2-hydroxyethyl methacrylate (HEMA) gels to increase ocular residence time obtaining promising results [279].

The feasibility of ME sterilization by gamma irradiation has been described by Bosela et al. These authors observed that 5% and 10% water content in ME can protect prednisolone from degradation during gamma irradiation at increasing doses of 2, 5, 10, 15 and 20 KGy [159]

5.2. Niosomes for topical administration

Non-ionic surfactant vesicles (niosomes) have been present in the pharmaceutical field since the 80's decade, their utility for ophthalmic drug delivery is being explored in the recent years because of their ability to increase ocular bioavailability

Aggarwal et al., (2004) prepared niosomes to increase the ocular bioavailability of the poor soluble and permeable drug ACZ, employing sorbitan ester and cholesterol [40]. They evaluated different methods of preparation, being the REV method the best in terms of entrapment efficacy (43.75%). Authors included in the formulation DP or SA to obtain negatively and positively charged vesicles, respectively. They observed that although the positively charged niosomes offered the better entrapment efficiency and also a good corneal permeability, they resulted toxic for corneal cells. A bioadhesive niosomal formulation of AZC was also prepared by coating the niosomes with Carbopol®. The bioadhesive formulation resulted less toxic than positive niosomes. After instillation of 30 µl of the coated-niosomes suspension (0.5% ACZ), a comparable

physiological effect (intraocular pressure reduction) than a marketed eye drop formulation of dorzolamide 2% was observed, with the advantage of a 2-folds increment in the duration in comparison with the commercial formulation. These results were lately supported by pharmacokinetic studies using microdialysis [280]. It was observed that the niosomal formulation induced a more rapid penetration of the drug across the cornea. Furthermore the Cmax in the aqueous humor was two-folds increased and a significant broadening of peak from 80 to 120 min was also observed. In a similar study, these authors evaluated the use of niosomes coated with bioadhesive polymer such as Carbopol® and chitosan to increase the bioavailability of timolol maleate and compared them to timolol solution (0.25%) [41,281]. Authors observed that both coatings increased the antihypertensive effect of the drug. Among them, the chitosan-coated niosomes offered a more sustained effect up to 8 h (*versus* 6 h for Carbopol®-coated niosomes) and compared well with a timolol maleate 0.5% marketed gel formulation. It is interesting to remark that the niosome formulations showed less lowering of IOP in the contralateral eye (20-40% as compared to 100% in case of drug solution), indicating lesser systemic side effects. Abdelbary and El-gendy (2008) developed niosomes for the ophthalmic controlled delivery of a gentamicin sulphate by employing the thin film hydration technique[38]. They tested different commercial surfactants and included in the formulation cholesterol, as stabilizer, and dicetyl phosphate (DP) to get negatively charged surface niosomes. A preparation with 1:1:0.1 molar ratio of polysorbate 60, cholesterol and DP gave the best EE values (92.02%±1.43) and release results, showing a release of 66.29±1.33% at 8 hours of release study. In this work no *in vivo* studies were performed to evaluate the efficacy of the formulations. Abdelkader and co-workers (2011) described the development and *in vitro* evaluation of Span 60-based niosomes for ocular delivery of naltrexone

hydrochloride (NTX) [39]. They also tested the incorporation of different additives demonstrating that these compounds can influence the entrapment efficacy and also the size and shape of niosomes. *Ex vivo* transcorneal permeation studies conducted using excised cow corneas showed that niosomes were capable of controlling NTX permeation and enhance its corneal permeability. The prepared niosomal formulations were found practically non-irritant when applied onto the surface of a 10-day-old hen's chorioallantoic membrane [177]. The same authors observed that niosomal preparations were more viscous that drug NTX solution, suggesting better wetting and spreading abilities on the ocular surface. Interestingly, the prepared formulations were able to protect the encapsulated NTX from the photo-induced oxidation up to 3-folds in comparison to free NTX solutions [42].

6. DENDRIMERS FOR OPHTHALMIC ADMINISTRATION

Despite all the attractive characteristics of dendrimers as new drug nanocarrier systems, their use in ophthalmic routes remains almost unexplored. In contrast to other administration routes (oral, intravenous or transdermic) where interesting progresses have been already performed [50,53,282] only a few authors have paid attention to the potential of dendrimers as drug carriers at ophthalmic level.

6.1. Dendrimers for topical administration

In the past years, some bioadhesive polymers have been tested to achieve higher residence time on ocular surface and decrease the frequency of administration in topical ophthalmic treatment [283]. In this sense, dendrimers contain high density of chemical groups on the surface that could interact with ocular surface tissues, increasing the loaded-drug retention time. An additional advantage offered by dendrimers is that they

do not provoke blurred vision nor formation of any veil on the corneal region, problems typically associated with other bioadhesive polymers [284]. In a pioneer work published in 2005, Vandamme and Brobeck designed a study to investigate the potential of PAMAM dendrimers as ophthalmic vehicles in ocular formulations [180]. They tested PAMAM dendrimers with peripheral carboxylic, amine and hydroxyl groups. No ocular irritation or watering reflex were observed in any of the dendrimers evaluated in concentrations up to 2.0% (w/v), after instillation of aqueous solution of dendrimers in eyes of New Zealand albino rabbits, even for the cationic ones, typically the worst tolerated in studies in other tissues [178,179]. Authors found that the mean ocular residence time for COOH- and OH- PAMAM dendrimers evaluated after their instillation in a buffer solution (approximately 4 to 5 hours), were comparable to that of the 0.2% w/v Carbopo1® solution, a well-known bioadhesive polymer for ophthalmic dosage forms. Interestingly, the residence time of PAMAM dendrimer with amino surface groups was even significantly longer than that of Carbopol® or HPMC solutions. Authors suggested that the bioadhesive properties of PAMAM dendrimers could be explained by their interaction with ocular mucins depending on the structure, shape, and surface functional groups. Pilocarpine nitrate and tropicamide were employed as model drugs for miotic and mydriatic activity tests on rabbits. During the evaluation of dendrimer-drug interaction, a host–guest relationship was suggested leading to a sustained release of both drugs. The activity results obtained showed that the co-administration of PAMAM dendrimers with these drugs prolonged their pharmacological effect, indicating increased precorneal residence time, compared with solution of the drug in PBS. To profound on this aspect, in a recent work, our research group developed the first *in vitro* method based on biosensor chip technology (surface plasmon resonance) designed to evaluate the interfacial interaction phenomena between

human transmembrane ocular mucins and dendrimers. PAMAM dendrimers with NH_2, OH and COOH terminal groups were tested and compared with linear polymers typically used in ophthalmic clinical practise to increase the drug retention time on the ocular surface (HA, carboximethyl cellulose -CMC- and HPMC). Results demonstrated a stronger tendency of PAMAM dendrimers whatever their surface chemical groups (64 NH_2; 64 COOH or 64 OH) to originate interfacial interaction with human ocular transmembrane mucins, in comparison to HA, CMC and HPMC, showing a relevant importance of the co-operative effect of dendrimer due to the proximity of the active chemical groups. Interestingly, the amino and hydroxyl terminal groups PAMAM dendrimers showed statistically similar tendency to interact with transmembrane ocular mucins, suggesting the high importance of non-ionic interactions at that level. The results obtained reinforce the usefulness of dendrimers as polymers able to increase the retention time of drugs on the ocular surface and hence their bioavailability [62].

Yao and co-workers published some works focused on the potential of PAMAM dendrimers as corneal penetration enhancement agents [285,286]. They prepared complexes of PAMAM dendrimers by hydrogen bonding interaction with puerarin, an isoflavone compound with ability to low the intraocular pressure. Both, cationic PAMAM dendrimers and anionic PAMAM dendrimers were tested. *In vitro* release studies demonstrated that the drug was slowly released from the complexes for several hours, especially for the cationic dendrimers (G4 and G5). The quantification of puerarin on rabbits' tear after instillation of complexes in PBS (pH 7.4) showed longer ocular residence times for complex with mean values of elimination half-life of 1.01 hour and 1.8 hours for complexes prepared with anionic and cationic PAMAM dendrimers respectively, in comparison to values of 0.8 hours observed for puerarin eye drops. Authors evaluated the corneal permeation enhancement activity of PAMAM

dendrimers using the Valia-Chien diffusion cell with excised rabbits corneas. An enhancing permeation behaviour of PAMAM dendrimers was observed probably due to their interaction with corneal epithelium membrane cells or originated by their ability to relax the epithelium cell junctions to increase the drug flux. This point was not clarified in the text.

Not only PAMAM dendrimers but also new dendritic structures are being proposed specifically designed for ocular topic application. For example, Spataro and co-workers developed an interesting new series of phosphorus-containing dendrimers [287]. The core of the dendrimer was one quaternary ammonium salt and the terminal groups were carboxylic acids (3, 6 and 12 -COOH groups for G0, G1 and G2 respectively). According to authors' suggestion, the presence of a quaternary ammonium group as core of dendrimers could replace benzalkonium chloride, so no preservative might be needed in the final formulation. Once synthetized and characterized, dendrimers were used to form electrostatic complexes with carteolol, an ocular anti-hypertensive drug used to reduce the intraocular pressure (IOP) in glaucoma treatment. The catanionic dendrimer created presented high water solubility only when the G0 dendrimer was used, while for G1 and G2 solubility limitations were found. Complexes were instilled in rabbits' eyes to evaluate the benefit of these nanosystems to enhance the penetration of the drug to the aqueous humor. No irritation was observed, whatever the dendrimer used, for several hours. In spite of the low solubility of the conjugate prepared with the second generation of dendrimer, the quantity of carteolol penetrating inside the eye was larger (2.5 folds) than expected when compared with carteolol alone. Authors concluded that, even if the solubility of such systems is a limitation, the pharmacodynamic observations highlight the potential usefulness of phosphorous anionic dendrimers on the ocular surface.

In another interesting work, Durairaj and co-workers evaluated the use of dendrimeric polyguanidilyated translocators (DPTs), a novel class of dendrimers with triolyl branches and surface guanidine, as ophthalmic delivery nanocarrier of gatifloxacin for topical application in the treatment of bacterial conjunctivitis [288-290]. The model drug selected, gatifloxacin, was complexed with DPT dendrimers forming nanostructures (346 nm) thanks to ionic, no-ionic and hydrophobic interactions. According to authors, at these conditions the solubility of the drug was four-fold increased and its antibacterial activity against resistant strain of *S. aureus* was even enhanced. In a drug uptake *in vitro* study with corneal epithelial cells, it was observed that the complexes rapidly penetrated into the corneal cell in a few minutes, in comparison to drug alone. The drug-dendrimer nanosized complex was instilled in the eye of New Zealand rabbits at concentration 1.2% wt/vol. Results demonstrated that the complex of gatifloxacin with DPT dendrimers increased the bioavailability of the drug in targeted tissues in magnitude, with in 13-fold, and 2-fold higher areas under the curve (AUCs) for tissue concentrations in conjunctiva and cornea, respectively, when compared with drug solution. An increment in drug bioavailability in velocity was also denoted offering sustained aqueous humor and vitreous drug levels during the 24-hour study, with half-life values of 9 and 32 hours, respectively. Authors suggested that the systems created would allow the instillation of the drug in the complex only once a day to control the infection.

6.2. Dendrimers for intravitreal administration

Marano and co-workers have published several works concerning the use of cationic lipoaminoacids dendrimers to enhance cellular uptake of antisense-oligodesoxinucleotides (AS-ODNs) after intravitreal injection. Firstly, Wimmer and co-

workers designed and synthesized lipid–lysine dendrimers in an attempt to improve the delivery into the nuclei of retinal cells of ODN-1, a sense oligonucleotide that was reported to have potent anti-VEGF activity, potentially useful in the treatment of ocular disease such as ARMD and DR [291]. The new dendritic structure combined the presence of the lipidic part, with high degree of membrane-like character that might facilitates their crossing of biological membranes, and the poly-Lys part that could interact with ODN-1 offering protection of nucleic acids against nucleases. The transfection potential of the formed dendriplexes was confirmed in retinal pigmented epithelium cells (RPE D407) cultures. In a second work [292], authors increased the chemical family of lipid–lysine dendrimers and reported the ability of these lipid–lysine dendrimers to delivery ODN-1 into cells, causing a reduction in VEGF expression after intravitreous injection in choroidal neovascularisation (CNV) animal models (rats). The results showed that dendrimer/ODN-1 dendriplexes significantly suppressed VEGF expression in cell level studies during the first 24 hours (40–60%), some of them even remained active for up to 2 months after injection, verifying that these lipid–lysine dendrimers could protect the ODN-1 form nucleases and prolong its delivery *in vivo*. In a third study [293], the *in vivo* study was prolonged for six months, observing that the VEGF reduction remained active for four months. Furthermore, in that study authors also evaluated the dendriplex tolerance after *in vivo* administration. No significant toxicity and damage were observed on injected rat eyes after ophthalmological examinations. Finally, in 2006 a new family of lipid-lysine dendrimers were presented with galactose moieties on their surface [294]. Authors observed that this modification increased the solubility of the nanosystems and increased the transfection of oligonucleotides in RPE 51. However, they recognized that the chemistry of this new

family might be improved. Since then, to our knowledge, any new result has been published.

In a recent work, Izze and co-workers showed that PAMAM dendrimers with OH terminal groups have an intrinsic ability to selectively localize microglia, and can deliver drugs inside these cells for a sustained period [295]. Studies with fluorescent markers attached to surface chemical groups of the dendrimer demonstrated that this tropism was only observed in the retina of rats with retinal degeneration. Authors hypothesised that in case of neuroinflammation, activated microglia, very phagocytic, could endocytose the dendrimer, retaining them during four weeks at the experimental conditions. According to authors, this pathology-dependent biodistribution could be exploited to treat disease promoting retinal neuroinflammation like age-related macular degeneration and retinitis pigmentosa. Authors prepared conjugates of PAMAM-OH G4 with fluocinolone acetonide, a neuroantinflammatory drug. The conjugate released the drug in a sustained manner over 90 days. *In vivo* efficacy studies demonstrated that only one intravitreal injection (1 µg of drug/ 7 µg of dendrimer) was able to stop retinal degeneration, preserve photoreceptor outer nuclear cell counts, and attenuate activated microglia, for an entire month in a rat retinal degeneration model.

6.3 Dendrimers for periocular administration

Shaunak and co-workers evaluated the conjugation of anionic PAMAM dendrimers with glucosamine to prepare immuno-modulator systems and the conjugation with glucosamine 6-sulfate to obtain antiangiogenic systems. These two conjugates were administered by subconjunctival injection in a rabbit model of wound healing after glaucoma filtration surgery. Results showed that the co-administration of the two dendritic conjugated increased the long-term success of the surgery from 30% to 80%, reducing the inflammation and decreasing the angiogenic responses, compared to

placebo-treated animals. In addition, there was no evidence of any persistent inflammatory or neoangiogenic response 30 days post injection. Authors claimed the potential utility of the systems created in clinical practice [296].

Kang and co-workers [297] prepared host-guest anionic PAMAM-carboplatin systems that aggregates forming nanoparticles (258 nm). Authors proposed the administration of these nanoparticles by subconjunctival injection for the treatment of retinoblastoma, to reduce the important side effects connected to systemic or subconjunctival administration of drug solution. Results demonstrated that the mean tumour mass in eyes of retinoblastoma animal models (LHβ-Tag mice) was significantly reduced after injection of nanoparticles, in comparison to the non-treated group and also in comparison to the group treated with carboplastin aqueous solution. Authors explained the results on the basis of prolonged retention of nanoparticles and sustained drug delivery.

7. CONCLUSIONS AND PERSPECTIVES

The present review describes the usefulness of different pharmaceutical systems for ocular administration by different routes of administration.

Depending on the target site topical, periocular and intravitreal administration is chosen. Tolerance is critical in all formulations as the ocular tissues are extremely sensitive. For topical administration, liquid and semisolid formulations are the most preferred. Although nanoparticles have demonstrated to increase bioavailability of the drug by their penetration to the cornea, especial care has to be taken in order to avoid alteration of this physiological barrier.

One of the key challenges to effective ophthalmic therapy is to target the active substance to the specific ocular tissue. Liposomes, dendrimers and niosomes are promising mainly for topical administration. They can be used to augment penetration

of the active substance through the cornea and/or conjunctiva to reach the aqueous humor or anterior segment tissues and for diseases affecting the surface of the eye. Furthermore, their use in the ophthalmic field is not limited to drug delivery. In fact, liposomes are already proposed as components of artificial tears as their components are similar to the ones present in the preocular tear film.

Nano and microparticulate systems play an important role in the future of treatment of ophthalmic diseases affecting the back of the eye. It is clear that microparticles are more useful for the treatment of chronic diseases in which effective concentrations of the active substances have to be maintained during long periods of time. Nanoparticles are useful to protect substances for degradation and result more adequate to gen delivery and to be up taken by the RPE or other cells with phagocytic activity.

A greater focus is being placed on the combination of different pharmaceutical systems to design personalized therapies. Future directions will be further developments of effective topical formulations involving more sophisticated physicochemical systems (i.e. liposomes and niosomes dispersed in bioadhesive, or thermo-responsible polymers, ME with the addition of bioadhesive polymer). Dendrimers are promising platform for ophthalmic therapy as the functional groups on their surface can be readily controlled for enhancing cell permeability, targeting or drug delivery. Advances in nanotechnology will provide innovative nanoparticles useful for poor aqueous soluble, poor permeable or labile therapeutic molecules. Microparticles loaded with several active substances will allow the treatment of chronic and multifactorial ophthalmic diseases offering the advantage of more prolonged drug delivery compared to nanoparticles.

On-going research is being conducted to determine the efficacy and safety of the new advantageous ophthalmic formulations.

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FIGURES AND CAPTIONS

Figure 1: General non-scaled schema of the main micro and nanosystems intended for ocular drug delivery.

Figure 2: Different administration routes for ocular drug delivery.

Figure 3: Elaboration of liposomes by the thin-layer hydration method

Figure 4: Elaboration of PLGA microspheres loaded with dexamethasone by the O/W emulsion method

Table 1: Classification of the lipid vesicles depending on their structure.

Table 2: Main bibliography related to micro and nanosystems for administration.