Microspheres as intraocular therapeutic tools in chronic diseases of the optic nerve and retina

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ABSTRACT
Pathologies affecting the optic nerve and the retina are one of the major causes of blindness. These diseases include age-related macular degeneration (AMD), diabetic retinopathy (DR) and glaucoma, among others. Also, there are genetic disorders that affect the retina causing visual impairment. The prevalence of neurodegenerative diseases of the posterior segment is increased as most of them are related with the elderly. Even with the access to different treatments, there are some challenges in managing patients suffering retinal diseases. One of them is the need for frequent interventions. Also, an unpredictable response to therapy has suggested that different pathways may be playing a role in the development of these diseases. The management of these pathologies requires the development of controlled drug delivery systems able to slow the progression of the disease without the need of frequent invasive interventions, typically related with endophthalmitis, retinal detachment, ocular hypertension, cataract, inflammation, and floaters, among other. Biodegradable microspheres are able to encapsulate low molecular weight substances and large molecules such as biotechnological products. Over the last years, a large variety of active substances has been encapsulated in microspheres with the intention of providing neuroprotection of the optic nerve and the retina. The purpose of the present review is to describe the use of microspheres in chronic neurodegenerative diseases affecting the retina and the optic nerve. The advantage of microencapsulation of low molecular weight drugs as well as therapeutic peptides and proteins to be used as neuroprotective strategy is discussed. Also, a new use of the microspheres in the development of animal models of neurodegeneration of the posterior segment is described.

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high IOP values with no response to hypotensive therapy and in normotensive subjects.

Delivery of active substances in therapeutic concentrations to the targeted intraocular tissues is restricted by the effective static (corneal layers, sclera, retina, blood aqueous and blood retinal barriers) and dynamic barriers (tear dilution, conjunctival and choroid blood flow, and lymphatic clearance) as well as efflux pumps that are present in the eye [5] (Fig. 1). Instilled drugs have poor bioavailability in the intraocular tissues [6]. For this reason, the ophthalmic topical administration is restricted to the treatment of pathologies involving the ocular surface or whether the pharmacological target is located in the anterior segment of the eye. Intraocular and pericellular injections are used to release therapeutic molecules inside the eye. In the case of intravitreal injections the drug is injected close to the retinal tissues, which leads to the highest bioavailability in the posterior segment tissues compared with the other local routes of administration. Although pericellular injections are less invasive, the therapeutic molecule has to reach the posterior segment by diffusion through different tissues.

Intravitreal injections are regularly used in the clinical practice for the treatment of posterior segment diseases. However, even with the available access to different ocular therapies, there are some challenges in the management of patients suffering retinal diseases. One of them is the need of frequent interventions. Also, an unpredictable response to therapy has suggested that different pathways may be playing a role in the development of these diseases. This might the reason why mono-therapy is not enough to slow the progression of the degeneration [7].

One of the most challenging areas in ophthalmology therapy is dedicated to decrease the number of interventions. Under the technological point of view this objective can be achieved thanks to the development of intraocular drug delivery systems (IDDS) [8]. These devices are prepared with different biomaterials to provide sustained delivery of the active molecule close to the target site during long term. Depending on the properties of the biomaterial the IDDS can disappear from the site of administration (biodegradable) or remain during the lifetime of patients (non-biodegradable).

According to their size, IDDS are classified as implants (size > 1 mm), microparticles (size 1–1000 μm) or nanoparticles (1–1000 nm). The different drug delivery systems are useful for the treatment of neurodegenerative diseases affecting the posterior segment of the eye. The right choice of the most adequate IDDS depends on the target site, the ophthalmic disease and the duration of the treatment. While implants and microparticles deliver the drug for long periods of time, nanoparticles have the advantage to be internalised by cells being useful for gene therapy [9,10].

Implants are currently used in the clinical practice to provide long term delivery of therapeutic molecules. Several non-biodegradable (Vitrasert™, Retisert™ and Medidur™) and one biodegradable (Ozurdex®) macrodevices are commercialised [8].

Microparticulate systems are able to encapsulate and deliver the drug for several months. Attending to their structure, they received the name of microcapsules if the drug is surrounded by a polymer or polymer mixture (reservoir) and microspheres if the drug is dispersed in the polymer network (matrix). Injection of microparticles can be performed by using small gauge needles (25–32 G). Personalised therapy is possible as different drug doses are administered depending on the amount of administered microparticles. Biodegradable microparticles are preferred as they gradually disappear from the site of administration [5].

Biodegradable polymers (gelatin, albumin, polylactoesters, polyanhydrides and polyesters) have been explored to prepare microspheres for intraocular administration. Among them, the poly(lactic) acid (PLA), poly(glycolic) acid (PGA) and their co-polymers poly(lactic-co-glycolic) acid (PLGA) are the most employed as they are approved for clinical purposes. The use of injectable microspheres has become more popular over the last few decades [5].

The main objective of the design of microparticulate drug delivery systems has been to obtain long-acting injectable drug depot formulations (Fig. 2). Biodegradable microspheres are able to encapsulate low molecular weight substances and large molecules such as biotechnological products. Over the last years, a large variety of active substances has been encapsulated in microspheres with the intention of providing neuroprotection of the optic nerve and retina.

The purpose of the present review is to describe the use of microspheres in neurodegenerative diseases affecting the retina and the optic nerve. Also, a new use of the microspheres in developing animal models is discussed.

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**Fig. 1.** Eye and retina elementary structure.
2. Chronic diseases of the optic nerve and retina

Advances in the understanding of pathogenesis that lead to retinal cell death have been essential to gain new insights about neurodegenerative diseases like age-related macular degeneration, diabetic retinopathy or glaucoma. These pathologies share a common pathophysiologic pathway that includes oxidative stress, axonal transport impairment, neuroinflammation, and excitotoxicity, which are the major cause of retinal cell death.

2.1. Age-related macular degeneration

Age-related macular degeneration (AMD) accounts for 8.7% of all blindness worldwide and is the most common cause in developed countries, especially in people older than 60 years [11]. This pathology is becoming more prevalent in the developing world due to an exponential population ageing. Differences in prevalence from diverse populations may offer insights into possible environmental and genetic causes of AMD [12]. Environmental and behavioral factors for AMD include cigarette smoking, obesity, low dietary intake of vitamins A, C and E, zinc, lutein and omega-3 fatty acids, and an unhealthy lifestyle related to cardiovascular risk factors [13].

AMD is characterized by a central vision loss due to degenerative alteration in the macular region of the retina. The disease can be classified as dry or wet AMD, depending on the absence or presence of choroidal neovascularization, respectively. In wet AMD, the growth of new blood vessels from the choroid into the sub-retinal space and sub-retinal pigment epithelium is responsible for the vision loss [13]. In addition, in both dry and wet AMD, the pathology manifests as pigmentary irregularities of the retina and deposits called drusen, which are accumulations of extracellular material immediately beneath the retinal pigment epithelium. Although drusen are a manifestation of the normal ageing process, they can represent an important sign associated with retinal disease [14]. Patients usually develop rapid visual loss...
when neovascular AMD occurs. Typically, they describe sudden worsening of central vision with distortion of straight lines, a dark patch in their central vision, or both. Treatment of wet AMD includes laser photocoagulation, photodynamic therapy, anti-VEGF therapy and visual rehabilitation.

Laser photocoagulation is effective in reducing long-term severe visual loss, but is limited by lack of vision gain and high recurrence rates (50%), and a risk of immediate moderate visual loss (41%) [15]. Photodynamic therapy consists of a two-stage process involving intravenous infusion of verteporfin, a green photosensitizing dye that accumulates preferentially in neovascular membranes, followed by a dye activation with infrared light [16]. This process generates oxygen-free radicals that damage the endothelium, promoting closure of newly formed vessels. This therapy is not exempt from adverse events that include photosensitivity, headaches, acute severe visual loss (in 4% of patients), and chorioretinal atrophy able to lead to gradual visual deterioration [17].

Progress in understanding the key role of vascular endothelial growth factor (VEGF) in choroidal neovascularization has led to the development of molecules that by blocking VEGF are able to partly revert some of the pathological mechanisms shown in neovascular AMD. Anti-VEGF drugs are typically administered by intravitreal injection, and include pegaptanib, ranibizumab, bevacizumab and aflibercept [18–24].

### 2.2. Diabetic retinopathy

Diabetic retinopathy (DR) is a leading global cause of vision-loss. In 2010, it was estimated that around one third of 285 million people with diabetes mellitus worldwide had signs of DR [25]. In approximately one third of people with DR, the pathology was vision-threatening, defined as severe retinopathy or macular edema [26]. For progression of vision loss, the most clinically important risk factors are duration of diabetes, hyperglycemia and hypertension. Emerging evidence supports a genetic component for DR, showing a heritable tendency independent of shared risk factors [27–29].

On the early stages of diabetic retinopathy, localized dilatations of the eye microvasculature appear in patients, which are assumed to be related with weaknesses in the vessel wall, pressure disturbances, or glial retraction/death [30]. An increment in the appearance or disappearance of these microaneurysms has been found to be related with the progression of retinopathy and also with eventual visual function decrements [31]. Other lesions in early stages of the pathology, which are also important in the eventual progression to neovascularization, include capillary non-perfusion and degeneration [32,33]. Both stimulate the release of hypoxia-regulated vasoproliferative factors, such as VEGF, correlated with an increase in neovascularization and vascular leakage. In advanced stages of DR, mechanisms of DR-related vision loss include vitreous hemorrhage, tractional retinal detachment from proliferative DR, development of a fibrovascular membrane in the vitreous, and macular edema [34] (Fig. 4).

To inhibit the development or progression of DR, several approaches are currently used in the clinical practice. In early stages of the pathology, interventions are directed towards control of blood pressure, hyperglycemia, and lipids. However, maintaining these parameters under control is challenging in many diabetic patients [35–38]. In advanced stages of DR, treatments include pan-retinal laser photocoagulation, or intravitreal injections of anti-VEGF agents and steroids. Laser can largely control neovascularization and prevent blindness, however this treatment cannot restore vision, and, as mentioned before, it has...
vision-impairing effects of its own as well [39]. Regarding the use of anti-VEGF or steroids, they can significantly reduce macular edema [40,41]. Unfortunately, their beneficial effects have been found to be temporary in comparison with the effects of standard photocoagulation, and they are also associated with vision-threatening complications related to repeated intravitreal injection [41]. Hence, given the limitations of these therapies, vision loss from DM is a significant healthcare concern [42].

2.3. Retinitis Pigmentosa

Retinitis pigmentosa (RP) is considered a group of related eye disorders that cause progressive vision loss by degeneration of the visual cells and alterations in retinal pigment epithelium. In people with RP, vision gradually deteriorates being irreversibly lost after several decades [43]. It is a common form of inherited retinopathy, with a prevalence of approximately 1 in 3500. More than 3000 mutations in over 57 different genes and 61 loci are currently known to cause the non-syndromic form of RP (www.orpha.net).

The first manifestation of RP is usually a loss of night vision, which becomes apparent in childhood. Night blindness is caused by an impairment of rod photoreceptors. Later, it is followed by a progressive death of these cells, causing blind spots in the peripheral vision, generating “tunnel vision” (Fig. 5). Due to the gradual cones death, patients undergo a progressive deterioration of visual acuity in the central field as well as a decline of chromatic discrimination, which leads RP patients to a condition of legal blindness [44].

Degeneration mechanisms of RP are complex, still poorly understood, and reveal multiple molecular targets, which make difficult the development of therapeutic strategies, maintaining this pathology as an orphan disease. However, although there is no cure for RP, there has been outstanding advance in understanding genetic and molecular mechanisms to understand the pathology, which has led to restoration of vision in specific cases, such as in patients with a mutation in a gene coding for the retinal pigment epithelium protein RPE65, essential for photoreceptor physiological activity [45,46]. Other strategies include the implantation of prosthesis in advanced stages of RP, when most rods and cones have been inevitably lost. The progress achieved in this field is extraordinary, and clinical trials for different types of implants have proceed successfully or are expected soon [47,48]. Molecules studied for RP have been designed for increasing neuroprotection with the use of neurotrophic factors, decreasing cellular oxidative stress, inhibiting photoreceptor apoptosis, or attenuating retinal inflammation. Unfortunately, pharmacological approaches to RP are still experimental and their main goal is not to repair RP, but rather to slow it down, enlarging the temporal window of useful vision [49].

2.4. Glaucoma

Glaucoma comprises a group of disorders characterized by a distinctive optic neuropathy that leads to progressive asymptomatic visual field loss. It is thought that loss of vision in glaucoma is associated with damage to the optic nerve and retina that results in irreversible retinal ganglion cell (RGC) damage. Glaucoma is currently the leading cause of irreversible blindness worldwide. It has been estimated that the total number of patients with glaucoma will be close to 80 million by 2020 [50]. By the time patients are aware of vision loss, the disease is commonly quite advanced [51]. Patients usually experience blurry or missing spots in their peripheral view (Fig. 6).

Increased IOP and IOP variability are recognized as significant risk factors both for the development and progression of glaucoma. For that reason, the majority of primary open-angle glaucoma treatment options focus on the management and reduction of IOP [52–54]. Current treatments involve the use of eye drops and gels, laser treatment, or incisional surgery to achieve a lower IOP by either decreasing aqueous humor production or improving aqueous outflow [51]. It is important to remark that not all glaucomatous patients experience high IOP and in many cases, the reduction of IOP does not avoid the progression of the disease.

Whatever the origin of the disease, in all glaucoma patients visual field loss is produced by RGCs death. Normal physiological ageing is responsible for an estimated rate of 0.4% RGC loss per year over approximately 1.5 million RGCs present in a healthy retina [55]; in glaucoma, this percentage is increased to 4% [56]. Considering the complex and multifactorial nature of glaucoma, multiple mechanisms are thought to contribute to RGC loss, such as apoptosis, necroptosis, axonal transport failure, inadequate neurotrophic factor support, excessive depolarization, mitochondrial dysfunction, excessive short-wave blue light, excessive glutamate, oxidative stress, and inappropriate RGC/glial cell interactions [55,57–61]. Over the past years, many publications have related glaucoma to neuroprotection.

3. Microencapsulation of small molecules for the treatment of chronic diseases of the optic nerve and retina

The clinical application of small drug molecules has been extensively studied for the treatment of neurodegenerative pathologies affecting the posterior segment of the eye. However, their clinical application is restricted due to the fast clearance they experience that requires frequent administrations to reach therapeutic levels in the target tissues [62]. This limitation can be overcome by encapsulating these drugs into delivery systems such as polymeric microspheres, which allow sustained drug release, and subsequently a reduced-dosing indication.
Microspheres can be administered locally without surgery. Such locally injected depots provide long-term sustained drug release, thus enabling the safe dosing of drugs with pharmacokinetics issues such as a rapid systemic clearance or a narrow therapeutic window [64]. Many neuroprotective agents exhibit significant side effects when administered systemically. These side effects are severe enough to limit the dose, and ultimately, the efficacy of the drug. Successful treatment strategies may involve local, sustained delivery of these drugs in a way that maximizes the efficacy while limiting side effect to an acceptable level. Drug loaded-microspheres are being developed with this intention as well (Table 1).

### 3.1. Corticosteroids

Retinal vasculature diseases have become more prevalent as a result of ageing population [65]. Even with the growing use of anti-VEGF therapeutic agents, corticosteroids are still drugs of choice for the treatment of some of these disorders, and still are a big actor in the medical care due to their confirmed efficacy. It has been demonstrated that corticosteroids can increase tight junction integrity, protecting the blood-ocular barrier and decreasing leakage. Additionally, they have shown an antiapoptotic effect, and can protect retinal photoreceptors via activation of glucocorticoid receptors [66].

Dexamethasone (Mw 392.5 g/mol) is a cortical steroid that has a very short vitreous half-life [67]. Since the approval from the US Food and Drug administration of Ozurdex (Allergan), a long-acting implant containing dexamethasone for the treatment of posterior segment eye diseases, the development of microparticulate sustained release systems containing steroids has gained considerable attention. Contrary to the use of the implant Ozurdex, that has to be applied with a large needle (22 gauge), microparticulate formulations can be delivered though needles from 27 to 34 gauge, avoiding invasive surgery or the use of larger devices for their application. Several studies have been made to deliver dexamethasone from microparticles prepared with biodegradable polymers. Hou H. et al. investigated pharmacokinetics and pharmacodynamics of the engineered oxidized porous silicon (pSiO2) particles loaded with dexamethasone (width 20 ± 5 μm, thickness 14.2 ± 0.3 μm, porosity 50 ± 3%) in vitro and in vivo [68].

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day in vitro release, particles demonstrated a 92% decrease by weight, indicative of pSi degradation and dexamethasone delivery in the dissolution chamber at 37 °C over time. Dexamethasone concentration was at a therapeutic level at the end of the 60-day in vitro release study (12.4 ng/mL). In vivo ocular pharmacokinetics in New Zealand Red Rabbits revealed a controlled drug release sustained for at least 8 weeks without apparent ocular toxicity. The study demonstrated the utility of porous silicon dioxide microparticles as carriers for controlled release of corticoids in ophthalmology. AndrésGuerrero V et al. [69] prepared dexamethasone-loaded microspheres based on poly(ester amide)s (PEAs), which are amino acid containing biodegradable polymers combining ester an amide groups in the polymer chain. Their work focused on the study of the ability of PEAs to form microspheres for ophthalmic drug delivery purposes. In this study, authors showed the synthesis of the PEA polymers and the preparation, sterilization, in vitro tolerance and delivery characteristics of microspheres loaded with dexamethasone. Authors built a kinetic ocular model with in vitro release data to predict in vivo drug concentrations in a rabbit vitreous model. Then, they analyzed the behavior of PEA microspheres after injection in the subtenon space and vitreous humor of rats. Amino acid based polyesters were successfully formulated into microspheres (∼15 μm), which showed good tolerance in macrophages and retinal pigment epithelial cells. Loading and release studies with dexamethasone revealed high drug encapsulation efficiency (∼85%) and a controlled drug delivery for 90 days. Pharmacokinetic simulations indicated that these microspheres would provide a release of the drug in rabbit eyes up to 3 months. After 24 h of the administration, the injected PEA microspheres retained their morphology and coalesced into depots in the vitreous and in the subtenon space, successfully reaching the injection site, showing the utility of polyesteramide microspheres as an alternative delivery system for controlled delivery of drugs to the eye.

Triamcinolone acetonide (TA) is a synthetic corticosteroid (Mw 434.5 g/mol) with a 7.5-fold higher anti-inflammatory potency than cortisone. It is one of the most extensively used drugs in the treatment of several ocular diseases which are characterized by inflammation, edema and neovascularization [70]. Triamcinolone acetonide is a water insoluble drug so its use in ophthalmology is available as an injectable suspension. Suspensions are required to be made with the insoluble drug in a micronized form, and are thought to be acceptable as a depot since it is assumed that the solid drug persists in the injection site giving rise to a sustained-release effect thanks to a slow dissolution rate of the poor soluble drug. However, suspensions have some disadvantages, such as not being possible to manipulate the concentration of dissolved drug due to their relative insolubility in the vehicle. For this reason and, due to the increment on the use of intraocular injections of triamcinolone in ophthalmology, the development of intravitreal drug delivery systems loaded with triamcinolone has increased in the past years [5].

Cardillo el al. presented a case report with 9 patients that received intraocular injections of RETAAC, a controlled-release system based on microspheres loaded with 1 mg triamcinolone. Authors compared the use of this system with a single intravitreal injection of 4 mg triamcinolone for the treatment of diffuse diabetic macular edema [71]. Quantitative measurements of central macular thickness showed a reduction from baseline exceeding 59% (RETAAC) and 56% (triamcinolone) at the 1- and 3-month follow-up visits respectively. The difference in central macular thickness between RETAAC and TA injected eyes was not significant because of a parallel initial pattern of regression of the macular edema. On the contrary, at 6 (p = 0.002) and 12 months (p = 0.002), RETAAC-injected eyes had thinner central macular thickness measurements compared to 4 mg triamcinolone injected eyes. According to the authors, the findings from this preliminary report neither advocate nor support the use of triamcinolone-microspheres for the treatment of diabetic macular edema, but imply that both RETAAC and triamcinolone injections may be well tolerated with long-term performance clearly favoring the RETAAC (1 mg) over TA (4 mg) for the anatomic and functional aspects of improvement tested in the study.

Blatiós et al. also evaluated a controlled-release system based on triamcinolone-loaded poly(DL-lactide) (PLA) microspheres [72]. Authors prepared several batches with different ratios of drug/polymer (5/100 and 10/100 TA/PLA) and two preparation methods (single and double emulsion) which gave spherical microspheres (2.09 ± 1.11 μm) with smooth surface. Encapsulation efficiency varied depending on the method used from 50.3%, to 57.9% and 77.3% for the single emulsion 10/100 and 5/100 TA/PLA, and double emulsion method 10/100 TA/PLA, respectively. The in vitro release study showed an initial burst of about 10–15% in the first day, followed by a sustained release until day 20 in all cases. Microspheres were compressed to mini-tablets (diameter 4 mm, thickness 0.8–1 mm) and episcleral implanted in rabbit eyes ex vivo. The drug diffused into the vitreous and aqueous humor rapidly during the first five days, showing a plateau in the aqueous humor at day 10. Nonetheless, although these systems showed promising results for transcleral drug delivery, authors agreed that further studies are needed to complete the evaluation in this setting. Kadam et al. have also evaluated the use of microspheres to sustain transcleral choroidal and retinal delivery of triamcinolone acetonide [73]. Triamcinolone-loaded PLA microparticles (loading 29.5%, size 2.09 μm) were spherical and had small pores on the surface, being able to delay in 120 days the complete release of triamcinolone in vitro. In vivo delivery was assessed in control and neovascularization (CNV)-induced Brown Norway rats after a single injection in the posterior subconjunctival space using a 30-gauge needle. Results showed drug levels in ocular tissues for 2 months post injection in both groups, and microparticles maintained therapeutic concentrations of triamcinolone in choroid-retinal pigment epithelial cells (RPE) and retina during that period of time as well. Entrapped microparticles were still observed in the periocular tissues at the end of 2 months post injection.

Zarei-Ghanavati et al. [74] evaluated the use of triamcinolone-loaded poly(lactic-co-glycolic) acid (PLGA) 50:50 (Mw 40,000–75,000 g/mol) microspheres (1.9–2.5 μm size, 50–85% encapsulation efficiency) in normotensive New Zealand albino rabbits, which received an intravitreal injection of 0.1 mL microspheres suspension containing 4 mg of drug. At pre-set times (3, 7, 14, 28 and 56 days after the injection) triamcinolone acetonide levels in eye tissues were analyzed. After 1 week, vitreous drug concentration was 0.047–0.012 and 0.083–0.067 mg/mL in eyes that received microspheres and triamcinolone suspension, respectively (p = 0.4173). Although, microspheres released triamcinolone in a sustained manner, the vitreous concentration declined faster in the microparticles group in comparison to the suspension form. Authors suggested a migration of smaller microparticles throughout the vitreous cavity as a possible explanation of the results.

Budesonide (Mw 430.5 g/mol) is a potent non-halogenated corticosteroid that has the ability to inhibit the expression of several proinflammatory genes, inhibiting inflammatory symptoms such as edema and vascular hyperpermeability at nanomolar concentrations [75]. Kompella et al. have successfully encapsulated budesonide in PLA microparticles (3.60 ± 0.01 μg) [76]. Authors showed release of budesonide from PLA microparticles over a 6-week study period and a cumulative budesonide release at the end of the study of 23% of the initial drug loading. Microparticles were subconjunctivally injected in rat eyes to determine retina, vitreous, lens and cornea drug levels up to 14 days. Authors detected drug levels in all tissues of the injected eye but not in the contralateral eye, suggesting that the contribution of systemic absorption and recirculation was minimal. About drug levels on day 14, they were higher in the microparticle group compared to the solution group in all cases, showing the utility of these systems in sustaining drug delivery of budesonide in eye tissues.

3.2. Nonsteroidal anti-inflammatory drugs (NSAIDs)

Several studies have shown that diabetes-induced prostaglandin expression can be significantly inhibited with the administration of

cyclooxygenase-2 (COX-2) selective inhibitors, contributing to the reduction of retinal inflammation and blood-retinal barrier breakdown during diabetes [77–79]. In rats, the oral administration of celexib, a potent COX-2 inhibitor, has shown to reduce VEGF mRNAs and vascular leakage in a streptozotocin-induced diabetic rat model [80]. However, since long-term oral administration of high doses of celexib may also lead to systemic toxicity [81], other alternatives have been proposed, such as intravitreal and periocular administrations, which are able to provide significantly higher local drug levels in the retina. In this regard, Amrite et al. [82] developed biodegradable celexib-loaded PLGA microspheres (mean particle size 1.11 ± 0.08 μm, ζ potential −38 ± 0.56 mV, loading efficiency 51.48% ± 0.42%). These particles released the drug in a biphasic manner in vitro over a period of 60 days, showing a release rate that was 38-fold lower for the microspheres than with the suspension dosage form. In vivo, drug levels in the ipsilateral eye were 0.66 ± 0.33, 0.79 ± 0.34, 0.23 ± 0.1, and 3.68 ± 3.01 ng/mg tissue, respectively, in the scera-choroid, retina, vitreous, and the cornea. The VEGF levels in the diabetic + celexib microspheres in the treated eye were reduced by 40% to 287 ± 92 pg/mg protein, and a 50% inhibition of the vascular leakage. No degeneration or atrophy of the retinal tissue were detected in the retina, when celexib-PLGA microparticle group and the control group were compared, revealing the safety and effectiveness of the pericocular celexib-loaded microparticulate delivery system developed in inhibiting diabetes-induced blood–retinal barrier leakage.

Ketorolac (Mw 255.3 g/mol) is a drug that belongs to the group of NSAIDs which has nonselective inhibitory activity of the COX enzyme and whose neuroprotective activity has been recently studied by Nadal- Nicolas et al. [83]. Authors evaluated the effect of an intravitreal solution of ketorolac or ketorolac-loaded PLGA microspheres on retinal ganglion cells survival after optic nerve crush in rats (at 0.5 mm from the optic disc). Microspheres (27 μm size, 11.43 μg drug/mg microspheres, encapsulation efficiency 88%) were able to release ketorolac in vitro for 30 days in a controlled fashion, showing an initial burst release effect during the first 24 h, and delivering the drug for 30 days following a zero order release kinetic (2.34 μg ketorolac/mg MP/day). Ketorolac-loaded microspheres were still observed in the ventral retina 14 days after intravitreal administration. Nonetheless, regarding retinal ganglion cells survival authors observed that low ketorolac doses slowly released from microparticles were not enough to protect retinal ganglion cells in an acute injury, suggesting that this type of injury probably requires higher doses of the active substance.

3.3. Photoswitch drugs

Photoswitch compounds are light activated and enable bi-directional photocontrol of neural membrane potential. The activation of these molecules consists on a transition from their trans to cis form in light responsive manner, providing drugs that can be delivered to cells to make them light sensitive. Progress has been made on several approaches to use these compounds to restore vision to a retina devoid of rods and cones, offering a substantial hope for vision restoration in the future [84]. In this regard, Grynon et al. have recently investigated the loading and delivery of quaternary ammonium–azobenzene–quaternary ammonium (QAQ) and diethylamine–azobenzene–quaternary ammonium (DENAQ), molecules with high light sensitive, in microspheres prepared with PLGA and PLA, comparing their loading efficiency and release profiles, for a possible utility in ophthalmology [85]. The highest amount of drug loaded into microspheres was when systems were prepared with PLGA 502H (51.15 ± 17.77 μg of QAQ/mg of microspheres and 62.37 ± 18.59 μg of DENAQ/mg of microspheres). This tendency was also observed in the release studies, where PLGA microspheres showed the highest cumulative release among all the systems for both QAQ and DENAQ. Approximately 87% of QAQ was released on the first 42 days, with no appreciable burst effect. For DENAQ, approximately 5% was released on day 1 in a burst release, followed by a plateau and relatively linear release from day 7 to day 42 leading to delivery of 95% of the drug. Although the loading and delivery of the photoswitch drugs from the biodegradable microspheres showed in this study can be optimized, these might be promising results for potential therapeutic applications in the ophthalmology area.

4. Microencapsulation of peptides and proteins for the treatment of chronic diseases of the optic nerve and retina

The encapsulation of peptides and proteins has been one of the most fascinating defiance over the last years in the pharmaceutical technology area (Table 2). In spite of their promising characteristics as therapeutic agents, they have some serious constraints. The main limit is their structural complexity and high molecular weight that have to be maintained with regard to keep their biological activity. Diverse methods like double emulsion, phase separation, hot-melt extrusion and spray drying, each one with their own conveniences and inconveniences are used to microencapsulate proteins. The method of preparation must assure the biological activity of the encapsulated biotechnological products not only during its elaboration, but also during storage and use of the microcapsules [86–89]. The W/O/W emulsion solvent technique is the most applied method for the preparation of these systems. Using this procedure, peptides and proteins are initially dissolved in the aqueous inner phase. However, the employment of macromolecules solution needs the evaluation of the specific assets through protein nature and further use, towards to avoid the associated potential leading dangers that might compromise its stability [90,91]. To bypass those risks, proteins and peptides can be microencapsulated in its solid state. It has been described that protein at solid state is kinetically cornered in the initial form, thus the conformational modification could be largely restricted [92,93]. In this regard, several authors are engaged on the protein microencapsulation following S/O/O methods in which the solid protein is suspended in the internal organic phase which is subsequently emulsified with an also organic external phase. Once created under these preparation conditions, protein unfolding is extremely slow [90]. Although issues may rise regarding residue of oily phases that could lead to regulatory and immunity problems [94], the S/O/W method might decrease these problems. Whatever S/O/O or S/W/O approaches are used, it is essential to use a protein powder with a small particle size at the internal S/O suspension step [95]. To obtain the desired protein powder this, lyophilisation of a water solution protein with additives is largely employed [96,97]. Moreover, the effect of lyophilisation on the biological active compound stability has been researched thoroughly, as denaturalization and aggregation are usual phenomena along freezing and subsequent dehydration, still in the presence of lyo-protectors [90]. The alternative employment of spray-drying also has its constraints. At the moment, spray-drying is applied for protein microencapsulation. In this method, lyophilisation of protein is needed again ahead dispersion and homogenization in an organic polymer solution. Then, the protein/polymer suspension created is sprayed over a heated nozzle and organic solvent is immediately removed by a rapidly vaporization by a hot gas flow. Even though the period of airing to high temperatures is usually short, it could have an adverse impact on protein stability [90]. In addition is attainable to direct spray the W/O emulsion where protein is soften in the water phase [98]. The benefit of this technique is that no high temperatures are used to eliminate organic solvent. However, the creation of a W/O emulsion does not assure proteins from the unfolding and aggregation development commented beneath.

Clinical use of peptides and proteins in neurodegenerative diseases has been under investigation in the last years. Macromolecules such as anti-VEGF, neurotrophic factors, matrix metalloproteinase, erythropoietin, serpin-derived peptides, connexin mimetic peptides among others have been encapsulated in microparticles for intraocular purpose.
### Table 2

Summary of peptides and proteins microencapsulated for the treatment of chronic diseases of the optic nerve/retina.

<table>
<thead>
<tr>
<th>Active molecules</th>
<th>IDDS</th>
<th>Preparation method</th>
<th>Duration for the active compound in vitro release from the IDDS</th>
<th>Optic nerve/retinal disease</th>
<th>In vitro or in vivo experiments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-VEGF agents</strong></td>
<td>Bevacizumab</td>
<td>PEG-PLA microspheres</td>
<td>O/A emulsion solvent evaporation methods</td>
<td>91 days</td>
<td>Age-related macular degeneration</td>
<td>In vitro technological studies</td>
</tr>
<tr>
<td></td>
<td>Ranibizumab</td>
<td>Trojan particles: ranibizumab-loaded chitosan (or chemically modified chitosan) nanoparticles included in PLGA (85:15) microspheres</td>
<td>Nanoparticles: ion gelation method. Microparticles W/O/W emulsion solvent evaporation methods</td>
<td>The best prototypes offer release periods higher than 180 days</td>
<td>Bioactivity and cytotoxicity in vitro studies (ARPE-19 and HUVECs cell lines)</td>
<td>Elsaid [101]</td>
</tr>
<tr>
<td></td>
<td>Ranibizumab/afibercept</td>
<td>No polymer employed (article focused on protein stability) BSA included in the inner phase to protect Ranibizumab/afibercept</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>Non performed</td>
<td>In vitro technological studies</td>
<td>Moreno et al. [93]</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab</td>
<td>Trojan particles: bevacizumab adsorption onto PLA nanoparticles included in PLGA (50:50) microspheres</td>
<td>Supercritical infusion and pressure quench technology</td>
<td>4 months</td>
<td>In vivo pharmacokinetic studies (healthy rats)</td>
<td>Yandrapu et al. [90]</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab</td>
<td>Trojan particles: bevacizumab adsorption onto PLA nanoparticles included in PLGA (50:50) microspheres</td>
<td>Supercritical infusion and pressure quench technology</td>
<td>4 months</td>
<td>In vivo pharmacokinetic studies (healthy rats)</td>
<td>Ye et al. [103]</td>
</tr>
<tr>
<td></td>
<td>Ranibizumab</td>
<td>PLGA microspheres: inner layer - protein and ethylene glycol outer layer -PLGA (50:50)</td>
<td>Coaxial electrospray method</td>
<td>Non performed (rhodamine-6G loaded microspheres were able to release the marker for 1 month)</td>
<td>In vivo tolerance studies with microspheres loaded with fluorescent marker (healthy chicken). No in vivo studies were performed with ranibizumab loaded microparticles</td>
<td>Zhang et al. [104]</td>
</tr>
<tr>
<td><strong>Neurotrophins</strong></td>
<td>Giall cell-line derived neurotrophic factor (GDNF)</td>
<td>PLGA (37.5/25) microspheres: PEG (400) included in the inner phase to protect GDNF</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>56 days</td>
<td>Retinitis pigmentosa</td>
<td>In vivo efficacy studies in rd/rd 1 mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLGA (50:50) microspheres: BSA included in the inner phase to protect GDNF</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>71 days</td>
<td>Glaucoma</td>
<td>In vivo efficacy studies in a spontaneous glaucoma animal model (DBA/2 J mice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLGA (50:50) microspheres</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>71 days</td>
<td>Retinal ischemia</td>
<td>In vivo efficacy studies in a pig model of acute retinal ischemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLGA (50:50) microspheres: GDNF in solid state. GDNF/oil additive (Vit E) suspension included in the internal phase</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>133 days</td>
<td>Glaucoma</td>
<td>In vivo bioactivity assays (photoreceptor explants and cultured RGCs). In vivo efficacy assays in glaucoma animal model (rats) of chronic IOP elevation</td>
</tr>
<tr>
<td><strong>Other therapeutic proteins and peptides</strong></td>
<td>Matrix metalloproteinase 2 (MMP-2)</td>
<td>PLGA (50:50) microspheres</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>One week</td>
<td>Retinal degeneration (cellular integration and promote retinal repopulation)</td>
<td>In vivo efficacy studies in Rho−/− mice</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>EPO-dextran nanoparticles in PLGA/PLA (PLGA (50:50):PLA ratio 4:6) microspheres</td>
<td>S/O/W emulsion solvent evaporation methods</td>
<td>80 days</td>
<td>Non specified</td>
<td>In vitro bioactivity assays (retinal explants). In vivo efficacy assays in retinal damage animal model (rats) due to optic nerve crush</td>
</tr>
</tbody>
</table>

(continued on next page)
Table 2 (continued)

<table>
<thead>
<tr>
<th>Active molecules</th>
<th>IDDS</th>
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<th>Duration for the active compound in vitro release from the IDDS</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Serpin-derived peptide “SP6001”</td>
<td>PBAE nanoparticles. Trojan particles; peptide-loaded PBAE nanoparticles included in PLGA (65:35) microspheres</td>
<td>Nanoparticles: complexation with PBAE. Microparticles: W/O/W emulsion solvent evaporation methods</td>
<td>140 days</td>
<td>Choroidal neovascularization</td>
<td>In vivo efficacy assays in a laser-induced choroidal neovascularization mice model</td>
<td>Shmuelli RB et al. [132]</td>
</tr>
<tr>
<td>Connexin43 mimetic peptide</td>
<td>PLGA (50:50) micro and nanoparticles</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>63 days (from nanoparticles), 112 days (form microparticles)</td>
<td>Chronic retinal and choroidal inflammation</td>
<td>Bioactivity in vitro studies (ARPE-19 cell line. In vivo efficacy studies in a retinal ischemia-reperfusion rat model)</td>
<td>Chen et al. [133]</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>PLGA (50:50) microspheres</td>
<td>W/O/W emulsion solvent evaporation methods</td>
<td>45 days</td>
<td>Retinitis pigmentosa (RP)</td>
<td>In vivo efficacy studies in rd10 mice</td>
<td>Isiegas et al. [134]</td>
</tr>
</tbody>
</table>

4.1. Anti-VEGF therapy

As mentioned before, VEGF is a protein that plays a critical role in angiogenesis and vascular hyper-permeability associated with wet AMD [99]. VEGF family consists of various ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PI GF), VEGF-A being the dominant mediator of pro-angiogenic signaling. Several therapies have been developed with the objective of inhibiting VEGF for the treatment of ocular pathologies. These therapies include VEGF inhibitors such as bevacizumab, ranibizumab and afibbercept.

Currently, the treatment of wet AMD with anti-VEGF therapies is based on one-month injections of the active compound in solution. Unfortunately, the effect is short for a chronic disease, with estimated intravitreal half-life values of several days in all cases. That is why the benefit of these new active compounds could be largely optimized by including them in drug delivery systems able to release the compounds for longer periods of time (ideally several months). Several research groups have explored the possibility to include these new active compounds in microspheres for intravitreal administration. Some of them have focused on the development of efficient microencapsulation methods for these anti-VEGF agents and in some cases the further evaluation of the microencapsulated protein activity in vitro has been also performed. For example, Li et al. [100] elaborated bevacizumab-loaded microspheres with PEG-PLA (PEG -5000 g/mol and PLA -5000 g/mol-) polymer by O/A emulsion solvent evaporation method using different emulsification techniques. The microparticles obtained (2–10 μm) showed a loading efficiency of 93.6% with an initial amount of active compound in the preparation of 5 mg and were able to sustain release the protein for 91 days. Unfortunately, no bioactivity studies were performed to confirm the integrity of the protein after the microencapsulation process. Elseid et al. [101] prepared “Trojan” microparticles loaded with ranibizumab. They first prepared ranibizumab-loaded chitosan (MW < 400,000 g/mol) nanoparticles (elaborated by ion gelification using plain chitosan, chitosan combined with hyaluronic acid or chitosan chemically modified by the inclusion of N-acetyl-Cys groups) The initial amount of protein included in nanoparticles was fixed in 5 mg. Subsequently the nanoparticles (17–350 nm) were included in PLGA (85:15; Mw 149,000 g/mol) microparticles by addition to the inner phase of a W/O/W emulsion. The best protein entrapment results were obtained when N-acetyl-Cys chitosan was used, reaching a 68%. Authors suggested that the presence of Cys might improve the protein solubility and also the protein entrainment by formation of disulfide bonds. When the in vitro release of the different prototypes was performed, the Trojan particles prepared with these nanoparticles showed the lowest burst effect and a prolonged release profile. Authors showed the absence of cytotoxicity of the systems proposed in ARPE-19 and HUVECs cells. Furthermore, they also demonstrated that the released protein maintained its antiangiogenic nature by evaluation migration in HUVECs cells and by performing capillary-like tubules formation studies. Authors concluded that the systems created might be considered interesting platforms for the delivery of active anti-VEGF proteins after intravitreal administration.

Moreno et al. [102] performed a deep analysis of different technological parameters that could influence the stability of ranibizumab and aflibercept after microencapsulation. To this, they evaluated different additives to be included in the inner phase of a W/O/W emulsion demonstrating the beneficial effect of bovine serum albumin (BSA) that partially avoided protein denaturalization during emulsification. Furthermore, they also studied the influence of different solvents and solvent combinations in O-phase of the emulsion. Among the different organic phases evaluated, triacetin and ethyl acetate resulted the best candidates for aflibercept microencapsulation while in the case of ranibizumab only triacetin showed significant better results in terms of protein stability.

Other authors have moved forward. Not only optimizing the microencapsulation processes of anti-VEGF compounds but also demonstrating the real utility of the systems proposed in vivo (in healthy animals or, even better, in some cases in retinal diseases animal models).

In this sense, Yandrapu and co-workers [99], published a very interesting work exploring the combination of nano- and microparticles for the sustained release of bevacizumab. Several “Trojan prototypes” were proposed. The most successful approach involved an initial protein adsorption onto PLA (1.0 dl/g) nanoparticles (2.5 mg protein/500 mg PLA; final mean particle size 265 nm) followed by their entrapment in PLGA (50:50; 0.67 dl/g) microspheres previous porosification by the supercritical infusion and pressure quench technology. The so-prepared particles (10% w/w PLA np content in the final formulation; microspheres final particle size 11.6 μm) led to in vitro release of the active compound in a sustained manner for 4 months. ELISA; SEC, CD and SDS-PAGE were employed to evaluate the protein stability after release demonstrating that the microencapsulation technique proposed do not alter neither the conformation nor the activity of bevacizumab. In vivo studies were performed in rats. 5 μL of microparticle suspension (30% w/v) were intravitreally injected in the right eye of healthy rats and
the same amount of protein (7.2 μg) was also administered in solution to the left eye of each animal for comparison. In eyes treated with encapsulated protein (Alexa Flour 488 conjugated bevacizumab) it was detected by noninvasive ocular fluorophotometry for two months while fluorescence disappeared two weeks after injection in eyes treated with the plain protein solution. Two months post-dosing eyes were enucleated and protein was quantified by immunoassay in different ocular tissues from the anterior and posterior segment. Bevacizumab was detected mainly in vitreous, retina and choroid-RPE only in eyes treated with microspheres. All these results confirmed the high potential of the new methodology proposed for microencapsulation of active proteins system for neurodegenerative diseases.

Ye and co-workers [103] used a different technological approach to create bevacizumab-loaded PLGA microspheres. In this case a S/O/hO emulation was performed. Authors achieved pharmacokinetic studies to “provide evidences for clinical application” of this kind of drug delivery system. The intravitreal injection of 12.75 mg of microspheres (protein loading of 0.098 mg of protein/mg of microspheres and mean particle size of 2–7 μm) was performed in the left eye of New Zealand albino-rabbits and a bevacizumab solution in the same concentration (125 mg/0.05 mL) was injected in the right eye. For 42 days, at several time points, three animals were sacrificed and the eyes were enucleated. The distribution of the active protein was evaluated in ocular tissues by immunofluorescence staining and its concentration in aqueous humor and vitreous was determined by ELISA. Results showed that the active protein was still present in ocular tissues 42 days post-injection, especially in retina, choroid, iris, ciliary body and anterior chamber angle. The concentration of bevacizumab in aqueous humor and vitreous after administration of microspheres was higher than that of bevacizumab in solution. The pharmacokinetic parameters calculated revealed area-under-the-curve values in the vitreous 2-fold higher from bevacizumab-loaded microspheres compared to the plain solution. Also important increase in Cmax values in the vitreous were observed (249 μg/mL for microspheres and 156 μg/mL for protein solution). Results showed the utility of PLGA microcarriers for anti-VEGF proteins long-term delivery in the vitreous in the treatment of chronic retinal diseases.

In an attempt to improve the microencapsulation process Zhang et al. [104] used a coaxial electrospray method to prepare PLGA microparticles loaded with ranibizumab. This method creates small drops with core-shell distribution, formed by an outer layer of or-
croparticles loaded with ranibizumab. This method creates small
lyophilisation, had a very uniform size distribution 2.4 μm, microglia activation and retinal cell death was observed at any time of the study. However, when the number of particles was increased to 0.2 mg, microglia activation and retinal cell death was observed in the short term study. Further studies with ranibizumab-loaded microspheres would be necessary to complete this interesting technological approach.

4.2. Neurotrophic factors

Neurotrophic factors are capable of attenuating or reversing neuronal degeneration. They are secreted by tissues throughout the body but are most abundant in the central nervous system. The retina contains Central Nervous System (CNS)-associated neurons making it an excellent potential site for neurotrophin therapies [105], such as BDNF (brain-derived neurotrophic factor), CNTF (ciliary neurotrophic factor) or GDNF (Glial cell-line derived neurotrophic factor), being the last one the most studied at the moment.

GDNF is a potent neurotrophic factor known to promote the development and survival of neurons. It was originally identified as a potent survival factor for midbrain dopaminergic neurons by Lin et al. [106]. This neurotrophin is the founding member of GDNF family ligands (GFLs), a subgroup of peptide trophic factors related to transforming growth factor-β (TGF-β) superfamily. Like other neurotrophic factors, GDNF belongs structurally to the cysteine knot proteins and thus, GDNF is a disulfide-linked homodimeric protein with a molecular weight of 30,400 g/mol and a 134 amino acid sequence [107].

GDNF is widely expressed in a large variety of central and peripheral neurons with an indispensable role in growth, differentiation, and survival [108,109]. In this regard, the use of exogenous growth factors as GDNF have shown to be an interesting therapeutic approach for neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and neurodegenerative ocular diseases [110–113]. In addition, recent studies have reported that long-term expression of GDNF in photoreceptors provides a neuroprotective effects in diseases like retinitis pigmentosa [114], retinal transplants [115], glaucoma [116] and diabetic eye disease [117]. In the retina, in vitro and in vivo studies have shown the potential of GDNF for rescuing retinal photoreceptors and ganglion cells functions during retinal degeneration [118]. Moreover, GDNF seems to protect RGCs after optic nerve transection [119,120] and following retinal ischemia [121]. Several authors have explored the use of GDNF-loaded microspheres for the treatment of ocular diseases.

Andrieu-Soler et al. [122] prepared GDNF-loaded PLGA microspheres by w/o/w emulsion solvent evaporation method using PLGA 374.5/25. Polyethylene glycol was included in the inner aqueous phase to protect GDNF during the emulsification process. A mixture of miscible and non-miscible solvents (3:1 methylene chloride:acetone) were used in the organic phase. Particles with a mean particle size of 27 ± 10 μm were able to encapsulate 1.26 μg GDNF/mg microsphere (encapsulation efficiency 92%). In vitro release studies performed with radiolabeled GDNF, demonstrated that the created systems were able to control the release of the protein. After a burst release of the 35% of the dose in the first 24 h, a slow delivery of the neurotrophic factor occurred at 10 ng/day for 56 days (end-point of the release study) when 60% of the total dose had been released. For in vivo experiments, rd1/mice were used. This animal model is characterized by very fast retinal degeneration, with massive loss of retinal cells by day 28 post-natal. Particles were suspended in a mixture of polysorbate 80, mannilitol and carboxymethylcellulose in water (1 μL containing 0.3 μg of microspheres, GDNF dose of 0.38 μg) and intravitreally injected. The extent of retinal degeneration was determined after animal euthanasia at post-natal day 28, coincident with the complete photoreceptors degeneration in this animal model, 17 days after the intravitreal injection. No vitreous reaction or retinal folds were observed, demonstrating the safety of PLGA microspheres. However, in two of the 44 eyes injected with microspheres (14 with GDNF-loaded microspheres, 18 with non-loaded microspheres and 12 with GDNF-loaded microspheres in which the protein had been previously denaturalized) microspheres agglomerated close to the retina promoting retinal folds but no retinal detachment. While non treated eyes showed only one layer of cells in the ONL (56 nuclei/400 μm), 3 to 5 rows of cells were observed in eyes treated with GDNF-loaded microspheres (123 nuclei/400 μm). Interestingly, a slight high amount of nuclei was observed in animals treated
with blank microspheres (91 nuclei/400 μm) compared with the non-treated animals. An increment in rods survival was also observed for eyes treated with GDNF-loaded microspheres, in comparison to non-treated eyes and with eyes injected with non-loaded microspheres of GDNF-loaded microspheres with denatured GDNF. Furthermore, after administration of GDNF-loaded microspheres it was observed a reduction of proliferation of retinal Müller in the subretinal space, in comparison to the other groups evaluated, demonstrating also a reduction in the inflammatory reaction. Finally, electoretinogram recordings were performed at shorter times (12 days after injection) to evaluate photoreceptors functionality. Rods from eyes treated with non-loaded microspheres or with microspheres loaded with denatured GDNF do not respond to light stimulation leading to b-wave amplitude very small. On the contrary, in the case of GDNF-loaded microspheres injected eyes there was a statistically significant increment in the b-wave amplitude, suggesting not only anatomical but functional rescue of photoreceptors. This strategy could be especially useful in diseases characterized by a loss of photoreceptors such as RP.

Ward et al. [123] achieved the protection of retinal ganglion cells in a glaucoma animal model (mice) after injection of GDNF-loaded microspheres. The particles were prepared according to the W/O/W emulsion solvent evaporation method using PLGA (PLGA Resomer® 503H 50:50 Mn = 25,000). Bovine serum albumin was included in the inner aqueous phase in combination with the active protein to protect it and a mixture of methylene chloride and trifluoroethanol (1:4) was chosen as organic solvent. Microspheres (mean particle size of 10 μm) released GDNF in a sustained fashion over 71 days, preceded by an initial burst release in which the 55% of the loaded protein was delivered within the first 24 h. The in vivo study was performed in a spontaneous glaucoma animal model (DBA/2 mice). This animal model suffers progression of retinal ganglion cells degeneration, with reduction of 30% at 8 months (called “early degeneration”) and of 80% at 10 months (“late degeneration”). 1 μL of microspheres suspension in PBS (0.02 mg of microspheres with a total GDNF dose of 0.707 μg) was tested in the animal model. Four protocols were performed. In three of them animals were exposed to several intravitreal injection of particle suspension each 2 months (3 or 4 injections). Animals were sacrificed and evaluated at 4, 6 and 8 months for protocol 1, at 13 months for protocol 2 and at 15 months for protocol 3. In a fourth protocol animals were injected only in the “late degeneration” time period (at 8 and 10 months) and were subsequently sacrificed at 12 months. Authors intended to cover both “early” and “late” RGC degeneration and “early” and “late” microspheres administration. The determination of RGC degeneration was performed by quantifying RGC densities. After intravitreal administration of GDNF-loaded microspheres at 2, 4 and 6 months, the “early degeneration” was evaluated, showing RGC rescue of 18.6% at 8 months. Furthermore, these animals underwent a prolonged neuroprotection without the need of further injections, so when they were evaluated at “late degeneration” stage of the disease (Protocol 3) the 15-month-old treated animals showed 3.5 times greater RGC survival in comparison with non-treated animals. Similarly, when the treatment was set at an early stage of the degeneration and it was maintained each two months until 13 months (Protocol 2) the RGC density was 2.9 higher in treated eyes. Interestingly, results from protocol 2 and 3 showed that the administration of GDNF-loaded microspheres promoted the shift in the onset of massive RGC degeneration from 8 to 10 months to 13−15 months. Finally, when GDNF-loaded microspheres were injected in the “late degeneration” step of the disease (at 8 and 10 months, protocol 4) also a neuroprotective effect was achieved although the RGC density in animals from this group was considerably less than densities from all others. In conclusion these authors demonstrated that GDNF released from GDNF microspheres significantly promoted RGC survival, being the early treatment alone sufficient for this survival effect. Furthermore, the RGC rescue results observed in long-term studies supported the potential of the systems created for the treatment of chronic neurodegenerative diseases such as glaucoma. One year later, Jiang et al. [124] evaluated GDNF-loaded microspheres (elaborated with the same protocol) in a rat model of glaucoma. This animal model is created by injection of hypertonic (1.9 M) saline solution into the episcleral vein, which promotes a chronically elevated IOP for eight weeks that simulates glaucoma events in retina and optic nerve. GDNF loaded microspheres were suspended (at 2 and 10%) in PBS and intravitreally injected. Animals treated with the higher microspheres concentration showed significant increase in RGC and axon survival, a reduction in the loss of retinal IPL (inner plexiform layer) thickness and a decrease in glial activation in both retina and optic nerve, compared with non-loaded microspheres. Furthermore, the treatment with loaded GDNF microspheres moderately reduced cupping on the optic nerve head. Later, Kyhn et al. [121] evaluated the neuroprotective effect of the same GDNF-loaded PLGA microspheres (prepared according to Ward et al. [123]) in a pig model of acute retinal ischemia. In this work microspheres (2 mg in 0.2 mL of PBS; total GDNF dose of 70.7 μg) were intravitreally injected three days after the ischemia insult. The left eye was injected with loaded and non-loaded microspheres and the right eye of each animal was left as control. Histological studies were performed after animals’ sacrifice at the end of the study (day 42−49 post injection) to evaluate potential retinal damage and to quantify cells in the RGC layer. In the animal model used, the pressure-induced retinal ischemia affects the inner retinal layer, the RGC layer becomes pathologically thin or almost absent with the nerve fiber layer thinned as well. Furthermore, edema appears in the plexiform layers and the inner nuclear layer. However, for animals treated with GDNF-loaded microspheres less destruction of the inner retinal layers, including the RGC layer was observed, in comparison to non-treated retina and with retinas treated with non-loaded microspheres. Quantitatively, the neuroprotective effect of the microsystems proposed was demonstrated by immunohistochemical detection of nuclei in the RGC layer. Eyes injected with GDNF-loaded microspheres showed RGC presence more than double that seen with blank microspheres. This increase in RGC cells was corroborated by an improvement in the retinal functionality, according to the multifocal electoretinography studies performed.

Checa-Casalenguia et al. [116] also evaluated the neuroprotective effect of GDNF-loaded microspheres Using PLGA 50:50 (Resomer® 503, 35,000 g/mol). In this case rats with chronic elevation of IOP, generated via episcleral vein injection of hypertonic saline were used as animal models. A new microencapsulation method for proteins was proposed by the research group based on the S/O/W emulsion method. The novelty of the technique was based on two facts. On one hand, the protein was not pre-treated before encapsulation and always remained in its solid state. On the other hand, an oily additive (Vit E) was included in the internal phase, promoting additional protein protection, release modulation and pharmacological activity itself (antioxidant) [125]. The microspheres prepared (mean particle size 19 μm; 25.4 ng GDNF/μg microspheres (MS)) released in a controlled manner the protein for 133 days. Initially, a burst effect was observed with 16 ng GDNF/μg MS delivered in the first 24 h. After that, several steps of relatively rapid and slow release appeared: at 117.3 pg GDNF/μg MS for the following 41 days, at 175.2 pg GDNF/μg MS from day 42 to day 77 and at 13.5 pg GDNF/μg MS form day 77 to the end of the assay. The biological integrity of the protein after the microencapsulation and release process was demonstrated by bioactivity assays using photoreceptor explants and cultured RGCs. For the in vivo studies 5 μL of a microspheres suspension in PBS (0.5% of microspheres, GDNF total dose of 0.64 ng) was intravitreally injected in animals one week after increase of IOP. Eleven weeks post-injection animals were sacrificed and RGC cells were quantified by counting anti-NeuN positive cells in the ganglion cells layer. In healthy rats values close to 67 cells/mm were observed, while in non-treated glaucomatous animals of animals receiving non-loaded microspheres this values drops to approximately 20 cells/mm. In eyes treated with GDNF/Vit E loaded microspheres values around 51 cells/mm were obtained, demonstrating the ability of the microsystem to rescue RGC. Furthermore, the treatment proposed...
was also found to have positive effect on optic nerve axonal preservation, with survival values of 73%, in contrast to values around 30% of survival obtained from non-loaded microspheres of with the same dose of GDNF administered as bolus in solution. No obvious side effects on the retinal integrity were noted.

4.3. Other therapeutic proteins and peptides

While most of the works developed until now are focused on the intracocular administration of anti-VEGF compounds and neurotrophic factors, other proteins and peptides have already emerged as interesting active macromolecules to be microencapsulated for the treatment of ocular diseases.

Yao et al. [126] microencapsulated matrix metalloproteinase 2 (MMP-2) and co-administered the particles in the subretinal space with retinal progenitor cells. MMP-2 is a protein (Mw approx. 72,000 g/mol) able to degrade cell adhesion molecules such as CD44 and neurocan involved in the maintenance of the inhibitory extracellular matrix subretinal barrier. The interesting strategy explored in this paper was to use a slow release of MMP-2 as coadjuvant to enhance the cellular integration and promote retinal repopulation. Particles were prepared using PLGA (Resomer® 502, 14,000 g/mol) by W/O/W emulsion solvent evaporation method. The microencapsulation conditions were optimized to create microspheres able to release the protein in one week, time necessary to degrade inhibitory extracellular material and to allow transplanted cells migration without disruption of host retina. Rho ×/× mice were used as retinal degenerative recipient animals in this study. 1 μL of microspheres (10–20 μm range of particle size) suspension (5 ng/mL of released active MMP2) was mixed with RGCs (2 × 10⁷/μL) and subretinally injected. CD44 and neurocan quantification was performed by immunohistochemistry during the following 5 days in eyes from different animal groups. Coincident with the in vitro peak MMP-2 release at day 3, an important reduction of both molecules was observed also in vivo at the same time. This fact was related with a significantly higher retinal progenitor cells migration into the retina, approximately 3-folds at day 3 and 2-folds at day 5 after injection in comparison to control groups, demonstrating that the strategy proposed can be an interesting tool for retinal repair.

Erythropoietin (EPO) is another promising candidate in the treatment of ocular diseases. It is 165 amino acid peptide with a 30,000 g/mol protein. It owns four glycosylation sites and thus controls the protein bioactivity. This glycosylation determines the peak of biological activity, which occurs after six weeks. The intravitreal injection of 1 mg of microspheres in PBS (total EPO dose of 0.125 μg) was performed immediately after the retinas’ insult. Also another animal group was treated with EPO solution in PBS every two weeks (EPO dose of 0.0312 μg/injection). Non-loaded microspheres suspension in PBS and PBS injections were also performed in control groups. Several analyses were performed to determine the retina status after optic nerve crushing for eyes treated and untreated. For the first two weeks after damage retinas were compared in terms of apoptotic activity via TUNEL study, showing significantly fewer apoptotic RGCs for both groups treated with EPO microspheres and with EPO solution, 11% and 12% of RGC apoptosis respectively one week after the insult. Furthermore, two weeks after the damage, a reduction of proliferation of retinal Müller in the subretinal space for groups treated with EPO (both microencapsulated and administered in solution) was observed after determination of glial cells activation in retina.

On the contrary, non-treated animals showed an increment in this parameter, related with inflammatory events, with a peak at two weeks after optic nerve crushing. Also, to determine the prolonged neuroprotective effect of the microspheres proposed on RGC survival, a retrograde labeling of RGCs was performed 4 and 8 weeks after insult. The mean density of survival RGCs in EPO microspheres and EPO solution treated groups increased in comparison to control and non-treated groups. Globally, this work demonstrated that EPO dextran-PLA/PLGA microspheres showed a prolonged neuroprotective effect by promoting RGC survival and partially delaying RGC apoptosis in the same extent that multiple EPO solution injections, regardless of the side effects caused by repeated injections. Two years later, the same authors published another work focused on the evaluation of the tolerance of the EPO-dex PLA/PLGA microspheres after intravitreal administration in rabbits (5 mg of microspheres/0.1 mL of PBS) [131]. Tolerance evaluation was performed 1, 2, 4, 8, and 12 weeks after injection. Microspheres remained in the lower part of the vitreous of the injected eyes and gradually degraded and disappeared at 11 weeks post-injection. No any RGC apoptosis or glial cell activation were observed in treated eyes. Furthermore, the retinal inner structure remained unaltered by the presence of microspheres. Neither retinal detachment nor inflammatory reactions occurred after injection. Not only structural, but also retina functionality evaluation was performed by electromyogram, showing no changes in the b-wave amplitudes after blank PLGA microspheres injection.

Some authors have explored the possibility of using serpin-derived peptides in the treatment of choroidal neovascularization [132]. These compounds are able to inhibit angiogenesis by both inducing endothelial cell apoptosis as well as decreasing their migration by increasing adhesion. One of these peptides, named “SP6001” was microencapsulated and evaluated in a laser-induced choroidal neovascularization mouse model (NVAMD) in vivo. Before the microencapsulation process, it was complicated with a biodegradable cationic polymer (poly-beta amino ester, PBAE) thanks to the negative charge of the peptide due to the presence of glutamate residues, in order to protect the peptide form degradation and to extend the delivery. The nanoparticles created (mean particle size of 119 nm) were tested alone or microencapsulated in PLGA microspheres prepared by the inclusion of the peptide nanoparticles in the inner aqueous phase following a W/O/W emulsion solvent evaporation method (PLGA 65:35 Wt 40,000–75,000). The so prepared microspheres (mean particle size 12 μm; peptide encapsulation efficiency around 70%, with an initial amount of 0.25 mg of peptide in the formulation) showed a cero-order kinetic release for 140 days (without burst effect) at a rate of 8 mg of peptide/mg of particle/day, followed by a slightly slow release phase for additional 60 days. The animal model used in this work undergoes Bruch’s membrane rupture, which induces neovascularization in the damaged zone. Two weeks after the
Insulin as new therapy in the protection of photoreceptors and conditions. In the treatment of chronic retinal and choroidal in diseases and has shown the ability to delay photoreceptors death in retinitis pigmentosa animal models [135,136]. Microspheres were prepared by W/O/W emulsion solvent evaporation method using PLGA (50:50 Resomer® RG503H) as polymer matrix. Particle size ranged from 10 to 32 μm with an active compound loading of 3.2 μg/mg microspheres.

The in vitro release profile of the formulation selected showed an important initial burst when >60% of the total amount of encapsulated protein was released, followed by a sustained delivery of proinsulin for 45 days with a final accumulated release of 90% of the total loaded molecule. According to authors, this initial important release might contribute to the neuroprotective effect expected for the systems created. The animal model selected for this study, rd10 mice, is characterized by a spontaneous mutation of the rod-phosphodiesterase (PDE) gene, leading to a rod degeneration that starts around P18 and is also followed by cones loss. The photoreceptor death reaches a peak around P25 [137]. Animals were intravitreally treated with loaded microspheres (total protein dose 22.42 ng) at P14 or P15, before the onset of any photoreceptor degeneration. As control, the contralateral eye was injected with non-loaded microspheres. Retinas were evaluated at P25 in terms of photoreceptor preservation by determination of outer nuclear layer thickness and the number of cell rows. Eyes treated with proinsulin-loaded microspheres showed higher ONL thickness and number of rows all over the different retina sections evaluated from nasal to temporal zones. The attrition of cell death for treated eyes was also observed in retinal organotypic studies at P22. Furthermore, authors evaluated retina functionality by electroretinography. In this animal model the electroretinography (ERG) reveals alterations in the physiology of the inner retina as early as P18 (before any obvious morphological change of inner neurons) and yet consistently with a reduced band amplification by bipolar cells [137]. Results demonstrated that the treatment with loaded microspheres significantly increased the b-cone, b-mixed and oscillatory potential amplitudes compared with values obtained for eyes injected with non-loaded microspheres. According to authors, these results might be explained by a reduction in rod cell death, leading to structural improvement in the retina and also a neuroprotective effect on synapses between rods and bipolar cells improving their functionality.

4.4. Other uses of protein microencapsulation involved in the study of optic nerve and retinal diseases. Animal models

Due to the difficulty of simulate adverse conditions in chronic diseases, different strategies have been assayed. Two pioneer research works from 1998 and 2001 already explored the utility of protein-loaded microparticules, not to treat ocular diseases but, on the contrary, to progressively induce damage in animal retinas simulating events typically occurring in ocular diseases, such as neovascularization. Kimura et al. [138] prepared cross-linked gelatine microspheres loaded with b-FGF (basic fibroblast growth factor) by polyion complexation. b-FGF is a prominent promoter of endothelial cell migration and proliferation that stimulates the angiogenic effect. After subretinal injection, particles (mean particle size of 40 μm) induced a foreign body reaction from the first week after injection. Two weeks post-implantation several events promoted the formation of new subretinal vascular vessels, not only due to the inherent effect of the loaded protein but also to the release of soluble compounds of infiltrating macrophages and RPE such as VEGF. Interestingly authors observed that the choroidal neovascular membranes created resulted immunohistochemically similar to those developed in AMD. Cleland et al. [139] prepared microparticles intended for the development of ocular diseases animal models as well. They performed poly-glycyclic acid microspheres loaded with VEGF. In a first step VEGF particles were prepared by spray-freezing procedure including different agents to improve the protein stability (trehalose or polysorbate). Subsequently, the so prepared protein was included in PLGA microspheres also prepared by spray-freezing and further organic solvent extraction (PLGA 50:50 RG502 and RG502H). Authors tested these microparticles with therapeutic purposes for the induction of neovascularization in ischemia regions; however authors also explored the potential of VEGF-loaded microspheres to increase angiogenesis after intravitreal and subretinal injection. In both cases an
increase in retinal vessels dilation and the appearance of tortuous new vessels was observed five days post-implantation.

5. Conclusions and future trends

Neurodegenerative diseases of the posterior segment of the eye are directly related with blindness. These pathologies have in common that the target site is the retina (or close to retinal areas), systemic and topical treatments are not effective (due to the presence of ocular effective barriers) and most of them are chronic, multifactorial and elderly related. Patients suffering these diseases can gain benefits from the use of drug delivery systems, to slow down the progression of the neurodegenerative events.

One of the most challenging areas in ophthalmology therapy is to decrease the number of invasive interventions. This objective can be achieved by the combination of active substances and/or the development of drug delivery systems. Currently, retina specialists are taking into consideration the utility of the combination of active substances that target different pathophysiological mechanisms in retinal complex pathologies. The combination of active substances in these therapies has been demonstrated to have benefits in comparison with monotherapy. For example in diabetic macular edema (DME), a pathology involving vascular endothelial growth factor (VEGF) and other inflammatory mediators [140]. Although DME is currently first-line treated with intravitreal anti-VEGF therapies, some patients are refractory to multiple anti-VEGF treatments. In these cases a combination therapy of Anti-VEGF with corticosteroids appears a promising alternative [7]. Similar therapeutic strategies have been also employed in the management of neovascular age-related macular degeneration. Thanks to this combination authors have described an overall reduction in the required ranibizumab re-treatments compared with ranibizumab monotherapy [141,142]. The use of combined therapy in AMD has been extended to non-steroidal antiinflammatory drugs. In fact, bromfenac combined with the anti-VEGF in the treatment of wet AMD has resulted more effective than single aflibercept [143].

Long term delivery of different active substances can be achieved with the use of multi-loaded drug delivery systems avoiding successive injections. Microspheres can contain different drugs being released in a controlled fashion. Thanks to this therapeutic strategy, an improvement in the chronic treatment of neovascular AMD and DR could be gained. Combined neuroprotection from biodegradable microspheres can be extended to glaucoma by lowering the degeneration occurring in subjects with high levels of IOP as well as in normotensive patients. The controlled release of several combinations such as biotechnological products with low molecular weight active substances or even several low molecular weight drugs with different mechanism of action will result of great interest in the coming future.

The use of microspheres could result beneficial as a combined therapy to be used in inherited neurodegenerative diseases. In this case, the combination of gene therapy with neuroprotective drug delivery would be of great interest. The advances in gene therapy and the on-going clinical trials support the fact that this kind of treatments could become available in the next coming future. With the help of the slow delivery of neuroprotective substances a more rational therapy is available.

The combination of several technological strategies in the same formulation can be an interesting tool to improve the benefit of active compounds as well. For example, Oswald and Kang-Mieler recently prepared PLGA microspheres loaded with anti-VEGF agents (ranibizumab or aflibercept) and included them in an injectable thermos-responsive hydrogel. This combination allowed the extension of the bioactive compounds in vitro release over almost 200 days at constant rate after initial burst release [144].

Microspheres are also emerging tools in the development of chronic animal models of degeneration that allow a more physiological approach to the events occurring in the clinical scenario.

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