THERAPEUTICS AND CARRIERS: THE DUAL ROLE OF PROTEINS IN NANOPARTICLES FOR OCULAR DELIVERY

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Summary

List of abbreviations	
EE	Entrapment/Encapsulation Efficiency (amount of drug loaded/initial amount of drug added)*100
LC	Loading Capacity (amount of drug loaded/amount of the carrier)*100
aFGF	acid Fibroblast Growth Factor
AMD	Age-related Macular Degeneration
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BRB	Blood-Retinal Barrier
BSA	Bovine Serum Albumin
CNV	Choroidal NeoVascularization
CNTF	Ciliary Neurotrophic Factor
DME	Diabetic Macular Edema
ELP	Elastin Like Polypeptide
HIP	Hydrophobic Ion Pairing
HSA	Human Serum Albumin
IOP	Intra Ocular Pressure
mAb	monoclonal Antibody
MPs	Microparticles
MPT	Multiple Particle Tracking
MW	Molecular Weight
NGF	Nerve Growth Factor
NPs	Nanoparticles
NV	NeoVascularization
PBAE	Poly(Beta-Amino Ester)
PBS	Phosphate Buffered Saline
PEDF	Pigment Epithelium Derived Factor
PEI	PolyEthylenImine
PLA	PolyLactic Acid
PLGA	Poly(Lacti-*co*-Glicolic) Acid
PS	PolyStyrene
RGD	arginine-glycine-aspartic acid tripeptide
RPE	Retinal Pigment Epithelium
SPI	Soy Protein Isolated
US	UltraSound
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide

ABSTRACT

Blindness and visual impairment affect millions of people worldwide and have a very important impact on ~~the~~ patients quality of life. Proteins and peptides represent nowadays an important therapeutic tool~~s~~ for the treatment of ocular diseases but, despite their potential, have significant limitations, as ~~and~~ the~~ir~~ administration of protein-based pharmaceuticals represents a real challenge. ~~Additionaly~~Moreover, ~~drug administration~~ administration of ocular medications ~~to the eye~~ is ~~a~~ difficult ~~task,~~ due to the peculiar structure of this organ and the presence of numerous barriers protecting the eye ~~internal tissues~~inner structure. Nanoencapsulation of peptides and proteins presents a number of advantages for their ocular delivery ~~could, in principle, be of help~~ since it can protect the drug from metabolic activity, control and sustain the release and increase drug bioavailability after topical ~~and~~ or intravitreal administration. In fact, nanoparticulate formulations are contributing to overcome ocular barriers, such as the corneal or the blood-retinal barrier, improve the residence time in the eye, increase local drug level, reduce the drug dosage and showing improved performance when compared to conventional formulations,

Besides, proteins ~~can also be used~~have also been proposed ~~as excipients~~ for the preparation of nanocarriers intended for ophthalmic administration, since they are highly biocompatible, ~~and~~ biodegradable and easily ~~amenable to modifications for the~~modified to ~~attachment of~~link surface ligands.

The present review focuses the attention on the use of ~~–~~protein~~s~~ ~~s~~in ~~in~~ ocular drug delivery nanotechnology:~~:~~ their dual role as both ~~-~~therapeutics and carriers has been critically evaluated and discussed.

1 Ocular drug delivery

Blindness and visual impairment affect millions of people worldwide and have a very important impact on the quality of life. The main causes in middle-income and industrialized countries are represented by glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy. These pathologies are prevalent in the elderly and in diabetic patients and, because of aging population and lifestyle change, the prevalence of these diseases is expected to rise significantly. Beside these, other pathologies with inflammatory, infective, genetic or degenerative causes involve different ocular structures.

In the past years, the introduction of new pharmacological strategies involving proteins (such as anti-vascular endothelial growth factor – VEGF - monoclonal antibodies – mAb -) has significantly changed the clinical outcome of some of these diseases, such as wet AMD. The continuous improvement of understanding the molecular basis of the pathologies will probably increase the number of possible drug candidates in the next years.

Despite the new available molecules, drug administration to the eye remains a difficult task, due to the peculiar structure of this organ and the presence of numerous barriers, static and dynamic, protecting the internal tissues.

1.1 Ocular anatomy

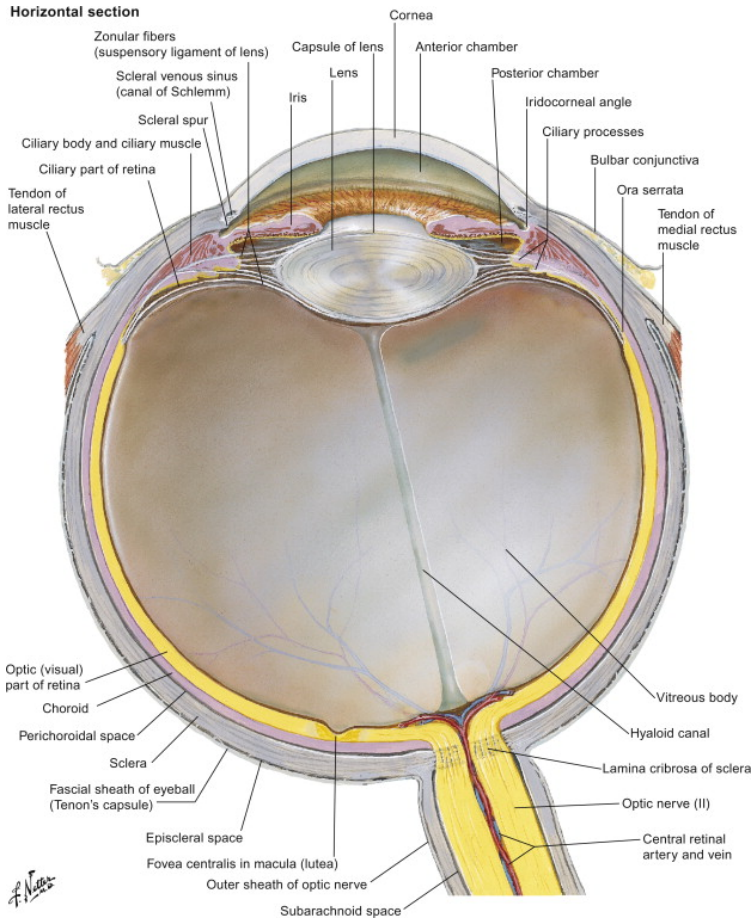

Figure 1. Schematic representation of human eye (with permission from [1]).

Figure 1 illustrates the structure of the human eye [1]. The eye bulb has three concentric tunics, one external, fibrous layer comprising the cornea and sclera; one intermediate vascular tunic comprising the iris, ciliary body, and choroid; and one internal, nervous tunic or retina. Additionally, the eye can be roughly divided into two segments, separated by the lens: the anterior segment (conjunctiva, cornea, anterior and posterior chambers containing aqueous humor, iris and lens) and the posterior segment (vitreous body, retina, choroid and posterior sclera). Anterior and posterior chambers are filled with the aqueous humor, while the vitreous cavity is filled with a gel-like fluid called vitreous humor. The conjunctiva is a transparent mucous membrane that covers the inner surface of the eyelids and the surface of the anterior sclera; it is continuous with the cornea and the junctional region is called limbus. The epithelium of the cornea and of the conjunctiva are covered by mucins (secreted mucins – both gel forming and small soluble - and cell surface-associated mucins) playing a lubricant and protective role and contributing to the hydrophilic character of wet-surfaced epithelia [2, 3].

For the treatment of anterior segment diseases and for the management of glaucoma, the application of eye drops, gels or ointments in the conjunctival fornix and/or on the ocular surface, is the simplest and generally preferred administration route. However, this route is characterised by low compliance (often due to the high administration frequency required) and low drug bioavailability (generally lower than 5%). The latter is due to the short residence time of the formulation on the ocular surface and to the low permeability of the cornea. The cornea is an avascular tissue consisting of a collagenous layer (stroma) enclosed by an anterior epithelium and a posterior endothelium. The epithelium, with tight junctions in its superficial layers, represents the main barrier toward the penetration of drugs.

The posterior segment of the eye is not accessible to drugs applied topically on the cornea or in the conjunctival cul-de-sac; on the other hand the efficacy of systemic administration is hindered by the presence of the blood-retinal barrier (BRB) and limited by possible systemic toxicity. BRB is composed by two distinct barriers, located in different regions: the outer BRB is located in the retinal pigment epithelium (RPE), while the inner BRB is located in the endothelium of retinal microvasculature.

For these reasons, the intravitreal injection (i.e. direct injection of the drug in the vitreous body) is, at present, the most efficient option for drug administration to the retina. However, due to the serious side effects, other, alternative and less invasive administration routes are in use or under investigation: trans-scleral (comprising subconjunctival, sub-Tenon, peribulbar, posterior juxta-scleral and retrobulbar injections), intra-scleral and subchoroidal delivery are among them. Using these routes the drug is not directly available to the retina, but must diffuse across the underlying tissues, overcoming static (sclera, choroid, Bruch’s membrane, RPE) and dynamic barriers, such as blood and lymphatic flux and choroidal circulation.

It is worth mentioning that the integrity, structure and efficiency the different barriers involved in ocular drug delivery can change as a function of two important factors: aging and disease. Since aging is the most important risk factor for several eye diseases, the changes produced by the two causes tend to overlap. Disease-induced barrier weakening or disruption had been shown to influence deeply drug distribution. When the epithelial barrier of the cornea is compromised by the presence of corneal neovascularization (NV), for example, the topical application of a mAb can be effective in the treatment of anterior segment diseases [5]. Concerning the posterior segment, as an example, celecoxib

accumulation in retina and vitreous, following periocular administration, was higher in diabetic than in healthy rats, because of the disruption of BRB produced by diabetes [6].

2 Nanoparticles and eye

Nanomedicines are medicinal products with at least one component at nano-scale size. Until a few years ago the size was the only classification criterion and the nano range was defined as the interval 1-100 nm. Presently, in the EMA website, nanotechnology is defined as “the use of tiny structures - less than 1,000 nanometres across - that are designed to have specific properties”, significantly widening the size range and emphasizing the role of nanometric dimension on the clinical advantages (i.e. specific organ/tissue distribution), more than the sole effect on chemical and physical properties (generally observed for materials with size below 100 nm) [7]. Similarly, the FDA has recently released a Guidance for Industry where the nanotechnology definition is extended to materials or end products “engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm)” [8].

Numerous and diverse are the contributions given (or that could be given in the future) by the nanotechnologies to the treatment of eye diseases [9]: sensors for biophysical measurement (such as wireless contact lenses for IOP - intra ocular pressure - measurement), biosensors for diagnostic and theragnostics [10], prosthetics (i.e. sight restoring therapy by retinal cell stimulation), regenerative nanomedicine [11] and nanocarriers, such as nanoparticles, liposomes and other vesicular carriers, micelles and dendrimers, for the delivery of drugs, peptides and genes. The use of nanocarriers in ocular drug delivery can address some of the the main unmet medical needs, such as the low bioavailability of antibiotics, anti-inflammatory and antiviral drugs, the treatment of retinal and corneal NV, degenerative diseases, hereditary retinal disorders and tumors. Additionally, refractive cornea surgery, healing process in the eye, gene therapy, regenerative medicine (growth factors delivery) could highly benefit from nanocarriers.

The possible advantages of using nanocarriers are due to the special physical and/or chemical properties produced by size reduction, that differ from those at macroscopic scale. In particular, in the case of nanocarriers for pharmaceutical use the most important property is the increased surface area-to-volume ratio. This leads to an increasing dominance of the behaviour of atoms on the surface of a particle, affecting all the surface

phenomena and the interactions with other materials, such as those present in the biological environment in which the particle finds itself after administration. In this perspective, it is clear that size, shape, surface charge, surface coating and functionalization, as well as the nature of the constituent material, highly influence nanocarriers performance and biocompatibility. These characteristics can often be controlled and tuned thanks to parameters variation in the preparation procedures.

In general, NPs have been proposed as carriers to protect the encapsulated drug from degradation, control drug release, increase residence time on the ocular surface, facilitate the transport of drugs into cells and finally increase drug bioavailability. Additionally, due to the very small dimensions, NPs can be administered through any of the routes previously mentioned.

However, the usefulness of this carriers in ocular drug delivery is not “universal” and the benefits depend upon several factors, such as the ocular pathology involved (the different localization inside the ocular bulb but also the target that can be both extra and intra cellular), the administration route and the characteristics of the drug (in particular its potency, its physico-chemical characteristics and its stability in a biological environment). Furthermore, some issues about NPs for ocular delivery must be highlighted. When considering the ocular anatomy, for instance, it is evident that there are important limitations on the volumes that can be administered. For example, maximum one drop (50 µl) is considered suitable for topical application, 20-100 µl for intravitreal injection, 500 µl for subconjunctival injection, 35 µl for intra-scleral injection and 35-100 µl for suprachoroidal delivery [12, 13]. It follows that, in order to administer a significant amount of drugs, NPs should have an high drug loading (not easy to achieve) and/or be highly concentrated, especially in the case of drugs administered at high doses. NPs are not always physically stable and can aggregate when present at high concentration; as a consequence, despite the immune privilege of the eye, inflammatory and/or immunostimolatory events could occur. The issues related to nanomaterial toxicity are a hot topic, that is presently the subject of numerous paper and public discussions. Reflection papers can be found in the website of regulatory agencies, but very few data are available on the specific toxicity on ocular tissues. NP toxicity is related not only to the polymer used (for instance, PLGA - Poly(Lacti-*co*-Glicolic) Acid - is already used for the intravitreal implant Ozurdex® and demonstrated good tolerability), but also to the size that can promote cell-uptake, with possible accumulation inside the retinal cells and damage of cellular function [14].

3 Administration routes

3.1 Topical administration

Topical administration is used when the target is the conjunctiva, the cornea or the anterior and posterior chambers. The limitations of this delivery route, i.e. short formulation residence time, eye irritation, undesirable systemic absorption, low corneal permeability, could, in principle, be overcome by using particulate carriers. Because of the small size, in fact, NPs dispersed in an appropriate liquid can be applied just like eye drops without the sticky feeling or blurring of vision caused by ointments. Additionally, they do not cause scratching, nor foreign body sensation; they control drug delivery, reducing administration frequency, and protect the drug from chemical or enzymatic degradation meanwhile reducing the irritating potential on the ocular surface. Finally, nanoparticulate systems give the opportunity to administer compounds with poor water solubility using an aqueous vehicle.

The main advantage that can be achieved using nanoparticulate carriers is an improved drug bioavailability in the anterior chamber, generally due to increased residence time and, in some cases, enhanced trans-corneal permeability.

The increase in residence time in the cul-de-sac and on the ocular surface can be obtained by using mucoadhesive excipients. The presence of mucoadhesive polymers allows NPs to adhere to the pre-corneal or conjunctival mucus layer by non-covalent bonds and to remain on the ocular surface for as long as mucin is present. This can ultimately lead to a reduction of the frequency of drug administration and thus increase patient compliance. A detailed review on this topic has been recently published [15]. When the constituting polymer is not mucoadhesive, NPs can be superficially coated with mucoadhesive polymers such as chitosan, hyaluronate or alginate [16].

The improved drug bioavailability reported using nanocarriers can also be ascribed to an enhanced trans-corneal transport, due to NPs permeation. This phenomenon has been reported with different polymers and/or coatings and has been ascribed to different phenomena: opening of tight junction in the corneal and conjunctival epithelia, so as to improve the transport across the paracellular pathway, NPs endocytosis, cellular lysis due to NP degradation products [17-19].

Apparently, the possibility for NP to cross the epithelial layers depends upon NP size and surface properties such as charge, composition (hydrophilicity/hydrophobicity) and

coating/functionalization. One of the most studied polymers is chitosan, a natural polysaccharide characterized by biocompatibility, biodegradability, mucoadhesion, and the ability to transiently enhance the permeability of mucosal barriers [20]. At the same time, also NPs unable to permeate the cornea have been reported: a recent paper from Mun *et al.*, for instance, demonstrated on an *ex vivo* bovine eye model that neither thiolated nor PEGylated (0.75 and 5 kDa) silica NPs with a mean diameter between 20 and 70 nm could penetrate the epithelium [21].

In the literature several recent reviews are present on the use of nanoparticulate carriers for anterior segment diseases. Cholkar [22] analyses in particular the results related to anti-inflammatory drugs, while a recent review from Gonzalez *et al.* provide an overview of the possible use of nanotechnology in corneal NV [23]. Kim [24] and Pita-Thomas [25] describe the results obtained and the potential implications for the glaucoma treatment.

3.2 Intravenous administration

Intravenously administered NPs have been studied to target and treat posterior segment diseases, based on the assumption that they can pass through the BRB. Sakai [26] demonstrated, in an experimental uveoretinitis model in rats, that betamethasone loaded in PLGA NPs accumulated in the retina after intravenous injection and was more effective than when injected alone. Kim *et al*. found that intravenously administered gold nanoparticles can cross the BRB depending on particle size. In particular, 100 nm NPs were not found in the retina, while 20 nm NPs passed the BRB and were detected in all retina layers [27]. The possibility to cross the BRB is often related to the presence of ocular diseases. For instance, PLGA NPs loaded with recombinant Flt23k intraceptor plasmid for gene therapy were injected intravenously in a rodent model of laser-induced choroidal neovascularization (CNV): NPs accumulate in the neovascular eye, but not in the control eye, indicating that drug ocular bioavailability following intravenous injections depends upon the integrity of BRB. These NPs were also functionalised with the tripeptide arg-gly-asp (RGD) that can bind integrin receptors, and/or with transferrin that can bind transferrin receptor on the retinal cells surface. In both cases the surface functionalization increased retinal uptake and inhibit the progression of the disease [28].

3.3 Intravitreal injection

The main goal of the intravitreal injection is to deposit the drug in close contact with the target site (retina/RPE/retinal blood vessels/vitreous). Despite its efficacy, this route of administration has significant drawbacks and risks, such as bleeding, pain, infection, IOP increase and retinal detachment. Additionally, for the treatment of chronic diseases such as AMD, monthly intraocular injections are needed, with a corresponding increase in the risk of side effects. Another limitation of this dosage regimen is an initial very high drug concentration in the vitreous that can be responsible for important side effect, in particular in the case of corticosteroids.

In order to reduce these problems, intravitreal implants have been proposed [29, 30]. In this perspective, NPs can be of help since they can be easily injected with a conventional needle and form a reservoir inside the vitreous; due to the small size they should not interfere with the vision (or maybe limit the blurring of vision compared to microparticles); they protect the drug from the environment, increasing the drug half-life in the vitreous and can control the release of the drug thus avoiding peak and valleys in the vitreous concentration (even though they are less efficient in controlling drug release compared to microparticles). In this way they could also reduce the amount of drug that needs to be administered and possibly the volume to be injected (thus reducing IOP increase).

An additional property attributed to nanoparticulate carriers is the ability to cross cell membranes. The first evidence that polymeric NPs can enter the cells is dated 1977 when Patrick Couvreur studied cell internalization of 200 nm nanocapsules [31]. Presently, many papers have demonstrated NPs uptake into cells also in the case of ocular delivery, even though the mechanisms involved are not always known. Mechanisms elucidated till now are endocytosis, clathrin and caveolin mediated endocytosis [32] and endocytosis mediated by transferrin/deslorelin receptors with NPs functionalized with transferrin and deslorelin [33, 34]. The NP cell-uptake can be particularly useful in the case of intracellular diseases and gene therapy, for which NPs have also demonstrated to enhance the transfection efficacy [35].

The fate of NPs injected in the vitreous depends upon the size and the NP surface characteristics, since these properties mediate the interaction with the vitreous humor components, i.e. collagen fibrils and glycosaminoglycans [36]. Bourges *et al*. in 2003 injected PLA (polylactic acid) NPs loaded with fluorescent probes and found a quick trans-retinal passage and an accumulation in RPE cells up to 4 month after injection. Neither the size (140 *vs* 310 nm) nor the zeta potential (-60 mV *vs* -6 mV) seems to influence the

distribution [37]. The importance of surface chemistry on intravitreal movement was evidenced by different authors [38-40]. Kim found that cationic nanoparticles diffuse more slowly in the vitreous than anionic NPs (probably because of a ionic interaction with hyaluronan) and that the positive charge hinders movement more than the size. This data was also confirmed using self assembled polymeric NPs in a murine model: strongly cationic PEI (polyethylenimine) nanoparticles interacted tightly with the vitreous matrix, while cationic glyco chitosan NPs penetrated the vitreous and reached the inner limiting membrane but could not cross it. On the contrary anionic NPs of hyaluronic acid and human serum albumin (HSA) showed the ability to penetrate across the whole retina and accumulate in the RPE [39].

The importance of both size (100-1000 nm) and surface chemistry was also investigated *ex vivo* using polystyrene (PS) amine-modified (cationic PS-NH_2), carboxy-modified (anionic PS-COOH) and PEG coated (not charged, PS-PEG) nanoparticles [41]. A summary of the results obtained is illustrated in Figure 2 and highlights: (i) steric hindrance for 1000 nm particles; (ii) electrostatic hindrance for positively charged NPs; (iii) hydrophobic hindrance for concentrated negatively charged NPs; (iv) free diffusion for non-adhesive (PEG coated) NPs smaller than 1000 nm. These results, obtained *ex vivo* using real-time multiple particle tracking (MPT), can be of great help in the future design of NPs for intravitreal delivery.

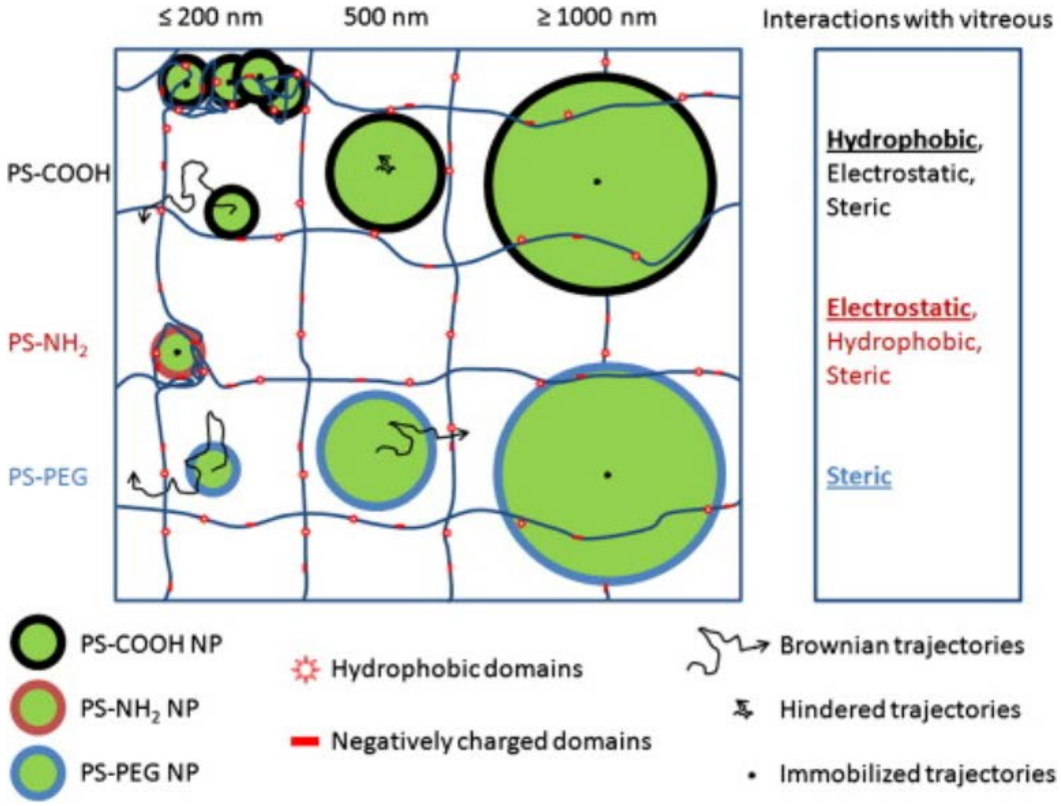

Figure 2. Schematic illustration of the effect of size and surface characteristics on NPs transport in the vitreous. Hydrophobic, electrostatic and steric effects can hinder particle transport in vitreous meshwork. Particles with size ≥ 1000 nm were immobilized due to steric hindrance; dilute PS-COOH NP (≤ 200 nm) diffused freely within vitreous, while concentrated PS-COOH NP aggregated because of hydrophobic interactions. 500 nm PS-COOH NP was greatly hindered due to both hydrophobic and steric effects; PS-NH_2 NP aggregated because of electrostatic interactions with the negatively charged vitreous; non-adhesive PS-PEG NP diffuse freely within vitreous if their size was no larger than 500 nm. From [41] with permission.

3.4 Trans-scleral, intra-scleral, suprachoroidal administration

These new administration routes have been proposed as an alternative to intravitreal injection to reach the posterior segment of the eye without the side effects and risks of direct injection. In the trans-scleral delivery, the drug must cross the whole sclera to reach the underlying tissues; this happens when the drug is applied in the conjunctival fornix, but also in case of subconjunctival, sub-Tenon, peribulbar, posterior juxta-scleral and retrobulbar injections. NPs can be used in these administration routes mainly to create a drug reservoir in order to control and sustain the delivery of drug for long periods, thus reducing administration frequency. NPs should in this case be retained as long as possible at the injection site so as to deliver all the loaded dose However, apparently, microparticles

(MPs) seems to be a better solution, because of the higher drug loading, lower burst effect and better control of drug release [42]. Additionally, MPs are characterized by a slower clearance in the periocular space, compared to NPs. The fate of nanoparticles (20 and 200 nm and 2 µm carboxylate polystyrene NPs, negatively charged) has been investigated after periocular administration and the 20 nm NPs were rapidly cleared by blood and lymphatic circulation, while 200 nm and 2 µm particles was still found in the periocular tissues after 60 days [43]. The fast clearance of small NPs was also recently confirmed by Feng *et al.* using pRNA NPs 10 nm in size [44], while a significantly longer retention was demonstrated by latanoprost-loaded NPs of 80 nm that sustained drug level in the aqueous humor for at least 6 days [45].

In the intra-scleral delivery, a drug reservoir is injected in the sclera thickness using an hollow microneedle: Jiang *at al*. demonstrated *in vitro* the relatively easy injection of solutions and nanoparticles (PLA, 280 nm) in human sclera, while MPs (latex, 1 µm) could not be delivered in the same way, due to the blockage by the fibrous microstructure of scleral tissue [46]. Despite the interesting results obtained, there is a limited space accessible for fluid infusion into the sclera (max 10-35 µl) and this limits the amount of drug that can be delivered. An alternative approach, that could in principle allow the delivery of higher volumes, is represented by the suprachoroidal injection, that deposes the drug between the sclera and the choroid. A solution injected in the suprachoroidal space spreads circumferentially around the eye. Data obtained *ex vivo* and *in vivo* with different animal models demonstrate the possibility of easily injecting particles with diameters between 20 nm and 10 µm. These particles (non-biodegradable), administered *in vivo* to New Zeland white rabbits, remained in the suprachoroidal space for at least 2 months without any sign of clearance [12, 47].

4 Nanoencapsulation of therapeutic proteins

Proteins represent nowadays very important therapeutic tools. The total global market for protein-based therapies is growing very fast, with mAbs dominating. At present, more than 240 mAb and 120 proteins are in clinical trials or under development for the treatment of cancer, immune disorders, infections, and degenerative diseases [48]. Proteins are used also for the treatment of several eye diseases. Together with the well known bevacizumab, ranibizumab and aflibercept, anti-VEGF agents for neovascularization-related eye diseases, other proteins are in use or under development [49]. Table 1 presents the therapeutic proteins in use or under investigation for the treatment of eye diseases.

Despite their potential, proteins have important limitations as therapeutics and their administration represents a real challenge, for several reasons [50]:

(i) permeability across endothelial and epithelial structure is very low. For instance, they show very poor ability to penetrate the blood-brain barrier and the blood-retina barrier; additionally, the intracellular targeting is very difficult and this is the reason why, at present, protein therapeutics are mainly limited to cell surface or extracellular targets;

(ii) they can have stability problem (aggregation) and/or can be degraded by enzymes naturally present in the body;

(iii) all protein therapeutics are potentially immunogenic in patients;

(iv) they are generally very expensive.

Nanoencapsulation of peptides and proteins could be of help for solving some of the previously mentioned issues. NPs can protect the drug from metabolic activity that is present in ocular tissues [51] and in the vitreous body [52] thus prolonging their half-life, and can control and sustain the release of the active. These actions will also reflect in a reduction of the required dose, with a possible positive impact on costs. Finally, NPs can promote drug retention on ocular surface (mucoadhesion), penetration across epithelia and cell internalization.

However, the complications related to protein nanoencapsulation cannot be neglected. The problems are strictly linked to the encapsulation method used, nevertheless, some general consideration can be made. First of all the method chosen must preserve protein structure and activity; this is a very delicate aspect, also considering the extensive use of heat and/or organic solvents in NPs production. Sometimes, to preserve drug activity, the therapeutic protein is loaded after the nanoparticle/liposome production procedure. Due to the high drug cost it is very important to optimize the production procedure so as to increase as much as possible the encapsulation efficiency. In general, it is known that the encapsulation efficiency (EE) is inversely related to the size of the particles (the procedure and the polymer being the same, EE is generally higher for MPs compared to NPs). In analogy, by reducing the size of the particles, also the control of the release is reduced, with the appearance of an important burst effect. Together with EE, also the drug loading (LC) is of the outermost importance, in particular in the case of highly dosed protein, so as to reduce as much as possible the amount of excipient to be used. This aspect is significant, also considering the very small volumes that can be delivered in the case of ocular administration (see section 2). All these consideration justify the reason why at the

moment no protein-loaded nanoparticles are present on the market, also in the case of ophthalmic application.

In the next paragraphs, a review of scientific works describing ocular delivery of NPs containing therapeutic or model proteins is presented. The data have also been summarised in Table 2. It is worth noting that all the paper found on this subject are very recent and demonstrate a growing interest on this subject driven by the recent discovery of the molecular basis of some ocular pathologies, by the increased possibility of protein production thanks to the DNA recombinant techniques and by the undeniable relevance and impact that sight problems have in our society.

4.1 Anti-angiogenic drugs

Bevacizumab (Avastin^{TM}) is a recombinant humanized mAb of 149 kDa. Bevacizumab blocks the angiogenic process by binding strongly to human vascular endothelial growth factor. Despite the lack of approval for ophthalmic therapy, it is currently used, by repeated intravitreal injections, for the treatment of retinal NV. It is also reported to be efficacious to treat corneal NV, administered as eye drops. The use of NPs can represent a convenient strategy for bevacizumab delivery and several examples can be found in the literature.

Liposomes loaded with bevacizumab were prepared following the dehydratation-rehydratation method, using phospholipid and cholesterol in molar ratio 1:1. Bevacizumab was added to blank multilamellar liposomes and the mixture was freeze-dryied: the encapsulation efficiency was 45% and bevacizumab remained stable during the preparation procedure. The intravitreal injection of bevacizumab-loaded liposomes in rabbits *in vivo*, allowed to achieve a higher drug concentration-time curve and a slower clearance compared to the antibody solution [53].

The preparation of PLGA NPs using the emulsification/solvent evaporation technique is reported by different authors [54-58]. Pan has described bevacizumab containing NPs with an average size of 819 nm and a LC of 2.2%. Inside NPs, which were evaluated for the treatment of CNV in a rat model, the mAb retained its activity [55]. Concerning the antibody stability during the preparation procedure, Varshochian reported the positive effect exerted by albumin, which, at the same time, can be of help also in the control of drug release [56]. Hao and colleagues have prepared bevacizumab-PLGA NPs, which, unlike previous examples, were intended for trans-scleral delivery [57].

In order to avoid one of the more important drawbacks of NPs, i.e. the limited control of drug release and the presence of a burst effect, a system composed of nanoparticles entrapped in porous MPs was prepared and evaluated. In particular, bevacizumab-coated

PLA NPs were encapsulated into porosyfing PLGA microparticles using supercritical carbon dioxide [59]. Supercritical carbon dioxide enabled the rapid expansion of PLGA, while produced no effect on crystalline PLA: the final entrapment of PLA NPs into PLGA microparticles was confirmed by SEM images (Figure 3).

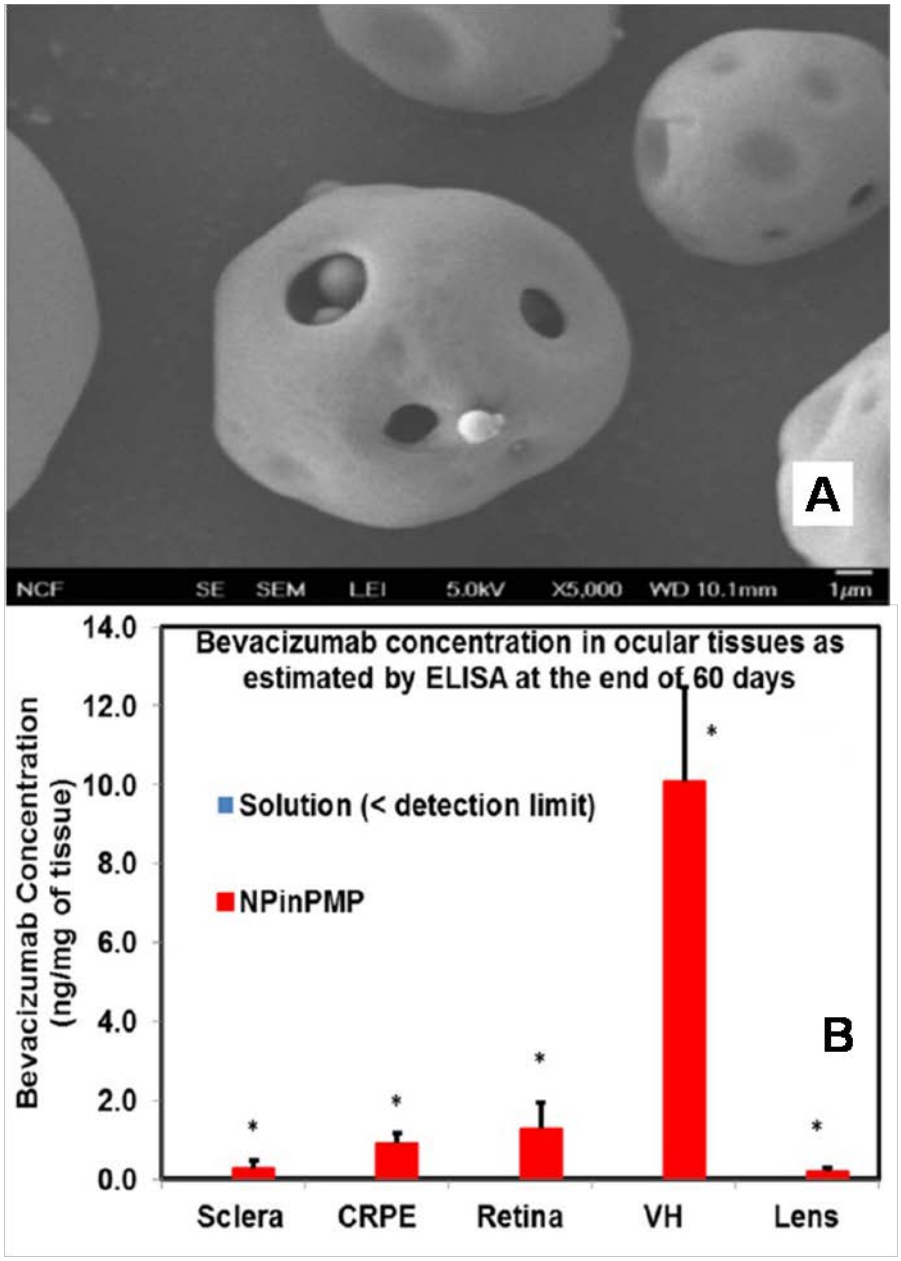

Figure 3. Bevacizumab-loaded PLA NPs in porous PLGA MPs. Panel A. Scanning electron microscopy image (5000 X). Panel B. bevacizumab concentration in rat ocular tissues 2 months after intravitreal injection of bevacizumab solution and bevacizumab-NPinPMP. In case of NPs in MPs, bevacizumab was not detected in conjunctiva, cornea and aqueous humor; in case of the solution, bevacizumab was not detected in any of the ocular tissues analysed (from [59] with permission).

Despite the supercritical treatment, bevacizumab maintained its activity. When the MPs were injected in a rat model, the antibody was constantly released in vitreous over a period of 2 months (Figure 3); the authors hypothesized that PLA NPs behaved as plugs by blocking PLGA pores, thus delaying bevacizumab release.

Recently, bevacizumab was loaded in annexin-associated liposomes intended for application on the ocular surface, but with the final aim to target the retina [60]. 100 nm unilamellar liposomes, negatively charged (approximately -5 mV) were prepared by the lipid film hydratation method and the resulted EE was about 22-25%. The presence of a functionalizing protein as annexin A5 on the liposome surface enhanced the transport across epithelial barriers, allowing bevacizumab to reach the posterior chamber in *in vivo* experiments on rats and rabbits (see also section 6).

Widely used in therapy for treating retinal diseases is also **ranibizumab**. Ranibizumab (Lucentis®; 48 kDa) is the Fab fragment of a humanized mAb approved for intravitreal injection. Ranibizumab exerts its anti-angiogenic activity by binding to three VEGF isoforms, i.e. $VEFG_{165}$, $VEGF_{121}$ and $VEGF_{110}$ [61]. Being one third in weight if compared to bevacizumab, ranibizumab is a good candidate for the development of NPs. An example of ranibizumab NPs was reported by Patel and colleagues, who successfully prepared PLGA NPs loading a ranibizumab-dextran sulphate complex, obtained via hydrophobic ion pairing (HIP) complexation (i.e. a complex based on the formation of an hydrophobic reversible interaction between macromolecules showing opposite charges). After complex formation, the NPs, 150 nm in size, were prepared by nanoprecipitation and had a maximum entrapment of *approx.* 85% [62]. HIP complexation has been previously described by the same research group, who developed PLGA NPs loaded with BSA [63] or lysozime [64], chosen as model proteins, in order to set-up the preparation of NPs able to guarantee not only the sustained delivery of the protein loaded, but also to preserve its therapeutic activity.

In 2011 FDA approved the use of **aflibercept**, a fully human recombinant fusion protein that exerts an anti-angiogenic activity by “entrapping” VEGF and thus preventing the interaction with VEGFR. From a clinical point of view, aflibercept has demonstrated a comparable efficacy to ranibizumab [65]; however, to date no data concerning aflibercept loading NPs are available.

Two recent examples of new anti-angiogenic peptides are C16Y, a 1.6 kDa integrin-antagonist peptide with hydrophilic properties, active in blocking integrins involved in NV

process, and SP6001, a serpin-derived peptide negatively charged. **C16Y** was successfully encapsulated in polylactic acid/polylactic acid-polyethylene oxide (PLA/PLA-PEO) NPs (approximately 300 nm, negatively charged -38 mV), in order to improve the peptide intravitreal half-life as well as retinal penetration. *In vivo* studies performed using a model of NV in rats, demonstrated the higher efficacy of C16Y-loaded NPs compared to the peptide solution [66]. For **SP6001** intravitreal delivery, poly(beta-amino ester) (PBAE), a cationic polymer, was used to obtain self-assembled polymer-peptide NPs (119 nm), that were than encapsulated inside PLGA microparticles (approx 10 µm) to extend peptide release [67]. *In vitro*, the system showed approximately zero order release of SP6001 for more than 6 months; *in vivo*, after injection in mice with CNV, angiogenesis decreased for at least 3 months: a better result than for peptide alone, which controlled the pathologic condition for less than one month.

4.2 Neuroprotective factors

Basic fibroblast growth factor (**bFGF**) is a neuroprotective agent involved in the prevention of photoreceptors degeneration. In order to protect the peptide and avoid complications associated to the direct injection of naked bFGF inside the ocular bulb, the protein was loaded on gelatin NPs. bFGF was incorporated by dropping a drug solution on freeze-dried gelatin NPs [68]. The intravitreally injected NPs (*approx*. 600 nm in size) were retained inside RPE and retina for at least 8 weeks after administration, allowing a sustained release of bFGF, which, being direct towards photoreceptors, has inhibited their apoptosis (Figure 4).

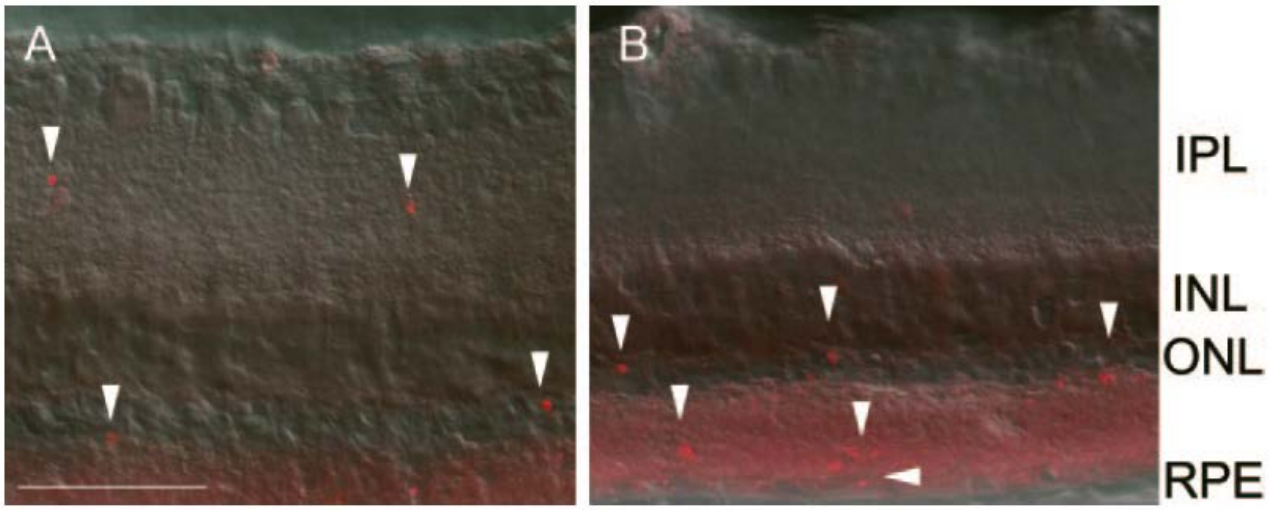

Figure 4. Confocal images of the retina of RCS rat 6 (A) and 8 (B) weeks after injection of rhodamine-labeled bFGF-NPs. NPs are present in the inner plexiform layer (IPL), and in the outer nuclear layer (ONL) at 6 weeks. At 8 weeks after injection, the NPs were in the inner nuclear layer (INL), ONL, and RPE apical surface. Arrowheads: NPs. Scale bar, 50 μm. From [68] with permission (to be obtained).

A **small signal peptide**, made only of serine, threonine and tyrosine, was conjugated to low molecular weight chitosan NPs, and proposed to treat macular degeneration thanks to its ability to act as a transduction agent inside RPE cells [69]. To prevent RPE cells apoptosis, recently Wang and colleagues proposed the use of a **mini-peptide**, made of 20 amino acids, derived from the heat shock protein αβ-crystallin and having chaperone properties [70]. Despite its neuroprotective activity, the small size makes the peptide highly exposed to intraocular degradation, with consequent very low viability. In order to minimize this aspect, an innovative bioengineering approach involving NPs was adopted. The mini-peptide was fused to an elastin-like polypeptide (40 kDa), used as carrier protein. This fusion-construct, expressed in *E. coli* using the DNA recombinant technique, self-assembly into small NPs following a temperature-dependent process. The resulted NPs, about 30 nm in size, were tested in human RPE cell culture, in which an oxidative stress was induced. The anti-apoptotic activity exerted by mini-peptide was preserved; furthermore, cell internalization, probably via clathrin-mediated endocytosis, took place.

4.3 Anti-inflammatory agents

Among anti-inflammatory agents, the introduction of anti TNF-alpha in ophthalmology for the treatment of uveitis and retinal diseases represents a recent fact and several studies are ongoing in order to demonstrate their efficacy and safety [71]. Efficacy and safety have been assessed for adalimumab and infliximab, two IgG mAbs, while the soluble TNF receptor fusion protein etanercept is no longer a candidate because of its tendency to worsen uveitis [72]. Among others anti TNF-α, **certolizumab** and **golimumab** have recently been approved for systemic use but they have not yet been tested in ocular inflammation affections [73].

Since the systemic administration of TNF-α inhibitors can result in several side effects (chiefly, an increase tendency to develop infectious diseases) [71], an alternative treatment based on local application is preferred.

At the present time no literature data are available concerning ophthalmic NPs containing **infliximab**; on the contrary, the development of adalimumab NPs has been reported. **Adalimumab** (Humira^{TM}) is a 148 kDa recombinant human IgG mAb, approved for the treatment of several rheumatic diseases (among which, rheumatoid arthritis and Crohn's disease) by means of subcutaneous injection [74]. It binds both soluble and membrane forms of TNF-α, a pleiotropic cytokine involved in the development and maintenance of inflammatory processes having an autoimmune origin. In ophthalmology, adalimumab has displayed its ability in controlling and preventing anterior and refractory uveitis as a consequence of subcutaneous administration [75]. Romero *et al*. have developed PolyElectrolyte Multilayers (PEMs) PLGA) NPs to control adalimumab delivery [76]. PolyElectrolytes used were alginate (negatively charged) and polylysine, which has a polycation nature. Blank PLGA NPs were prepared using the emulsion/solvent evaporation method and were then covered by using the layer-by-layer (LbL) technique. The positively charged antibody was added to alginate, in order to obtain a negatively charged complex (zeta potential *approx.* -25 mV). Then, polylysine and antibody/alginate complex were alternatively layer-by-layer deposited onto NPs up to 11 or 25 levels, in strictly controlled conditions of pH and ionic strength. Since the outer layer was always polylysine, the zeta potential of the final PEMs NPs corresponded to +13 mV. *In vitro* adalimumab release in PBS followed a first order kinetic after an early burst effect. After 1 and 5 days, the percentages of antibody released were respectively of 50% and 60-70%. Even if the described NPs have been proposed for local delivery, they have not been yet tested specifically for ocular application; however the preliminary data suggest their potentiality in ophthalmologic field, also for the delivery of different proteins.

Vasoactive intestinal peptide (**VIP**) is an endogenous ocular peptide, consisting of 28 amino acid residues, with recognized immunomodulatory properties. Peptide-loaded liposomes (between 300 and 600 nm in size) were prepared by thin layer hydration and loaded by lyophilization-rehydration. These carriers were developed in order to protect VIP against degradation, when intravitreally injected in rats with endotoxin-induced uveitis [77]. The study demonstrated the effectiveness of liposomes in controlling VIP release as well as their ability in protecting the peptide for 14 days. In this case the presence of the nanocarrier is essential to ensure the stability of VIP, which, when directly injected inside vitreous, is rapidly degraded and thus unable to produce a therapeutic effect.

4.4 Model proteins

Bovine serum albumin (**BSA**) is universally recognized as model protein. BSA is a 67 kDa hydrophilic and globular shape protein, with a hydrodynamic radius of 36 Å; the deep characterization, the high versatility, the reproducible behaviour, the good stability in solution, as well as the relatively low cost, justify its wide use as model compound in the pharmaceutical research field. BSA is therefore often chosen as reference protein in several type of NPs intended for mucosal application. For instance, hyaluronic acid/chitosan NPs, obtained by the ionic gelification technique [78] have been proposed for topical application, because the excipients are mucoadhesive, biocompatible and biodegradable. Xu *et al.* prepared PEGilated NPs PLGA-PEG NPs, via emulsification method, in order to obtain mucus penetrating properties [79].

Recently, fluorescently labelled BSA was also loaded on silk fibroin NPs [80] intended for the treatment of retinal diseases. The NPs are intended for trans-scleral delivery and their penetration should be enhanced by ultrasound (US) application. NPs were prepared following the anti-solvent precipitation method. A silk fibroin solution (2.5% w/v) was injected in acetone: immediately, silk fibroin NPs were formed and precipitated. After a purification by centrifugation, NPs were add a FITC-BSA solution and mixed. Glutaraldehyde solution was added dropwise and cross-linking reaction took place in 8 hours. Silk fibroin NPs, both blank and BSA-loaded, were about 200 nm in size. The EE resulted 76%. Passive trans-scleral diffusion of BSA loaded NPs resulted 8 time higher than BSA solution. As expected, the penetration efficiency of NPs showed a further increase when associated to US: almost 14-fold higher with respect to albumin solution and fibroin NPs demonstrated their stability in presence of US [80]. The work is a very good example of how the combination between NPs and a physical enhancer can lead to targets not achievable until few decades ago.

5 Carrier proteins

Proteins can be used as excipients for the preparation of NPs and have demonstrated to be suitable for the delivery of both small and high molecular weight active ingredients. Protein nanocarriers allow to preserve drug activity, to sustain drug release and enable the targeting to specific substrates; in fact, proteins are easily amenable to modifications to allow the attachment of targeting ligands. Proteins, being in most cases of natural origin, show high biocompatibility and biodegradability, are well characterized and may be considered safe enough, at least from a general point of view. The natural origin can also

be a drawback because of the possible immunogenicity (particularly due to aggregation), the high variability and sometimes the need to remove from the starting material all the contaminants that may produce toxicity in human [81] as, for example, the possible presence of transmissible spongiform encephalopathy vectors in bovine gelatin [82]. Despite these limitations, the use of protein as excipients for NPs preparation have interesting perspectives. It is worth mentioning, for instance, that one of the more successful examples of nanoparticle formulation on the market is made of albumin (Abraxane®).

Nanoparticles have been prepared using both water-soluble (serum albumin) and water-insoluble proteins (such as zein and gliadin). The next paragraphs review the use of proteins as nanocarriers for ocular drug delivery.

Fibroin is a high MW protein, composed of a 25 kDa light chain and a 325 kDa heavy chain, joined by a disulfide bond; the two chains are non-covalently connected to P25, a 25 kDa glycoprotein [83]. Fibroin is extracted from cocoons of *Bombyx mori* (mulberry silkworm) and purified from sericin, a protein coating single fibroin filaments. Silk fibroin has been used to prepare ocular NPs via anti-solvent precipitation method [80]. *In vivo* mucoadhesion tests performed on rabbit demonstrated the ability of the silk fibroin NPs to quickly adhere on sclera surface, while, as previously reported in the present paper, *in vitro* permeation studies confirmed the diffusion enhancement of BSA loaded in fibroin NPs, particularly when US were associate, with respect to BSA solution.

Being **BSA** highly versatile, it is used as a model of therapeutic protein (see section 4.4), but also as a carrier protein. Especially thanks to their biocompatibility and the biodegradability, many examples of albumin NPs for ocular use can be found in the literature. Most of them are intended for topical application and are loaded with small drugs such as antiglaucoma agent pilocarpine [84], anti-inflammatory compounds, such as hydrocortisone [85], aspirin [86], and meloxicam [87], antiviral drugs like acyclovir [88].

Zimmer and collaborators used a slightly modified desolvation method to prepare hydrocortisone-loaded albumin NPs, starting from a micellar solution of the lipophilic drug. The NPs obtained, having a size ranging between 100 and 300 nm, displayed an EE up to 70% and demonstrated their efficacy in both *in vitro* and *in vivo* studies [85]. Following the same method, also albumin NPs loaded with the hydrophilic pilocarpine were prepared and characterized [84]. In this case, the use of NPs instead of the solution has increase 1.5-1.9-times the miotic effect, with a consequent significant reduction of the IOP.

Desolvation method was used by Noomwang to prepare bovine albumin NPs of spherical shape and average size of 130 nm [88]. Acyclovir was successfully loaded with 20% of EE and 1% of LC.
Using the desolvation based coacervation technique Das [86] prepared aspirin-loaded albumin NPs having a size of 65 nm, with low polydispersity index, and a positive zeta potential (+25 mV). The average drug entrapment was 80% and *in vitro* studies showed the ability of NPs to release the NSAID for as long as 3 days, without any burst effect. Furthermore, DLS analysis demonstrated the stability of the lyophilized NPs for at least 6 months. Based on their experimental data, the authors hypothesized the use of the above-mentioned NPs, intended for topical administration, also for the treatment of posterior segment affections.

Thanks to the optimal tolerability, albumin represents also a suitable carrier for NPs intended to be administered intravitreally. NPs injected inside the ocular bulb enable a sustained release of the active inside the vitreal chamber, with consequent reduction in the number of injections needed. The hydrophilic drug ganciclovir, antiviral treatment of cytomegalovirus retinitis, was firstly loaded in albumin particles by El-Samaligy, who obtained spherical particles having an average particle size of 1.2 μm [89]. Afterwards, other several successful attempts were done in order to optimize the nanoparticulate formulation, by using the coacervation method and loading ganciclovir not only inside NPs, but also on their surface [90]: the entrapment efficiency resulted 40% for loaded NPs and 20% for those with the drug adsorbed on the superficial layer. The presence of ganciclovir on NPs surface resulted in a more effective antiviral activity since, once NPs are internalized by cells, the drug is released in a more rapid way [91].
As antiviral activity against human cytomegalovirus can be explicated also by antisense oligonucleotide, both phosphodiester and phosphorotioate oligonucleotides were either adsorbed on the surface or incorporated inside albumin NPs [92]. The entrapment efficiency was *approx.* 74% for phosphodiester while 59% in the case of phoshorotioate, reflecting the different affinity of the two oligonucleotides for albumin. The average size (250 nm) as well as the charge (-22 mV) were independent from the loading method and oligonucleotide type; [92]. Even if the NP preparation procedure preserved oligonucleotide hybridization capability, the simple adsorption onto NPs surface does not efficiently protect the oligonucleotide against enzymatic degradation. However, BSA NPs improved the internalization of oligonucleotides inside cells.

In order to deliver more effectively and to protect genetic material intended for ophthalmic treatment, some authors described the use of **HSA** NPs as non-viral vector for both intravitreal [38, 93] and intracorneal [94] administration. HSA NPs enabled the sustained release of the gene loaded and were rapidly uptaken from cells via receptor-mediated endocytosis. Furthermore, they demonstrated complete lack in cytotoxicity [93]. Human serum albumin NPs showed their safety also with regard to corneal keratocytes [94].

Gelatin is a composite of purified protein fractions derived from porcine and bovine collagen, available as type A gelatin, obtained by partial acid hydrolysis, type B gelatin, produced by partial alkaline hydrolysis, or a mixture of the two [95]. Gelatin is largely used in pharmaceutical technology as gelling agent, film-forming agent, viscosity enhancer and different grades of gelatin, with different particle size, molecular weight and other properties, are commercially available [95]. As nanocarrier, gelatine offers, among otherpolymers and biopolymers, a peculiar characteristic, which consists in the polyampholyte nature, due to the presence of cationic and anionic group together with hydrophobic groups [96].

Thanks to the high biocompatibility shown, several examples of gelatin NPs intended for ophthalmic use can be found in the literature. Gelatin allows the preparation of both positive and negative NPs, with smooth surface and spherical shape [97], to be administered either topically [97, 98] or intra-vitreally [68] and loaded with small molecules [98] as well as with high molecular weight compounds [68]. In order to increase ocular bioavailability of pilocarpine HCl and hydrocortisone, two drugs with different solubility, they were encapsulated in gelatin NPs via desolvation method [98]. NPs encapsulating pilocarpine showed that the type of gelatine as well as pH conditions did not affect the particle size and charges, that resulted slightly negative; on the contrary, hydrocortisone NPs showed different zeta potential, as a consequence of use of different gelatine type, even if the charge was negative in any case.

Since the ocular surface is negatively charged, because of the presence of sialic acid residues in the mucus, some authors suggested that cationic NPs may better interact and/or penetrate cornea and conjunctiva than the anionic ones [97, 99]. For this reason, cationic gelatin NPs were prepared and loaded with a plasmid designed to encode human MUC5AC [100]. MUC5AC is a mucin protein that plays a role in the homeostasis of lacrimal fluid and is thus reduced in dry eye syndrome. NPs were prepared by ionic gelation technique starting from cationized gelatin; plasmid and an anionic polymer (chondroitin sulphate or dextran sulphate) were added to the cationic gelatine solution in

the presence of tripolyphosphate. NPs obtained were characterized by a small size (less than 150 nm) and a positive surface charge (between +20 mV and +30 mV). The administration of associated plasmid NPs to rabbit cornea was followed by a an increase of the MUC5AC levels detected inside the precorneal tear film.

Gelatin NPs have been also proposed for intravitreal administration. In fact, freeze-dried gelatin NPs, obtained by UV cross-linking, were used as nanocarrier for basic fibroblast growth factor (bFGF) [68] (see also section 4.2). An electrostatic interaction occurred between negative gelatin and positive bFGF: the growth factor was released *in vivo* from the complex as a consequence of environmental changes, such as an increase of ionic strength and enzymatic degradation of gelatin. The study clearly demonstrated the ability of gelatin NPs to preserve bFGF from degradation, and, at the same time, to guarantee the anti-apoptotic effect thanks to a sustained release targeted to photoreceptors.

In the ophthalmic field, a recent example of nanoplatform based on **elastin like polypeptide** (ELP) was described for the intravitreal administration of a chaperone, as previously discussed in the present review [70]. Elastin like polypeptides are biopolymers derived from hydrophobic domain of tropoelastin, the soluble precursor of elastin. As well as tropoelastin, ELPs are soluble at low temperature, but, as a consequence of thermal raising, the solubility progressively decreases and self-assembly takes place [101]. Even if ELPs are commonly known to be termoresponsive, they may be designed to react to other physical change, such as pH variation [102]. Several characteristics can be controlled, among which MW is surely the most important, especially when ELPs are intended for the preparation of platforms, or even nanoplatforms, for drug delivery. The use of recombinant silk-elastin like protein for NPs production was also reported [103], even if not yet used for ocular formulations.

6 Peptides and proteins as functionalizing and coating agents

Finally, it is worth mentioning the role of proteins and peptides as active coating agents, that can enhance the cell adhesion and promote cell uptake. The idea, very simple but at the same time highly effective, consists in the conjugation of NPs with peptides or proteins, whose receptors are expressed on ocular tissues. Some examples are reported in the literature: among them, the conjugation of deslorelin and transferrin to polystirene NPs [33]. **Deslorelin** is a 1.3 kDa synthetic nonapeptide LHRH agonist, while **transferrin** is a glycoprotein having a MW of 80 kDa; they were chosen to functionalize NPs surface because of the expression of their receptors on both corneal epithelium and conjunctiva. In

fact, the presence of specific receptors on ocular tissues justified the increase of the transport of conjugated NPs of *approx.* 70% across cornea (Figure 5) and 50% through conjunctiva in an *ex vivo* bovine model, if compared to conventional NPs.

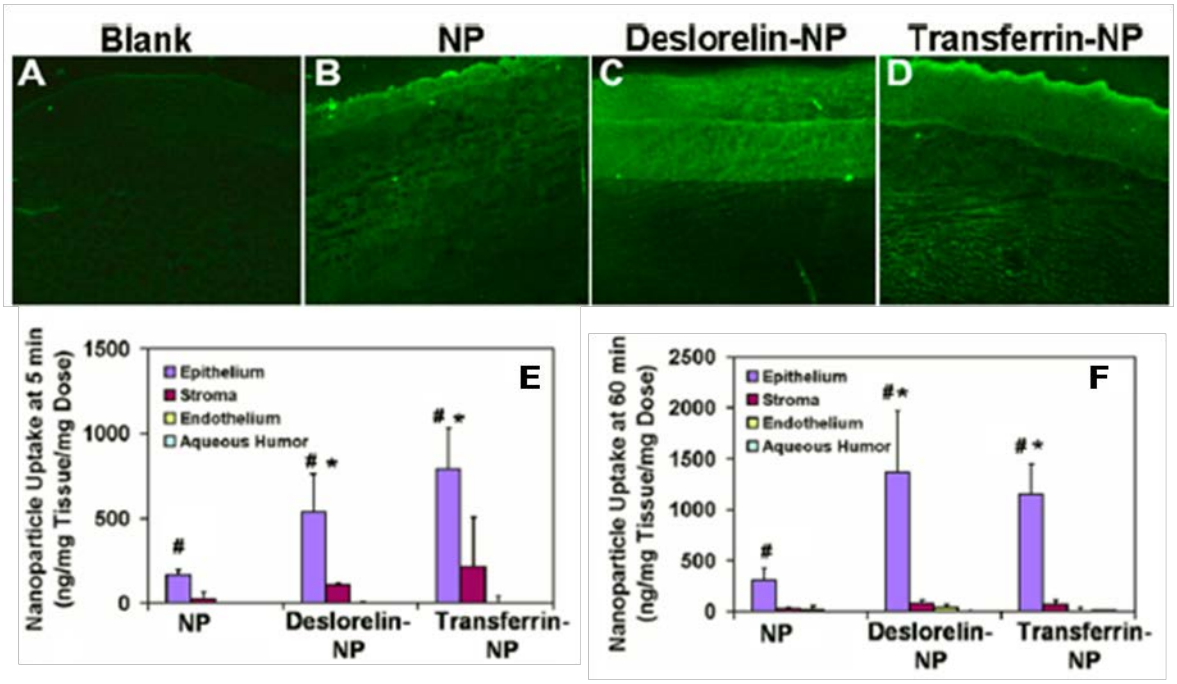

Figure 5. Ex vivo accumulation of coated and uncoated fluorescent nanoparticles (85-100 nm) in bovine cornea. Panels A-D: confocal images showing the uptake of nanoparticles in bovine cornea epithelium after 4 hours application using a modified Ussing chamber. A. blank tissue, B. uncoated NPs; C deslorelin-NP; D. transferrin-NPs. Panels E-F: Nanoparticles content in the corneal tissues and acqueous humor 5 and 60 minutes after instillation in an ex-vivo bovine eye model (with permission from [33]).to be obtained

Furthermore, confocal studies demonstrated the ability of deslorelin/transferrin NPs to be uptaken by epithelium unmodified, as well as the NPs accumulation in the stroma, with the formation of a reservoir [33]. Recently, another protein was selected as active coating of liposomes intended for corneal administration. **Annexin A5** is a calcium-dependent phospholipid binding protein, having a MW of 36 kDa. Thanks to its ability to interact with cell and biological membrane, annexin A5-associated liposomes were prepared in order to promote their transport across the corneal barrier [60]. The presence of annexin enhanced the delivery of topically applied bevacizumab-loaded liposomes to the posterior segment of both rat and rabbit eyes *in vivo*. This result, that authors ascribed to the ability of annexin in increasing the contact time as well as in enhancing transcytosis across epithelium, is a

an example of how the simple use of a protein as functionalizing coating agent can lead to switch from an invasive approach to a well-tolerated one.

A peptide active coating was also suggested to help the targeting of an anti-VEGF agent for the therapy of CNV, after intravenous injection of NPs: in fact, Luo *et al.* reported the use of the cell adhesion motif **RGD tripeptide** (arginine-glycine-aspartic acid) [104]. In this case the intravenous administration route was proposed as a valid alternative to the intravitreal injection of anti-VEGF, the latter procedure being poorly tolerated and sometimes injurious (see also section 3.2).

Finally, proteins can be useful in NPs preparation to promote physical stability and control the drug release rate. Some authors have suggested the use of albumin as coating agent of meloxicam nanocrystals (*approx.* average particle size 100 nm) intended for the treatment of postcataract endophthalmitis [87]. Furthermore, bovine serum albumin, as well as human serum albumin, showed stabilizing effects on therapeutic proteins when the NPs procedure involved an emulsification method [56].

7 Future perspectives

7.1 Potential drug candidates

The number of therapeutic proteins proposed for the treatment of ocular diseases is constantly growing, thanks to the considerable contribution of biotechnology (Table 1). Among these, the newly developed therapeutic proteins are briefly detailed below.

Recombinant MP0112 is an anti-VEGF, belonging to DARPins, a new generation of designed low MW therapeutic proteins made by repeated ankyrin domains, that recently demonstrated its safety and bioactivity following intravitreal injection in the treatment of diabetic macular edema [105] and exudative AMD [106].

Plasminogen kringle 5 (K5) is an 80 amino-acids natural peptide having proved anti-angiogenic properties, effective in prevention and block of retinal NV. After intravitreal administration, despite its effectiveness, it shows a very short half-life [107]. To minimize this low bioavailability, an expression plasmid of K5 was included in PLGA/chitosan NPs and then administered via injection in rats *in vivo,* with consequent K5 expression in all the layers of the retina [108]. Due to the high potential of the peptide, the possibility to prepare K5 loaded NPs should be take into account.

ESBA105 is a 26.3 kDa single chain variable region antibody fragment (scFv) direct against TNF-α. ESBA105 reached high concentration levels inside the anterior and posterior ocular tissues when topically applied in *ex vivo* and *in vivo* experiments

performed on rabbits eye, thanks to both trans-corneal and trans-scleral permeation routes [109]. Recently, ESBA105 was successfully applied as eye drops in an *in vivo* study carried on human volunteers, reaching therapeutic levels inside anterior chamber, even if penetration inside the vitreous was low [110]. Even if no penetration enhancers are required [109, 110], this scFv antibody should be considered a suitable candidate for NPs not to improve the permeation, which is quite high, rather to better control the amount permeated and to increase ESBA105 half-life inside ocular chambers, which is only 25 hours inside the vitreous [109].

The neuroprotectant agent **GDNF** (glial cell line-derived neurotrophic factor) exerts a neuroprotective effect by increasing the retinal ganglion cells survival. Since PLGA microspheres intended for intravitreal delivery were developed and successfully administered to rats *in vivo* [111], this positive result may suggest a similar desirable effect using NPs.

Recombinant human Hsp70 (rhHsp70) is a 72 kDa chaperone that has recently demonstrated its ability in prevent RPE degeneration, when studied *in vitro* on cellular lines [112]. In view of *in vivo* studies, the authors hypothesize that the formulation of the protein as NPs will provide a sustained release of the chaperone after intravitreal injection.

7.2 Carriers

Among animal proteins, **casein** and **β-lactoglobulin** from milk appear very promising carriers for drug delivery. Caseins constitute a family of four phosphoproteins (ranging in weight between 19 and 25 kDa), which appear very rich in proline residues, heat stable and amphiphilic, that can lead to formation of self-assembly micellar structures in aqueous solutions [113]. Casein has been already approved as excipient in tablet preparation and, as a consequence of its versatile physico-chemical properties, extensively used for the preparation of hydrogels, floating beads, microparticles [113] and more recently for nanoparticles [114-116]. Similarly, β-lactoglobulin, MW 18.4 kDa, is widely used in both pharmaceutical and nutraceutical fields and several examples of β-lactoglobulin NPs were reported [117-119]. Even if generally described as emergent nanocarriers, neither casein, nor β-lactoglobulin NPs has been yet proposed for ophthalmic administration, but certainly they deserve to be further investigated for this purpose.

Anyway, from a general point of view, some examples of ophthalmic NPs based on animal proteins are available and albumin is the main example, as previously seen in the present paper; the same thing can not be said for plant protein. Although generally used for

preparation of nanocarriers, plant proteins are still missing in ocular formulation even if, several candidates are available: among others legumin, gliadin, soybean and zein.

L**egumin** is a 360 kDa protein contained in peas. Proposed as carrier for NPs, thanks to the evidence that, by using the pH coacervation method, NPs of 250 nm in average diameter may be obtained [120]. Another possible source of carrier protein for NPs preparation is wheat gluten, from which the insoluble protein **gliadin** can be isolated; literature reports gliadin NPs intended for oral drug delivery [121].

Soybean is a source of proteins, the so-called soy protein isolated (SPI), mainly consisting of two globular proteins, glycinin (360 kDa) and β-conglycinin (180 kDa). Since they show both hydrophilic and lipophylic properties, they are, at least in principle, suitable for active compounds having different properties [122]. As often noticeable for biopolymers, being SPI very versatile, it was proposed their use for realizing films, as devices for controlling the release of drugs [123], and preparing NPs [124], but also as nanosuspension stabilizers for poorly water-soluble compounds, in analogy with β-lactoglobulin [119]. Nevertheless, to date no examples in ophthalmic delivery are available.

Finally, the relative small **zein**, a 38 kDa prolamine protein, contained in the seeds of maize, showing marked hydrophobic properties. Zein is commonly used in pharmaceutical technology field as tablet coating agent, tablet sealer, as well as wet granulation binder [95] and it has been studied as protein carrier starting from few years ago [125-127], also in association with another protein, i.e. β-lactoglobulin [118]. However, no one has yet reported the use of zein-based NPs in ocular drug delivery. Even if no data for ocular application are available, in perspective, zein use could be take into account, being mucoadhesive, having a self-assembling nature and the ability to form both solid core and hollow NPs; furthermore, the possibility of loading biomacromolecules, such as proteins and peptides, and the protective effect exerted against enzymes have been already reported [125]. Gamma irradiation could be taken into account as effective sterilization technique [128].

Table 1 Therapeutic proteins in use or under investigation in ophthalmology

PROTEIN	MW (kDa)	TARGET/DISEASE	REF.
~~anti~~Anti-angiogenic factors			
aflibercept	115	wet AMD	[65]
anti-HER2 rhuMAb	148	NV	[49]
bevacizumab	149	NV	[129]
C16Y	1.6	CNV	[66]
K5 (plasminogen kringle 5)	14	NV	[107]
MP0112 (DARPins, ankyrin repeat proteins)	15-18	DME, AMD	[105, 106]
PEDF	50	CNV	[49]
ranibizumab	48	AMD	[61]
rituximab	145	intraocular lymphoma	[49]
sFlt-1	110	CNV	[49]
SP6001 (serpin-derived peptide)	1.3	NV	[130]
~~neuroprotective~~ Neuroprotective factors			
aFGF (also called FGF-1)	17	photoreceptor degeneration	[49]
αβ –crystallin derived small peptide	2.4	AMD	[70]
BDNF	28-37	photoreceptor degeneration	[49]
bFGF (also called FGF-2)	17-23	photoreceptor degeneration	[68]
CNTF	23	glaucoma	[49]
GDNF	20	glaucoma	[111]
NGF	26	glaucoma	[49]
rhHSP70 chaperone (recombinant hHsp70)	72	RPE degeneration	[112]
small signal tripeptide	0.4	AMD	[69]
~~anti~~Anti-inflammatory agents			
adalimumab	148	uveitis	[74]
infliximab	149	uveitis, CNV	[49]
ESBA105 (single chain Ab fragment)	26	AMD	[110, 131]
VIP	3.3	uveitis	[77]
~~model~~ Model proteins			
BSA	67	-	-
cytochrome c	12	-	-
HSA	66	-	-
lysozyme	14	-	-
ovalbumin	45	-	-

Table 2. List of therapeutic and model proteins loaded in NPs for ophthalmic use

PROTEIN	MW (kDa)	CARRIER* *Written in italics, in the case of protein*	NP SIZE (nm)	TARGET	ADMINISTRATION ROUTE	REF.	YEAR
anti-angiogenic factors							
bevacizumab	149	liposome	-	NV	intravitreal injection	[53]	2009
		PLGA	230	NV	trans-scleral	[57]	2009
		PLGA	819	NV	intravitreal injection	[55]	2011
		PLGA	200-1000	NV	intravitreal injection	[54]	2012
		PLGA	165-1631	NV	intravitreal injection	[56]	2013
		PLA NPs in PLGA MPs	NPs 265 MPs 11610	NV	intravitreal injection	[59]	2013
		annexin-associated liposome	100	NV	trans-corneal	[60]	2014
		PLGA	168-362	NV	subconjunctival	[58]	2014
ranibizumab	48	PLGA	150	AMD	intravitreal injection	[62]	2012
C16Y	1.6	PLA/PLA-PEO	300	CNV	intravitreal injection	[66]	2010
SP6001 (serpin-derived peptide)	1.3	PBAE NPs in PLGA MPs	119	NV	intravitreal injection	[67]	2013
neuroprotective factors							
bFGF (also called FGF-2)	17-23	*gelatin*	585	prevention of photoreceptor degeneration	intravitreal injection	[68]	2007
small signal tripeptide	0.4	chitosan	200	AMD	intravitreal injection	[69]	2012
αβ –crystallin derived small peptide	2.4	*ELPs* (Fusion protein)	29	AMD	intravitreal injection	[70]	2014
anti-inflammatory agents							
adalimumab	148	PLGA/PLL	-	uveitis	topical	[76]	2013
VIP	3.3	liposome	300-600	uveitis	intravitreal injection	[77]	2007
model proteins							
BSA	67	hyaluronic acid/chitosan	312-1360	-	topical/transmucosal	[78]	2008
		PLGA/PEG	160-218	-	topical/transmucosal	[79]	2013
		silk fibroin	210-220	posterior ocular tissues	trans-scleral US	[80]	2014

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