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Polymeric Nanoparticles for Drug Delivery to the Posterior Segment of the Eye

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Abstract: Diseases of the posterior segment of the eye account for most cases of irreversible blindness worldwide. Drug delivery to this closed compartment remains a challenge because of the internal and external blood retinal barriers that selectively control drug penetration into the retina. Direct intraocular (intravitreal) delivery is currently used to achieve high drug concentration in the vitreous and the retina but is usually associated with several side effects. Alternatively topical, periocular, and systemic routes of administration can be used but are associated with low bioavailability and specific side effects. Therefore, intraocular sustained drug delivery systems are being designed to overcome these limitations. Polymeric nanoparticles loaded with therapeutic compounds are under investigation to provide new tools of administration to the eye. The first part of this paper will briefly review the barriers to ocular delivery of drugs and the advantages of using polymeric nanoparticle carriers as drug delivery systems. In the second part, the results in terms of preparation and characterization of polymeric nanoparticles loaded with nucleic acids, the study of the transretinal pathway of intravitreally injected nanoparticles and the assessment of their ability to efficiently deliver plasmids and oligonucleotides will be discussed.

Keywords: Drug delivery systems · Eye · Gene therapy · Intravitreal administration · Nanoparticles · Retinal pigment epithelium

1. Intraocular Drug Delivery Challenges

The anterior segment of the eye is an attractive target for pharmacological, surgical, and gene therapies due to its relative immune privilege as a closed system with multiple barriers [1–3]. In clinical practice, the eye is generally divided into two parts: the anterior segment and the posterior segment. The anterior segment includes cornea, anterior chamber, iris, crystalline lens, and ciliary body. The posterior segment includes the vitreous, retina, choroid, and optic nerve head. Posterior segment diseases, whether of degenerative, infectious, inflammatory, proliferative or neovascular nature, account for most cases of irreversible blindness worldwide.

Retinal pigment epithelium (RPE) is the outermost retinal layer; its integrity and functions are essential for neural retina homeostasis. It also plays a major role in ocular diseases associated with senescence such as age-related macular degeneration and diseases associated with dystrophies of the photoreceptors. RPE cells, therefore, are potential targets for drug delivery and gene transfer aiming at stopping or reversing the processes leading to these diseases.

Long-term delivery of biologically active molecules to the posterior segment is challenging. Indeed, it requires the development of drug delivery systems to improve drug availability at a target tissue confined in a closed system allowing the use of lower doses, more distant administrations in time, and subsequently fewer adverse effects. Four approaches are available to deliver drugs to the posterior segment of the eye: topical, systemic, periocular, and intraocular routes.

Topical delivery of drugs either in solution, suspension, gel or ointment has been the main route of drug delivery for the treatment of ocular diseases due to the simple application. Despite the relatively low bioavailability to the anterior segment of a topically applied drug, topical formulations remain effective and widely used. To reach the posterior segment, topically applied drugs have many barriers to cross. First of all, the continuous turnover of tears clears away most of the applied formulation. Then, the drug has to cross the cornea, relatively impermeable to most xenobiotics, the anterior chamber and the crystalline lens area before reaching the fundus of the eye. This way is further slowed down by the counterdirectional intraocular convection [4][5]. Therefore, high drug concentrations in the formulations are needed [6].

Alternatively, systemic delivery such as parenteral or oral administrations may be used. However, systemic delivery requires, again, large doses in order to overcome the blood-aqueous and blood-retinal barriers; this is usually associated with significant side effects.

Periocular routes (including sub-conjunctival, sub-tenonian, and retrobulbar routes) rely ideally on transscleral diffusion

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(in addition to the systemic absorption and recirculation) and can achieve therapeutic levels in the posterior segment. The transscleral diffusion rate can be further enhanced by iontophoresis that induces local breaks in the barriers and may enable drugs to penetrate the chorioretinal tissue and the vitreous cavity [3][4][7][8].

Direct intravitreal delivery *via* the *pars plana* is currently used to achieve high drug concentration in the vitreous and the retina. Depending on the clearance from the vitreous, large doses and frequent administrations may be required to ensure therapeutic levels over an extended period of time. Frequent injections may lead to complications such as vitreous hemorrhage, infections, and lens or retinal injuries and are inconvenient for the patients [9]. Sustained drug delivery devices offer an excellent alternative to multiple intravitreal injections: this approach increases drug bioavailability, ensures controlled and long term release of molecules, and avoids repeated intraocular procedures [10][11].

2. Polymeric Nanoparticles for Drug Delivery to the Posterior Segment of the Eye

2.1. *Preparation and Characterization*

Polymeric particles used for drug delivery are defined as colloidal systems made of solid polymers that may be classified according to their size and preparation processes [12]. Nanoparticles (NPs) are submicronic particles made of a polymeric matrix in which the drug is either adsorbed at the surface or dispersed in the core of the particles. Particles may be produced by polymerization of synthetic monomers, or dispersion of natural macromolecules or synthetic polymers. The preparation methods have been extensively reviewed in the literature [13–15] and will not be detailed in this paper.

The preparation of particles from preformed polymers is based on polymer precipitation. Basically, an organic solution of the polymer is emulsified in an aqueous solution with or without a surfactant. In a second step, the organic solvent is removed by methods such as evaporation, diffusion or salting out under stirring to allow particle formation. With these techniques, the drug has to be at least partially soluble in an organic solvent to be encapsulated. This is a major limitation to the encapsulation of hydrophilic compounds such as peptides, proteins or nucleic acids. Therefore, alternative methods have been developed to increase the encapsulation rate of hydrophilic molecules. It is possible, for example, to derivatize a hydrophilic compound to form a hydrophobic complex [16][17]. A more common way is to use a double emulsion technique in which an aqueous solution of the hydrophilic compound is first emulsified in an organic solution of the polymer. The first emulsion is then suspended in a large aqueous volume to allow particles formation after solvent removal [18][19]. The double emulsion technique has fairly good encapsulation efficiency for hydrophilic compounds; however, particle size is usually larger than with single emulsion technique.

Different polymers and macromolecules either from natural or synthetic origin can be used for the preparation of nanoparticles. Among these, slowly degradable aliphatic polyesters such as polylactides are of particular interest. The polylactide (PLA) and polylactide-co-glycolide (PLGA) copolymers are biocompatible and can be synthesized in various molecular sizes and conformations allowing for the encapsulation of different drugs. Their degradation rate depends on the polymer molecular weight, conformation, and copolymer composition. Furthermore, the use of these polymers is authorized by the FDA in humans as suture thread, for instance. Encapsulation of molecules within PLA or PLGA preserves the molecular integrity of the encapsulated drug and prevents their rapid *in vivo* degradation. Encapsulation of DNA plasmid or oligonucleotides (ODN) within PLA has also been shown to preserve their *in vivo* functional integrity [20][21].

Numerous studies have focused on the use of polymeric particles as drug delivery systems for ocular treatment [10][22]. A large part of the work was dedicated to topical administration and will not be discussed here.

2.2. *Transretinal Pathway of Intravitreally Injected Polymeric Nanoparticles*

The study of particle administration to the posterior segment of the eye has been reported by several groups. The migration of the nanoparticles after intraocular injection has been documented with fluorescently labeled polystyrene [23] and PLA [24] NPs. The presence of NPs was observed in the intravitreal cavity for as long as one month after administration of the polystyrene particles, the smaller particles having a longer residence time. Only particles with a diameter smaller than 200 nm were observed in the RPE layer [23]. With PLA NPs, a residence time of at least two months was reported [24]. The influence of the particle size was confirmed *in vitro* on ARPE-19 cells, a human retinal pigment epithelial cell model [25]. PLA and PLGA nano- and microparticles are also described as being well tolerated by the intravitreal ocular tissue [24][26]. Intravitreal efficacy and biodistribution of drug loaded nanoparticles have been described with different kind of polymers loaded for example with tamoxifen [27] or ganciclovir [28].

Recently, our team has shown that PLA NPs of different sizes and surface charges are able to migrate through the retinal layers and to accumulate in the RPE cells after intravitreal administration in rat [24]. Using environmental scanning electron microscopy, confocal microscopy, histology, and immunohistochemistry, the nanoparticle pathway was followed from the injection site onto the internal limiting membrane (ILM) to the ganglion cell layer (GCL) and the external retinal layers. Six hours after the injection, NPs were observed at the RPE level with a significant accumulation within the cell cytoplasm at 24 h. These observations demonstrated that a transretinal movement of the NPs took place. Furthermore, the presence of the NPs within the RPE cells four months after a single injection shows that a steady and continuous delivery of drugs can be achieved.

These results are a proof of concept that biodegradable NPs can be used *in vivo* as controlled and sustained release drug delivery systems targeting the outer retinal layers by means of a simple intravitreal injection.

2.3. *Polymeric Nanoparticles for Genetic Material Delivery to the Posterior Segment of the Eye*

The better understanding of the molecular bases of ocular diseases has provided new approaches based on nucleic acid administration. These approaches involve the administration of plasmid DNA, or the use of antisense oligonucleotides. For instance, subretinal injection of a recombinant adeno-associated virus (AAV) carrying wild-type RPE65 plasmid restored visual function in a large animal model (RPE65-/- dogs) of childhood blindness [29].

However, nucleic acids are very unstable in biological media and suffer from a poor intracellular penetration. Intravitreal injection of naked genetic material is poorly efficient due to the fast degradation of naked DNA in the vitreous, and the subsequently limited transfection to the inner retinal layers. Therefore, viral or non-viral vectors are needed. Subretinal delivery of virally vectorized genetic material has been used in most published experimental trials [1][29][30]. This route relies on the creation of a localized retinal detachment, and is hazardous in fragile human retinas. In spite of relatively reduced transfer efficiency, it is important to develop non-viral gene delivery systems in order to bypass the hazards and immune reactions associated with viral vectors [31].

Among the non-viral vectors, nanoparticles may offer several advantages for ocular administration of these drugs, such as intravitreal administration, controlled release,

injectable and sterilizable formulations, and long shelf-life after lyophilization, in addition to possible targeting and uptake by retinal pigment epithelium. Encapsulation of plasmid DNA and oligonucleotides within PLAs or PLGAs preserves their molecular integrity and prevents their rapid *in vivo* degradation [32][33].

2.3.1. *Plasmid Delivery to the Posterior Segment of the Eye*

Encapsulation of DNA plasmid within PLGA NPs has been shown to preserve their *in vivo* functional integrity, and produce efficient transfer and sustained expression of an alkaline phosphatase gene [34].

We have established that green fluorescent protein (GFP) and red nuclear fluorescent protein (RNFP) loaded PLGA NPs can effectively transfect human and bovine RPE cells *in vitro*. The levels of expression initially detected after 48 h of incubation remained unchanged during the following 8 days in culture [35]. These NPs were administered to rats and a preferential RNFP expression within the RPE cell layer was observed after a single intra vitreal injection of RNFP plasmid loaded PLGA NPs (Fig. 1).

2.3.2. *Antisense Oligonucleotide Delivery to the Posterior Segment of the Eye*

2.3.2.1. *Vascular Endothelial Growth Factor Antisense Oligonucleotide*

Retinal neovascular diseases including diabetic retinopathy and age-related macular degeneration are the leading causes of blindness in developed countries. These pathologies are characterized by an abnormal blood vessel proliferation with vascular leakage and hemorrhagic lesions responsible for vision loss. Vascular endothelial growth factors (VEGF) play an important role in the development and leakage of neovessels within the eye. Inhibition of VEGF activity is currently under investigation for the treatment of vascular-related diseases. To modulate the expression and/or activity of VEGF and other factors involved in the development and phenotype of neovessels, specifically where pathological events take place is of the utmost importance. Targeting the regulation of gene expression in retinal and/or vascular cells can be achieved by antisense oligonucleotides.

It was shown in the ARPE-19 cell line that PLGA NPs enhance cellular delivery of an encapsulated VEGF antisense oligonucleotide, and inhibit VEGF secretion and mRNA expression, while no effect was observed with the free oligonucleotide [34][36].

We investigated the capability of PLGA NPs to enhance the delivery of ODN in bovine and human RPE cells in the prospect of a specific gene expression regulation *in vivo*. PLGA nanoparticles were loaded with both Nile Red (a lipophilic dye with high affinity for the polymer to follow the nanoparticle intracellular pathway) and 6-FAM-tagged anti-VEGF ODN (a covalently linked yellow label to follow the oligonucleotide localization). Using a double emulsion technique, particles of 300 nm were prepared and lyophilized. Particles were then suspended in Hank's balanced buffer saline (HBBS) at a polymer concentration of 4 mg/ml and incubated with bovine RPE for 18 h. Observations under the fluorescence microscope showed a rapid diffusion of the ODN out of the polymeric matrix to in the nuclear area (Fig. 2). The particles were seen in red in the cytoplasmic compartment. These results illustrate clearly the interest of nanoparticles for oligonucleotide delivery.

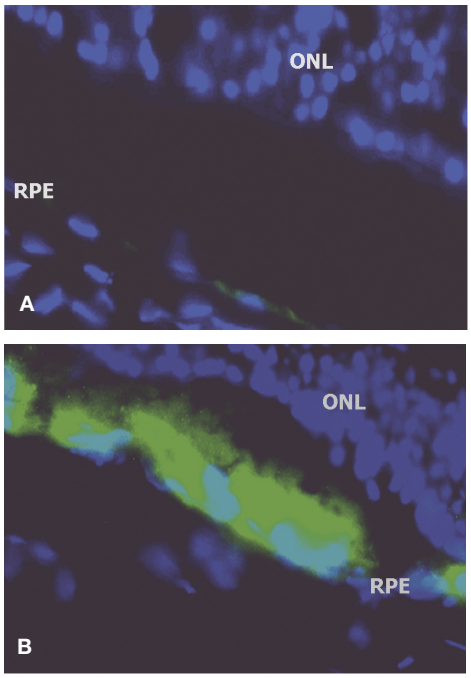

Fig. 1. GFP expression in Lewis rat retina after a single intravitreal injection of GFP plasmid-loaded PLA nanoparticles. Nuclei were stained with DAPI (in blue). A single intravitreal injection of 10 μl of GFP plasmid-loaded PLA NPs (2 mg NP/ml; 7.36 μg DNA/1mg NP) was applied in the eyes of 6–7 week old Lewis rats. GFP expression was preferentially localized in the Retinal Pigment Epithelium cell layer (plus occasional fluorescence in the vascular endothelial cells of the retina and ciliary body). Fluorescence at this level was observed starting 2 weeks, and up to 4 weeks after injection. A: Control. Intravitreal PBS. Very mild normal autofluorescence. B: Day 14 after intravitreal injection of 2 mg/ml of GFP plasmid-loaded NPs. GFP fluorescence is green; nuclei are DAPI stained (blue). RPE: Retinal Pigment Epithelium; ONL: Outer nuclear layer.

2.3.2.2. *Inducible Nitric Oxide Synthase Antisense Oligonucleotide*

Chronic inflammatory conditions are associated with an over-expression of the inducible nitric oxide synthase (iNOS) as a result of pro-inflammatory cytokines. The resulting high-output production of nitric oxide is often associated with cell damage or tissue destruction [37].

The inhibition of iNOS expression *via* antisense ODN represents a highly specific therapeutic approach as anti-inflammatory agents. The efficiency of oligonucleotide delivery was assessed by measuring the anti-iNOS activity after incubation with ODN loaded NPs. We studied the activity of an anti-iNOS ODN encapsulated in PLGA NPs. Chemical dosage showed that anti-iNOS ODN loaded PLGA NPs (320 nm in diameter, loaded with 0.03 μg ODN/mg NP) significantly inhibit *in vitro* nitrate secretion in primary rat RPE cells exposed to lipopolysaccharide (LPS) and interferon (INF)-gamma in order to stimulate their nitrate production (Fig. 3). These results were confirmed by Western Blot.

3. Concluding Remarks

Polymeric nanoparticles are able to migrate through the retina after intraocular administration where they can be internalized in RPE cells with a long residence time. Therefore, they offer an interesting approach for the long term targeted delivery of drug to the posterior segment of the eye, and may be a part of the answer to the challenge of intravitreal treatment of retinal pathologies. Work is in progress to further assess their clinical efficiency and also, from a technological stand point, to improve encapsulation efficiency of hydrophilic compounds such as proteins and nucleic acids.

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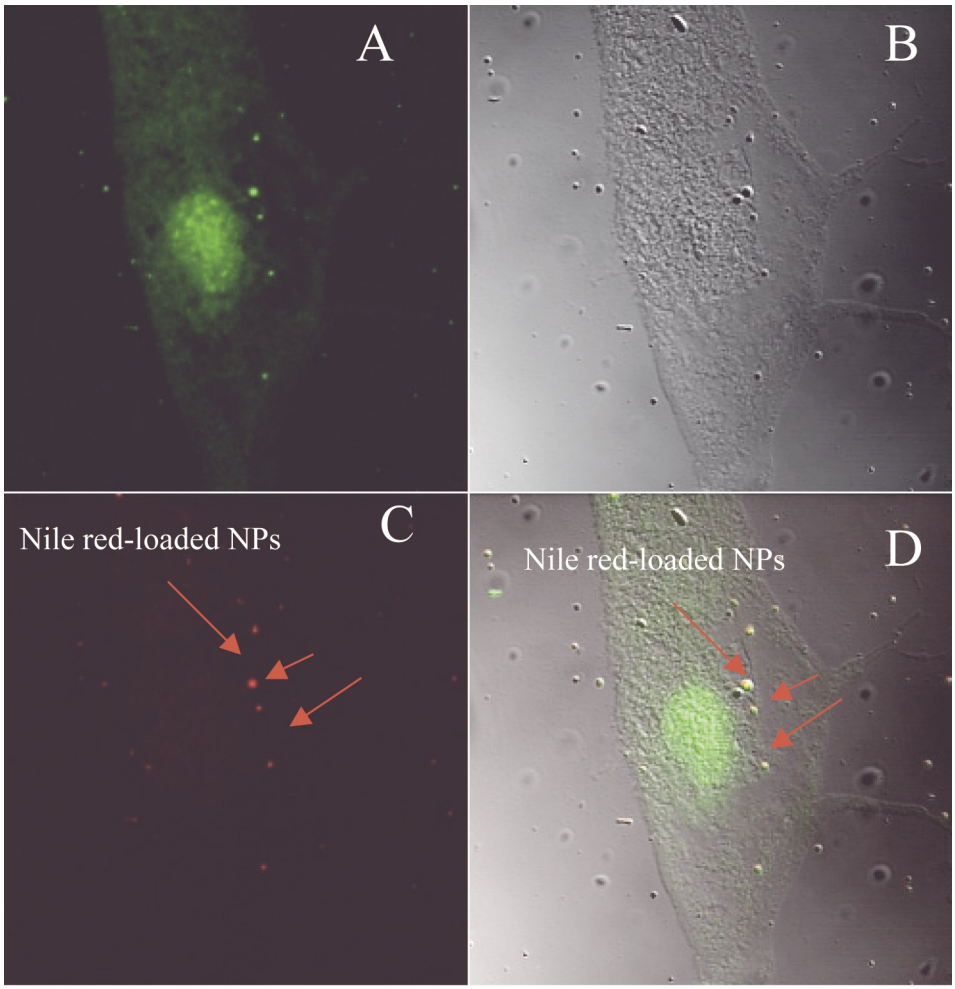

Fig. 2. Localization of Nile-Red labeled nanoparticles (in red) and 6 FAM labeled anti-VEGF-R2 ODN (in green) after incubation with bovine RPE. ODN-loaded NPs (2 mg/ml) were incubated for 6 h with cells. The observation took place 5 days after NPs removal. A: Red fluorescence; B: Phase microscopy; C: Green fluorescence; D: Combined phase, green, and red fluorescence.

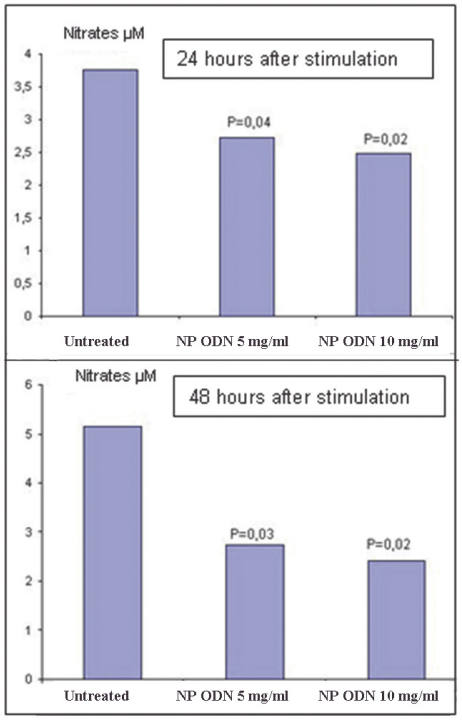

Fig. 3. Effect of anti-iNOS ODN-loaded PLGA nanoparticles on the nitrate secretion in stimulated primary rat RPE cells *in vitro*. Cells were incubated with 100 units/ml INF-Gamma plus 1 µg/ml LPS for 24 h. Treated cells were previously incubated for 24 h with iNOS antisense ODN-loaded PLGA NP (5 and 10 mg NP/ml; 0.03 µg ODN/1 mg NP). Nitrate production was measured using the Griess reaction method.

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