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Review article

Intravitreal hydrogels for sustained release of therapeutic proteins

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ABSTRACT

This review highlights how hydrogel formulations can improve intravitreal protein delivery to the posterior segment of the eye in order to increase therapeutic outcome and patient compliance. Several therapeutic proteins have shown excellent clinical successes for the treatment of various intraocular diseases. However, drug delivery to the posterior segment of the eye faces significant challenges due to multiple physiological barriers preventing drugs from reaching the retina, among which intravitreal protein instability and rapid clearance from the site of injection. Hence, frequent injections are required to maintain therapeutic levels. Moreover, because the world population ages, the number of patients suffering from ocular diseases, such as age-related macular degeneration (AMD) and diabetic retinopathy (DR) is increasing and causing increased health care costs. Therefore, there is a growing need for suitable delivery systems able to tackle the current limitations in retinal protein delivery, which also may reduce costs. Hydrogels have shown to be promising delivery systems capable of sustaining release of therapeutic proteins and thus extending their local presence. Here, an extensive overview of preclinically developed intravitreal hydrogels is provided with attention to the rational design of clinically useful intravitreal systems. The currently used polymers, crosslinking mechanisms, in vitro/in vivo models and advancements are discussed together with the limitations and future perspective of these biomaterials.

1. Introduction

Drug delivery to intraocular tissues is one of the major challenges faced by ophthalmologists and formulation scientists because the eye is a highly complex, isolated and specialized organ of the human body. The challenge of drug delivery to this organ is related to the presence of different barriers that prevent exogenous and harmful substances from entering the posterior chamber, in particular, the retina and optic nerve. Vision-threatening disorders are mostly related to abnormalities in intraocular tissues, especially in the retina. The occurrence of such diseases is rapidly increasing in industrialized countries, not only in the ageing populations but also in younger individuals, causing a substantial health problem in modern society. It is estimated that approximately 285 million people worldwide are visually impaired, and 39 million are completely blind.[1,2] These numbers are expected to double by 2050, representing a significant public health burden.[3,4]

Conventional eye drops containing low molecular weight drugs are the most commonly used ophthalmic drug formulations. Although they are not suitable for the delivery of therapeutic proteins, they represent 90% of the ocular products present in the market, which is due to ease of manufacturing and scale-up, stability and cost-effectiveness[5]. However, after topical instillation into the lacrimal fluid, drugs are rapidly removed from the ocular surface due to solution drainage and systemic drug absorption across the conjunctiva that lines the inner side of eyelids. Furthermore, the epithelia of the cornea and bulbar conjunctiva are major barriers for drug absorption into the eye. Therefore, only a minimal amount (< 5%) of the administered drug dose reaches the anterior part of the eye, and even a much smaller fraction (< < 1%) reaches the posterior eye segment after topical administration of the drug formulations.[6,7]. Thus, frequent administration is

Abbreviations: ESHU, (poly(ethylene glycol)-poly-(serinol hexamethylene urethane); RCE, rabbit corneal endothelium; HCEC, Human corneal epithelial cell lines; HRPE, human retinal pigment epithelial cell lines; dex-GMA, glycidyl methacrylate-derivatized dextran; HAMC, thermal of hyaluronan and methylcellulose; ICNPH, Chitosan nanoparticles/poly(lactic-co-glycolic acid)-poly(ethylene glycol)-poly(lactic-co-glycolic acid) hydrogel; PCM, Polycaprolactone dimethacrylate; HEMA, hydroxyethyl methacrylate; CNTF, Ciliary neurotrophic factor; HUVECs, Human Umbilical Cells; ARPE 19, Adult Retinal Pigment Epithelial cell line-19; DR, diabetic retinopathy; AMD, age-related macular degeneration; CNV, choroidal neovascularization

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needed to maintain drug concentrations within the therapeutic window.[4,8]

A wide range of posterior segment eye pathologies severely impacts vision. These disorders include neovascular age-related macular degeneration (AMD), diabetic retinopathy (DR), diabetic macular edema (DME), retinal vein occlusions (RVO) and diseases that originate from the alteration in the vasculature system of the retina, genetic disorders and eye tumours[9,10]. Many studies have demonstrated that vascular endothelial growth factor (VEGF) plays a crucial role in the pathogenesis of retinal diseases.[11,12] Therefore, many therapeutic approaches aim at blocking VEGF signalling by the delivery of intravitreally injected anti-VEGF proteins.[13,14] The effectiveness of antibodies (bevacizumab, Avastin®), antibody fragments (ranibizumab, Lucentis®), and soluble receptors (aflibercept, Eylea®) have been shown in the treatment of patients with neovascular AMD.[15] However, maintaining sufficient concentrations in the retina after intravitreal injection for an extended period is an important challenge. Monthly injections are burdensome and have resulted in impaired patient compliance[16,17].

In the past decades, tremendous efforts have been made to improve the disposition of drugs, especially bioactive proteins, in the retina by using different routes of administration and drug delivery vehicles. Several drug delivery technologies such as *in-situ* forming hydrogels, micelles, liposomes, nanoparticles and ocular implants have been developed for ocular applications.[18,19] *In-situ* forming hydrogels are considered attractive biomaterials, which can be engineered to offer several benefits, including less frequent administrations, patient comfort and cost reduction. Furthermore, hydrogels that jellify *in-situ* allow loading of therapeutically active compounds during network formation facilitating local delivery and release through a minimally invasive procedure. In the past years, the use of hydrogels have received increased attention as ophthalmic formulations that deliver drugs to the posterior segments.[20–24]

This present review discusses and highlights the clinical success of therapeutic proteins in the treatments for posterior eye diseases and the major limitations in protein delivery to the posterior segment of the eye. Anatomical and physiological barriers in ocular protein delivery are summarized and discussed. It is further conferred how *in-situ* forming hydrogels can improve the long-term release and subsequent exposure of protein-based therapeutics to the posterior segment of the eye, especially to the retina. Different, biodegradable, natural and synthetic hydrogels are presented together with diverse functional groups and crosslinking mechanisms employed to obtain hydrogels with suitable drug release profiles. Guidance on the rational design of ideal drug delivery systems to the posterior segment of the eye is provided in addition to the critical issues related to these delivery technologies.

2. Drug administration to the posterior segment of the eye

2.1. Anatomical and physiological components of the eye

The eye is a highly complex, isolated and specialized organ of the human body. The structure can be classified into two segments: anterior and posterior. The main structures in the anterior chamber are the cornea, conjunctiva, iris, ciliary body, aqueous humour, lens and supporting structures. The posterior chamber encompasses the sclera, choroid, retina, and vitreous body (Fig. 1). The vitreous cavity contains the vitreous body, which is a transparent, gelatinous mass located between the lens and the retina. The vitreous humour body is a highly hydrated three-dimensional network of hyaluronic acid (HA) ~0.5% and collagen ~0.5% with a water content of ~99%.[25,26] In order to perceive and recognize objects, light passes through the anterior chamber to the posterior chamber. Light 'travels' the eye through the cornea, the pupil and hits the lens, a convex and transparent disc that focuses and projects the light onto the retina, which is located in the inner layer of the back of the eyeball. The retina is loaded with millions of photoreceptors, which convert light energy into electrical signals that, in turn, the brain receives as impulses to produce images. Drug delivery to the retina can be achieved by various routes such as topical, systemic, intravitreal, sub-retinal, subconjunctival, intracameral and periocular administration. Unfortunately, most patient-friendly administration routes, among which topical and systemic, often result in poor drug bioavailability. Drugs administered to the eye can be restricted by various static and dynamic barriers, as discussed in section 2.2. Particularly, conventional formulations are unable to efficiently deliver proteins into the eye owing to these complex barriers and elimination mechanisms. Therefore, intravitreal injectable depot formulations may solve these limitations by bypassing these barriers and providing a sustained release of proteins reaching intraocular tissues.

2.2. Anatomical and physiological barriers in ocular drug delivery

In-depth knowledge of ocular barriers and pharmacokinetics is essential for the development of effective delivery technologies to the retina. Generally, two types of barriers can be distinguished: 1) anatomical/static barriers representing the different layers of cornea, sclera, conjunctiva, and retina blood-aqueous and blood-retinal barriers. 2) physiological/dynamic barriers including choroidal and conjunctival blood flow, lymphatic clearance, efflux transport, nasolacrimal drainage and tear turnover.

2.2.1. Topical administration

Topically administered eye drops are frequently used to treat anterior segment diseases therapeutically. However, this mode of drug administration is associated with low ocular bioavailability due to dynamic and anatomical barriers.[7] After installation to the eye, the solution drainage in tears and eye blinking results in rapid precorneal drug loss into the nasolacrimal duct, leading to poor absorption of small molecules (< 5%) even into the anterior eye tissues. Importantly, protein drugs have negligible absorption to the anterior eye tissues after topical administration.[22,27] Most of the dose of small molecular drugs after topical administration is absorbed into the systemic circulation via the highly vascularized nasolacrimal duct, which in turn can result in adverse systemic effects.[5,28–30] Depending on the drug and the technique of application to the ocular surface, these adverse effects encompasses low blood pressure, reduced heart pulse rate, fatigue, shortness of breath, headaches, allergic reactions and many more.[31] The delivery of topically applied drugs to the posterior eye segment is 1-2 orders of magnitude lower than to the anterior segment due to several reasons.[7,32] Firstly, the flow of aqueous humor from the posterior to the anterior chamber limits drug access to the intravitreal cavity.[23,33,34] Also, systemic drug distribution from aqueous humor to the blood circulation of the iris and ciliary body reduces distribution to the posterior eye segment.[7] Finally, the lens forms a dense barrier that limits drug penetration.[35]

2.2.2. Systemic administration

In ophthalmic therapy, systemic administration has been used to deliver antibodies, antibiotics and carbonic anhydrase inhibitors to treat various diseases like endophthalmitis, elevated intraocular pressure and uveitis.[29,36–38] The presence of the blood-retinal barrier (BRB) regulates the transfer of drugs from the blood circulation to the eye in both directions and can be a major limiting barrier. The blood-ocular barriers consist of a posterior BRB and an anterior blood-aqueous barrier (BAB), and together they represent an impenetrable tight barrier for proteins and other macromolecular therapeutics larger than 2 nm in diameter.[7] The BAB is formed by the inner non-pigmented ciliary epithelium, ciliary muscle capillaries and posterior iris epithelium. The BRB is made up of retinal pigment epithelium (RPE) and inner retinal endothelial capillaries (inner BRB). The RPE is a tight cellular monolayer that is located between the photoreceptors and choroid.[39] It

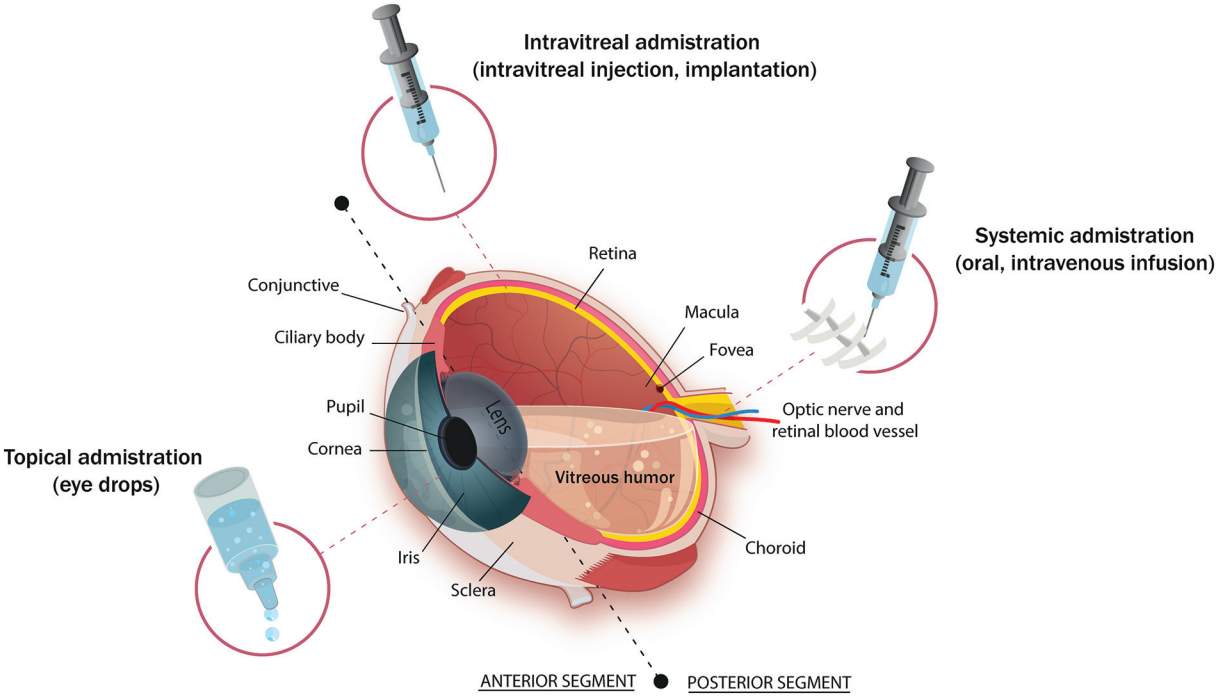

Fig. 1. Anatomical components of the eye and frequently used routes of drug administration.

regulates the homeostasis of the neural retina and the outer part of the BRB by controlling, e.g. epithelial transport, secretion, phagocytosis, spatial ion buffering and immune modulation.[40] It is, however, challenging to quantify the permeability of BAB and BRB separately because, after intravitreal injection or intravenous administration, as drugs can be eliminated from the eye through both of these barriers.[41] Furthermore, because of these physiological barriers, high drug doses and repetitive administration are necessary to achieve adequate therapeutic drug levels with the risk of adverse off-target effects.[42]

2.2.3. Intravitreal administration (IVT)

In clinics, intravitreal drug delivery is the only successful route of administration for proteinaceous drugs (such as anti-VEGF compounds) to the posterior segment of the eye, because of the proximity of the vitreous to the retina.[27,43–45] Intravitreal administration results in high retinal bioavailability since the drug is directly injected into the vitreous in the vicinity of the retina. After intravitreal injection, protein drugs distribute by Fickian diffusion from the vitreous over the surrounding ocular tissues, reaching the target sites in the retina.[7] The vitreous body does not act as a severe barrier for the diffusion of soluble proteins. However, it might limit the mobility of the administered delivery systems (e.g. drug-loaded nanoparticles) depending on their physico-chemical properties and design to deliver proteins to the posterior segment.[46] As mentioned before, one of the main components of the vitreous body, besides water, is hyaluronic acid, a hydrophilic polysaccharide with a molecular weight up to $2\text{-}3*10^6$ Da.[7,47] The vitreous humour has a loose and open structure that allows rapid diffusion of low molecular weight drug molecules since the mesh size in the vitreous network has been estimated to be ~500 nm.[7,48,49] Biologics generally administered through intravitreal injections do not exceed a diameter of 10 nm, e.g. bevacizumab (6.5 nm) and ranibizumab (4.1 nm), and therefore based on size only they should not display restricted mobility in the vitreous.[47,50] The diffusion of aggregates of self-assembled polymers, and nanoparticulate carriers can be restricted.[51] Additionally, positively charged molecules and particles can also be restricted because these cationic entities can bind to the negatively charged hyaluronic acid matrix.[52] Importantly, the movement of macroscopic hydrogels, microspheres and drug-loaded implants are significantly restricted or even absent in the vitreous as they are bigger than the average mesh size in the vitreous network of hyaluronic acid (500 nm). These systems can, therefore, be used as localized reservoirs for sustained drug delivery to the retina. Drug elimination from the vitreous cavity takes place either via anterior or posterior clearance. The anterior route involves drug diffusion in the vitreous to the posterior chamber, followed by convective elimination in the aqueous humor outflow.[33,53,54] The posterior route involves drug diffusion to the retina, followed by permeation across the blood-retina barriers into the systemic blood circulation.[33,55][40] Pharmacokinetic studies suggest that intravitreally administered biologicals are mostly eliminated from the eye by the anterior route, while a smaller fraction of the dose (≈10%) escapes across the blood-retina barrier.[41]

The posterior clearance is only relevant for therapeutic compounds that can cross the endothelial and epithelial of blood-ocular barriers (typically only small molecular drugs).[55] These barriers are selective, allowing passage of small molecules with lipophilic properties (smaller than 2 nm in diameter) while restricting the permeation of large molecules.[7] For this reason, the half-lives of low molecular weight molecules in the vitreous are typically in the range of 1-10 h, while those of proteins and other macromolecules are in the range of several days.[55] Unfortunately, ocular intravitreal therapies of therapeutic proteins presently applied in the clinics are associated with serious risks due to their frequent injections. This recurrence results in an increased risk of hemorrhage, retinal detachment, persistent discomfort, degeneration of photoreceptors (PRs), cataract formation, bacterial endophthalmitis and increased intraocular pressure (IOP).[7] New delivery systems are, therefore, urgently needed to prolong the injection intervals for improved treatments of retinal diseases and to reduce side effects. Particularly, tolerability and biocompatibility issues, biodegradability, sterility, reproducible manufacturing and eventually preclinical and clinical performance must be taken into consideration when developing such delivery systems for clinical translation.

2.2.4. Other administration strategies

Besides topical, systemic and IVT administration, some of the most actively investigated routes to bypass barrier functions in the eye and promote localization of drugs to the back of the eye are periocular,

subretinal, intrascleral and suprachoroidal routes.[56–58] Drug and gene delivery through each of these routes of administration can be limited by various static/dynamic barriers, as discussed by del Amo et al.[7] and Rowe-Rendleman et al.[59] Briefly, depending on the exact location, drug diffusion to the posterior segment after injections of drug formulations can be limited by static barriers such as sclera, choroid, Bruch's membrane, RPE tight junctions and retina. At the same time, dynamic restrictions can include choroidal circulation, retinal circulation, subconjunctival-episcleral lymph and blood vessels.[60–62] Despite these limiting barriers, many innovative drug delivery systems and strategies (such as nanoparticles, hydrogels, dendrimers, adenovirus, microspheres and microneedles) are being investigated in animal studies and clinical trials using periocular, subretinal, intrascleral, and suprachoroidal routes of administration.[46,63–67]

Minimally invasive microneedle-based ocular delivery is worth mentioning as it has the potential to revolutionize the way drug formulations are administered within ocular tissues. Solid and hollow microneedles were initially developed for drug delivery to the skin, where they are used to form micron-sized pores on body surfaces through which drugs can directly enter tissue layers and therefore increase drug permeation. In recent years this technology has been designed to be applied on the ocular surface (e.g. cornea, sclera and suprachoroidal space) to treat diseases in the anterior and posterior segments avoiding complications associated with ocular injections with conventional needles as discussed by Thakur Singh et al.[68] Importantly, this delivery strategy can significantly minimize damage to the ocular tissues, reduce patient discomfort/pain due to its micron-sized needle (typically 25-2000 μm in height) and allow precise localization of drug formulations, as shown by Park et al.[69] and Song et al.[70] Microneedles have been used together with drug delivery systems (e.g. gel formulations, nanoparticle and microparticle suspension) for sustained delivery.[71,72] The delivery of low molecular weight drugs via microneedles to the anterior segment and the posterior segment has been extensively investigated.[69,70,72] While delivery of therapeutic proteins has been developed primarily for the anterior segment, especially in the treatment of corneal neovascularization with limited information about the treatment of posterior eye diseases.[63,73] Furthermore, it is essential to note that drug distribution to the posterior segment after intrascleral or suprachoroidal microneedle injection can still be limited by the previously mentioned barriers. Issues concerning forces of injection, IOP, method of injection/retraction and the overall safety of the technology are to be systematically studied together with long term delivery of therapeutic proteins to examine the full benefits of microneedles for ocular applications.[68]

2.3. Clinical success of therapeutic proteins for intraocular diseases

During the last decade, the market of ophthalmic biologicals such as monoclonal antibodies, peptides, aptamers and recombinant proteins has been growing enormously. In 2017, the worldwide sales of monoclonal antibodies for various biomedical applications were estimated to be over 98 billion US$.[74] The success of these biologicals became possible due to the tremendous advances in the fields of genetic engineering, innovative biotechnology, pharmaceutics, identification of druggable targets, formulation and GMP production.[75,76] Particularly, attention has been given to the pharmaceutical development of anti-VEGF agents to rescue vision in retinal vascular diseases after the validation of the importance of the elevated intraocular levels of vascular endothelial growth factor (VEGF-A). These growth factors are responsible for the angiogenesis and neovascularization in the retina and, therefore, involved in the modulation of posterior segment eye diseases. These diseases include age-related macular degeneration (AMD), diabetic retinopathy (DR),[77] retinal vein occlusion with cystoid macular edema (CME), posterior uveitis.[78] AMD and DR are the leading causes of visual impairment worldwide.[1] The prevalence of these diseases is likely to increase with the rapid growth of the ageing population representing a major public health burden.[1,79]

AMD is an abnormality of the retinal pigment epithelium (RPE) that leads to the degeneration of the photoreceptors in the macula and consequent loss of central vision in elderly individuals. There are two main forms of AMD: neovascular (wet) and non-neovascular (dry), which affect over 16 million people in Europe and the United States.[80] The wet form represents 90% of cases of severe sight loss in AMD patients and usually affects both eyes. Wet AMD is known as abnormal neovascularization in the central region of the retina.[79] This abnormality results in vision loss due to retinal damage caused by fluid leakage and scar formation. The global cost of wet age-related macular degeneration (AMD) market is estimated to be $6.9 billion in 2018, and in the near future, it is expected to reach $10.4 billion by 2024.[81]

Diabetic retinopathy is the leading cause of blindness in diabetic patients and in working-age adults.[2] It results from damages of blood vessels in the retina, resulting in leakage of blood and other fluids into the retinal tissue followed by cloudy or blurred vision.[2,82] Diabetic macular edema (DME) is a swelling of the macula and a direct consequence of diabetic retinopathy. In order to cure or slow down the progression of these conditions, bolus intravitreal injections of therapeutic proteins and oligonucleotides such as bevacizumab, ranibizumab, infliximab, aflibercept, pegaptanib are administered.[83] These drugs are generally administered for the neutralization of cytokines and growth factors (Table 1). In this way, photoreceptors in the retina are

Table 1
FDA approved and off-label (*) proteins/biologics for intraocular medications and dosages.

Biologics	Type of biologics	Route	Clinical phase	Therapy. Indications	Mw (kDa)	Half-life after administration	Stand. Dose	Ref.
Bevacizumab (Avastin®)	Anti -VEGF	Ivt	Off-label drug (2004)	Wet AMD*, DR*, DM*	149	4.9 days human 4.3 days in rabbit	1.25mg/50μl	[45,93]
Ranibizumab (Lucentis®)	Anti-VEGF	Ivt	FDA-approved (2006)	Wet AMD, DME, DR*	48	2.88 days rabbit 9 days human	0.5mg/50μl	[93,94]
Aflibercept (Eylea®)	Anti-VEGF (VEGF Trap-eye)	Ivt	FDA-approved (2011)	Wet AMD, DME,CNV,DR	115	7.1 days human 3.63 days rabbit	2mg/ 50μl	[95,96]
Pegaptanib sodium (Macugen®)	VEGF inhibitor	Ivt	FDA approved (2004)	Wet AMD, DR*, DME*	50	10 days human	0.3mg /90 μl	[96–98]
Adalimumab (Humira®)	Anti-TNFα	SC	FDA-approved (July 2016)	Uveitis	148	2 weeks in human serum	40mg	[99]
Infliximab	Anti-TNFα	Iv/Ivt	Off-label for Iv	Chronic non-infectious uveitis*	149.1	8.5 days (rabbit vitreous), 7–12 days (systemic human)	1-1.7mg/ 100 μl (Ivt), 3-5mg/kg (Iv)	[89,90,100,101]
Brolucizumab (Beovu®)	anti-VEGF	Ivt	FDA-approved (October 2019)	Wet AMD	26	2.4 days (cynomolgus monkeys) in ocular compartments	1.0 or 6.0mg/eye	[91,92]

Abbreviations: Intravitreal (Ivt) injections; suprachoroidal (SC); intravenous (Iv) infusion.

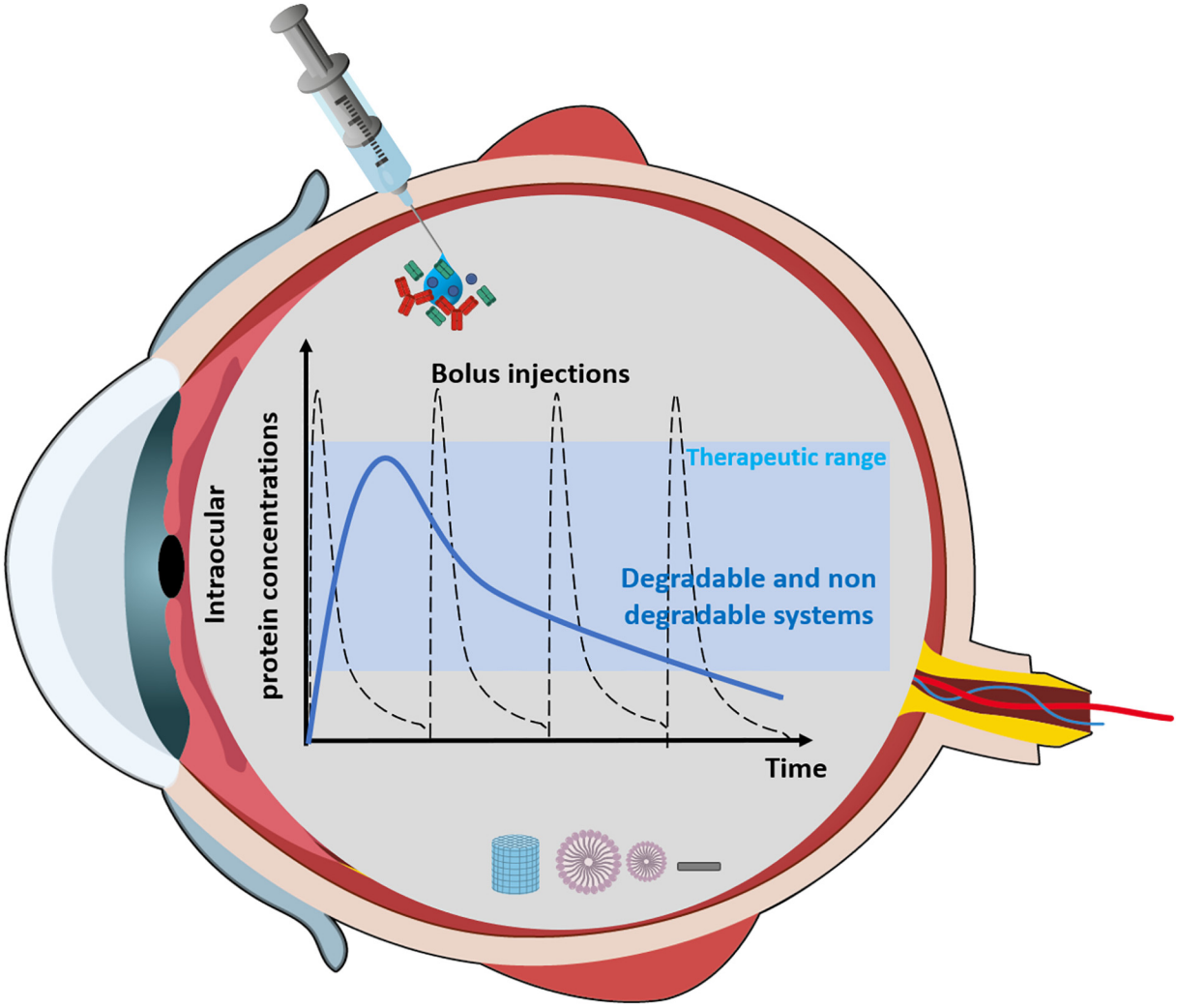

Fig. 2. Schematic representation of intraocular pharmacokinetics of drugs delivered by non-degradable and degradable sustained delivery systems compared to frequent bolus intraocular injections.

protected, and angiogenesis caused by the overexpression of vascular endothelial growth factor (VEGF) is prevented. In 2004, the first oligonucleotide-based anti-VEGF aptamer (Macugen®; Pegaptanib; Eyetech, Pfizer) received FDA-approval for neovascular AMD and choroidal neovascularization (CNV) treatments.[84] The monoclonal antibody Avastin® (Bevacizumab; Genentech) received FDA approval in 2004 for metastatic colon cancer treatment. Importantly, bevacizumab is currently used in ophthalmology as an off-label drug for AMD, although not yet FDA approved for these indications. Subsequently, in June 2006, a more effective monoclonal antibody (Lucentis®; ranibizumab; Genentech) was FDA-approved for neovascular AMD and DME therapy. Ranibizumab (48 kDa) is a Fab fragment of IgG1 and has a 17-fold higher binding affinity for the VEGF receptor when compared to bevacizumab (149 kDa). In recent years, ranibizumab has shown to prevent further vision loss in approximately 95% of patients and to improve vision in 40% of the patients suffering from wet AMD.[85] Likewise, in 2011, Eylea® (VEGF-TRAP-Eye; aflibercept; Regeneron)[86] received FDA approval for the treatment of CNV and wet AMD.[79] Aflibercept (97 kDa) is a recombinant fusion protein (decoy receptor for VEGF) that has a 200-fold higher affinity for VEGF than ranibizumab.[76] Moreover, anti-TNFα agents are also used in intraocular inflammation, edematous neurodegenerative and neovascularization diseases as TNF's play an essential role in the pathogenesis of these diseases.[87] Adalimumab (Humira®), a monoclonal anti-TNFα for uveitis, was FDA approved in 2016 and binds specifically to TNFα (pro-inflammatory cytokine produced by macrophages and T-cells) and therefore results in inhibition of the inflammatory response. Infliximab (INF) is also a monoclonal, chimeric IgG antibody that binds circulating and membrane-bound TNFα, but this antibody is not yet FDA approved for uveitis or ocular inflammation.[88–90] Beovu® (brolucizumab) developed by Novartis recently received FDA approval (October 2019) for the treatment of wet AMD (see table 1) by intravitreal injection. The protein is a potent anti-VEGF, which has a high affinity to all VEGF-A isoforms. Clinical studies by randomly used intravitreal doses of brolucizumab 6mg and 3mg versus aflibercept 2mg demonstrate that Beovu was non-inferior to aflibercept in visual function after 48 weeks.[91] Patients treated with Beovu overall showed a good improvement in their vision and health of the eye within one year.[92] Summarizing, anti-VEGF and anti-TNFα therapies have been very successful in the field of ophthalmology after their FDA-approval, changing the way vision-threatening diseases are currently treated in clinics.

2.4. Limitations of protein delivery to the posterior segment of the eye

Despite the tremendous clinical success of several novel therapeutic proteins for the treatment of vision-threatening disorders, frequent injection of these formulations cause discomfort and adversely affect patient compliance.[102] Importantly, this class of therapeutics is hard to formulate into long-acting delivery systems because of their structural complexity and undesired interactions with the delivery vehicle.

For instance, functional groups present in the hydrogel crosslinks can potentially react with OH, COOH, SH and NH_2 groups of the loaded protein. Further, there are protein stability issues due to protein unfolding, denaturation and aggregation, leading to loss of activity and even unwanted immune responses.[103–107] Intravitreal injection is the only route of administrations used today in clinics to deliver protein-based drugs to the posterior segment of the eye and eventually to the retina. However, this approach is painful, and often monthly repeats of injections are needed depending on the drug and disease progression.[16,108] Furthermore when delivering proteins using other routes of administration (e.g. topical, systemic), the poor bioavailability to intraocular target sites is caused by their inability to cross biological membranes as most of the therapeutic proteins are highly hydrophilic and have a high molecular weight.[46,109] Moreover, pharmacokinetic and cell studies have shown that the retina, cornea and sclera have tight junctions that limit the free diffusion of large hydrophilic molecules through these cell layers.[76,110] So far, various attempts have been made by formulation scientists to develop drug delivery systems that can bypass these intraocular restrictions. However, non-targeted delivery of biologicals into the ocular tissues by any route of administration may lead to drug distribution to other tissues, primarily through the circulatory system. Therefore, drugs eliminated from the eye to other tissues due to ocular barriers may eventually result in unwanted side effects and toxicity.[109]

2.5. Current delivery technologies for the posterior segment of the eye

Currently, in clinics, medical doctors have the choice between treating patients with intravitreal bolus injections (solutions and suspensions) or drug-loaded implants to treat posterior eye diseases.[111,112] Nevertheless, ophthalmologists consider current drug therapeutic options insufficient regarding effective delivery and reaching sustained therapeutic dose levels to the retina. Intravitreal pharmacokinetics data show relatively rapid ocular clearance of most intraocularly administered drugs. As a consequence of that, drug concentrations in the vitreous is oscillating above and below therapeutic levels in time with multiple bolus injections (Fig. 2).[113–115] Intraocular implants are currently the only delivery vehicles approved by the FDA for sustained release of intravitreally administered (small molecular) drugs to the retina. Monolithic (polymer matrix with homogeneous drug dispersion) and reservoir (drug particles loaded in a core with an outer shell of a certain polymer) type of intraocular implants have been produced, to treat both anterior and posterior segment eye diseases. For the fabrication of these implants, both biodegradable and non-biodegradable polymers have been employed. Biodegradable implants are generally based on poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), or poly(glycolic acid) (PGA), while non-biodegradable implants are mostly made of poly(dimethylsiloxane) (PDMS), poly(vinyl alcohol) (PVA), or poly(ethylene-co-vinyl acetate) (pEVA).[112,116–118] These formulations are used for the controlled release of low molecular-weight drugs, including hydrophobic steroids and hydrophilic drugs (e.g. ganciclovir), to yield therapeutic levels for an extended period of time. The currently FDA approved slow-release reservoir implants for intravitreal administration are summarized in table 2.

Unfortunately, most of these intraocular implants are generally not suitable as matrices for the sustained release of therapeutic proteins. The polymeric matrix's (e.g. EVA, PDMS) used to produce such intraocular implants have a high permeability for a variety of lipophilic drugs due to their hydrophobic characteristics.[111] However, therapeutic proteins are not easily released from these devices due to their relatively large size and hydrophilicity. Furthermore, interactions of the protein with these polymers may adversely affect protein stability.[103] In addition, the majority of these intraocular implants require invasive administration methods to place the devices at the target site, and subsequent surgical procedures are needed to remove non-biodegradable implants. This unmet clinical need triggered research towards innovative drug delivery products such as nanocarriers (NCs),[65,131] encapsulated cell technologies (cells embedded in a matrix that secrete therapeutic proteins)[132,133] and stimuli-responsive delivery systems.[19,134,135] These novel drug delivery systems are currently preclinically as well as clinically studied, particularly for protein/peptide delivery to the retina (Fig. 3). Mandal *et al.* discuss the major advantages and disadvantages of various preclinically/clinically developed implants and stimuli-response delivery systems for therapeutic proteins/peptides to treat ocular diseases.[76] Several products have shown the potential to prolong the residence time of proteins in the eye, thereby overcoming some of the limitations of ocular drug delivery. Biodegradable products are highly favoured because these systems do not need to be removed surgically after treatment, and their release profiles can be engineered to reach therapeutic levels over prolonged periods. (Fig. 2) Currently, many efforts are made by both pharmaceutical companies and academic groups to develop intravitreal implants for the sustained delivery of proteins.[136] Genentech reported the positive phase 2 outcome of a newly developed ranibizumab port delivery system (R-PDS),[137,138], which consists of a refillable permanent silicone-coated implant surgically inserted through a small incision in the sclera and pars plana. The loaded ranibizumab is released into the vitreous cavity by passive diffusion through a porous release control element based on stainless steel or titanium.[139] This implant was able to provide sustained VEGF inhibition during 9 months of clinical observation in neovascular age-related macular degeneration (nAMD) patients. Interestingly, R-PDS gave visual acuity in patients comparable with monthly intravitreal injection of ranibizumab while maintains the same anatomical results.[140] The product has the potential of being the first device for incorporating a therapeutic protein for posterior eye delivery.

Although there is a high demand for ground-breaking drug delivery technologies for ocular treatments, most of the developed and investigated technologies remain at preclinical levels and very few at the early clinical stage (Fig. 3). The slow progress of these technologies is due to challenges in reproducibility, safety, large scale production, long term stability. The design of an effective preclinical/clinical sustained release system that will be able to achieve regulatory approval is imperative to move from "Bench to Bedside."

3. Hydrogels for sustained intravitreal release of therapeutic proteins

3.1. Hydrogels: general features

Hydrogels are three-dimensional networks of synthetic or natural polymer chains crosslinked by physical and/or chemical bonds. Compared to other delivery systems that have been developed for posterior eye diseases such as colloidal nanocarriers (NCs) or polymeric implants, hydrogels offer several compelling advantages. Firstly, hydrogels embrace numerous biomedical and pharmaceutical applications due to their tissue-mimicking properties and desirable soft nature.[141–144] Secondly, hydrogels are able to absorb large amounts of water (up to 99% of their weight) while maintaining their structure due to the presence of the crosslinks between the hydrophilic polymer chains.[145] Therefore, hydrogels can serve as scaffolds that provide structural integrity to tissue constructs,[146–148] and can also serve as adhesives[149,150] or barriers between tissues.[151] Finally, as discussed in this review, hydrogels can be designed to control and/or sustain drug and protein release to tissues due to their network structure of crosslinked polymer chains.[152,153]

Interestingly, the first publication on hydrogels for biomedical applications was in the early 1950s, when Wichterle and Lim reported on crosslinked poly(hydroxyethyl methacrylate) (pHEMA). The aim was to create a new biomaterial for ophthalmic applications, which led later to the first soft contact lens.[154,155] Since this breakthrough, hydrogels

Table 2
FDA approved intraocular implants

Implants	Year of approval	Description	Ref
Yutiq™	2018	Non-biodegradable intravitreal implant using durasert™ delivery technology and loaded with fluocinolone acetonide (0.18mg) for posterior uveitis, engineered to release fluocinolone acetonide over 36 months at an initial rate of 0.25μg/day	[119,120]
Dexycu™	2018	Biodegradable intravitreal implant based on the Virisome® sustained delivery technology for the treatment of postoperative eye inflammation. This injectable implant provides sustained release of dexamethasone (103.4mg/mL in a single-dose vial) over 1-6 months.	[121]
Iluvien®	2014	Non-biodegradable intravitreal implant made of PVA matrix encased in a polyimide tube and loaded with fluocinolone acetonide (0.19mg) for the treatment of DME over 36 months.	[122–124]
Ozurdex®	2009	Biodegradable intravitreal PLGA implant loaded with dexamethasone (0.7mg) for DME and non-infectious uveitis with a six months release profile.	[125–128]
Retisert®	2005	intravitreal non-biodegradable (silicone/PVA) implant loaded with fluocinolone acetonide (0.59mg) to treat chronic non-infectious posterior uveitis used up to 3 years	[127,129]
Vitrasert®	1996	non-biodegradable (PVA/EVA) implant loaded with ganciclovir (4.5mg) for the treatment of viral retinitis, developed to release the drug over a 5 to 8 month period	[23,130]

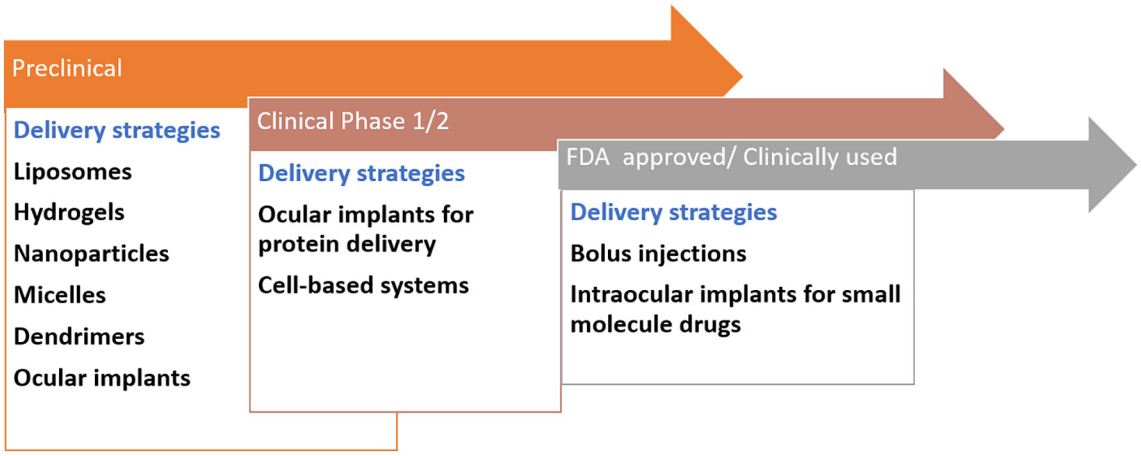

Fig. 3. Schematic representation of currently developed drug delivery strategies to the posterior segment of the eye. Many drug delivery systems are being investigated preclinically, and some technologies have even reached clinical evaluations. However, bolus injection and intraocular implants for small molecule drugs are still the most used therapy to treat posterior eye diseases.

have been of great interest to biomaterial scientists, and a great variety of hydrogels with interesting and tailor-made properties for different applications (drug delivery, wound dressing, tissue engineering, and hygiene products) have been developed until today as recently summarized by Cascone et al.[156] Hydrogels can be classified according to different criteria depending on preparation methods, source, physical properties, biodegradation and nature of crosslinking.[157] Additionally, hydrogels can consist of homopolymers, copolymers, and/or interpenetrating networks with different physical properties[158–160] and can also be designed using both charged and non-charged polymers. The net charge of the network might affect the release kinetics of the loaded drug due to electrostatic interactions between the polymeric matrix and the loaded protein.[161–163] Different types of ocular hydrogels with varying polymer architectures and crosslinking chemistry have been studied preclinically to deliver proteins to the posterior segment of the eye. (Table 3) In this review, we classify intraocular hydrogels based on the origin of the polymers and present how the studied hydrogels sustain the release of therapeutic proteins to treat intraocular diseases. The main advantages and disadvantages of polymer-based hydrogels that are currently evaluated in preclinical studies are presented and discussed.

3.2. Hydrogels under preclinical development for sustained intravitreal delivery of therapeutic proteins

To improve the current strategies of intraocular delivery of pharmaceutically active proteins, suitable delivery technologies should have the following characteristics:

1. Provide controlled and sustained release of therapeutic proteins for at least two to three months, therefore, resulting in reduced administration frequency.
2. Easy to administer (minimally invasive).
3. Safe and compatible with intraocular tissues with no- or very minimal irritation.
4. Do not obstruct the vision after injection and allow patient comfort.
5. Maintain effective local drug concentration with none to very low systemic effects compared to currently used delivery strategies in the clinic.
6. Maintain protein stability and activity during preparation, storage and release.
7. Undergo biodegradation; the degradation process can modulate the release of entrapped proteins.
8. Possess physical crosslinking and/or bioorthogonal crosslinking chemistry.
9. Allow easy manufacturing in terms of sterilization procedures, scaling up and GMP production.

Hydrogels are considered as one of the most promising preclinically ophthalmic biomaterials for the sustained release of bioactive proteins to intraocular tissues. One of the most interesting features of hydrogels is their physicochemical similarities with native tissues because they are generally very soft and have an elastic texture with high water content. Further, mild crosslinking conditions of polymers are presently available for preserving the activity of entrapped biopharmaceuticals such as proteins and peptides in the hydrogels.[152,164] The safety of intraocular hydrogels has been evaluated in different animal models

Table 3
Hydrogels for sustained and controlled release of intraocular proteins.

Hydrogel composition	Crosslinking method	Drug/protein encapsulated	Therapeutic indications	In vitro/vivo release	Animal tested	Toxicity test	Ref
POLY (NIPAAM-CO-DEX-LACTATEHEMA)	UV Photopoly-merization	Insulin	DR	5 months (vivo)	Rat	R28 retinal cells and rat eyes	[171]
PLGA-PEG-PLGA	Thermo-gelation	Bevacizumab	DR, DME, AMD	20% in 14 days (vitro) 6 weeks (Vivo)	Rat		[252]
N, *O*-CARBOXYMETHYL CHITOSAN (CMCS)	CMCS crosslinked with genipin	5FU, bevacizumab	Glaucoma filtration surgery	5FU 8h, 20% Bev. after 53h (in vitro)	Rabbit	Corneal endothelial cells	[177]
PNIPAAMPEG-DA	Free radical polymerization, thermo-gelation	(BSA), IgG, bevacizumab, ranibizumab	AMD, DR, DME	3 Weeks (in vitro)	Rat	HUVECs	[173]
4-ARM PEG-MAL & 4-ARM PEG-SH	Thiol-maleimide reaction	Bevacizumab	CNV AMD, DR, DME	14 days (in vitro)	-	L-929 cells	[241]
4, 8-ARM POLOXAMINE-MALEIMIDE & FURAN	Diels-Alder thermo-gelation	Bevacizumab	AMD, DR, DME	115 days (in vitro)	-	Mouse fibroblast L-929 cells	[285]
MPEG-PCL MICELLES & A-CYCLODEXTRIN(A-CD)	Inclusion complexation	Bevacizumab, dexamethasone Sodium Phosphate	CNV	5 days (65% Bev) and 1day (70% dex) (in vitro)	Rat and albino rabbit	L-929 cells, HCEC	[178]
HA-TZ & PEG-BISNOR-BORNENE	Diels – Alder (tetrazine & norbornene)	Fab1 protein	-	90% in 27 days	-	-	[172]
PEOZ-PCL-PEOZ	Thermo-gelation	Bevacizumab	AMD, DR, DME	40 μg/day for 11 days with 80% in 20 days (in vitro)	-	Human retinal pigment epithelial cells	[284]
HA-VS & DEX-SH	Thiol-ene reaction	Bevacizumab	AMD, DR, DME	6 months (vivo)	Rabbit	ARPE-19 cells	[165]
HA-TYR-INF/PEGDA-PNIPAAM-HA-INF	Enzymatically & Thermo-responsive	Infliximab	ocular inflammation	25% in 9 days (*in vitro*)	-	-	[169]
AUNPS & AGAROSE	Photo-modulation	IgG, BSA, bevacizumab, Ranibizumab, conbercept	Posterior eye diseases	On-Off release	-	RCE, HCEC, HRPE	[170]
ESHU	Thermo-gelation	Bevacizumab	AMD, DR, DME	17 weeks *in vitro* 9 weeks *in vivo*	Rabbit	Primary bovine CE and ARPE-19 cells	[286,314]
DEX-GMA	Radical polymerization	IgG	-	250 days (no dextranase) 5-10 days (0.001–0.03 U/g gel dextranase)	-	-	[174]
HAMC	Thermo-gelation	CNTF	Neuro- protection	7 days	Mouse	-	[153]
SILK BASED HYDROGELS	Physically crosslinked silk fibroin chains	Bevacizumab	AMD, DR, DME	3 months (in vitro & in vivo)	Rabbits	-	[21]
ICNPH (hybrid delivery system)	Thermo-gelation	Insulin (subconjunctival)	DR	2 weeks (in vivo)	Rat		[167]
PEG-PLLA-DA/NIPAAM (hybrid delivery system)	Thermo gel loaded with microspheres	Aflibercept	AMD, CNV	6 months (in vitro)	-	HUVECs	[315]
GLYCOL CHITOSAN AND OXIDIZED ALGINATE	Dynamic covalent Schiff-base linkage	Bevacizumab	AMD, DR, DME	3days	-	-	[223]
PCM-HEMA GEL	Light-activated copolymerization	Bevacizumab (suprachoroidal space)	AMD, DR, DME	20% burst release, ~ 4 months.	Rabbit	ARPE (in vitro), rats (in vivo)	[249]

(rabbit, rat, mouse) by using different techniques. Evaluation of the intraocular pressure after injection, fundus examination by ophthalmoscope imaging, histological analysis and assessment of retinal function (by electroretinogram analyses) have shown that generally speaking hydrogels are safe for intraocular use.[165–167] Furthermore, different cell lines have been used to investigate the cytocompatibility of hydrogels and their building blocks, and generally, no toxic effects have been observed with the used polymers (see Table 3).

To bypass the intraocular barriers that drugs have to pass after, e.g. topical or Iv administration (see section 2.2), drug-loaded hydrogels can be administered by intravitreal injection in a minimally invasive way. Importantly, hydrogels can be administered as "*in-situ*" forming formulations by injection using small gauge needles into the vitreous cavity. After intravitreal injection, the polymeric solution undergoes a sol-to-gel phase transition, entrapping and stabilizing therapeutic proteins in their hydrated network.[157,168] The gelation time, preferably, is in the order of seconds to minutes to limit the rapid and unwanted distribution of proteins and uncrosslinked polymers due to Fickian diffusion in the vitreous body. After gelation, the formed hydrogel functions as a reservoir, providing sustained release of the loaded protein for a prolonged period of time due to the polymer network that restricts protein mobility. Therefore, when properly designed, this reservoir can maintain effective local concentrations of the loaded pharmaceutically active protein in the vitreous and retina. Hydrogels have been successfully used for the controlled release of anti-VEGF (ranibizumab, bevacizumab), anti-TNFα (infliximab), and ciliary neurotrophic factor (CNTF) in the vitreous to protect vision as discussed in details below in section 3.3 and section 3.4. [21,153,165,169,170] Also, insulin has been released from hydrogel systems after subconjunctival injection to treat diabetic retinopathy.[171] Model proteins (BSA, Fab antibody fragment, IgG) have also been extensively used to study the tunability of hydrogel systems for release characteristics and possible unwanted or wanted interactions with hydrogel building blocks.[172–174] Therapeutic proteins have also been loaded together with low molecular weight drugs in hydrogels to obtain synergic effects.[152,175,176] To mention, the anti-metabolic agent 5-fluorouracil (5FU) has been co-released with bevacizumab from *N,O*-carboxymethyl chitosan (CMCS) hydrogels to modulate wound healing and prevent scar formation after glaucoma filtration surgery[177] as discussed in section 3.3.2. In another study, dexamethasone (Dex) was released in combination with bevacizumab (Avastin®) from supramolecular PEG-PCL micelles and α-cyclodextrin (α-CD) hydrogel to treat inflammatory corneal neovascularization in a rat model.[178] The hydrogel was obtained by mixing MPEG-PCL micelles with an aqueous solution of α-CD due to the "host-guest" interaction between MPEG and α-CD.[178,179] It was shown that 70% of the loaded Avastin® was released within 5 days, and 70% of Dex was released in 1 day. Despite the rapid drug release, this Dex/Avastin® hydrogel medication suppressed the corneal neovascularization in the studied rat model.[178]

Proteins loaded into a hydrogel can be released through different mechanisms, including diffusion-controlled and degradation-controlled release.[180–182] Formulation scientists have designed hydrogels capable of releasing proteins intraocularly from a hydrogel network in a predictable and controlled manner by tailoring the crosslink density, which depends on the polymer concentration, molecular weight, polymer architecture, and degree of polymer modification with reactive species.[152,183,184] In ophthalmology and other biomedical applications, biodegradable hydrogels are favored over non-degradable ones. Degradable ocular hydrogels have been designed in the last decades by using both natural and synthetic polymers and combinations thereof, selecting their building blocks as well as the applied crosslinking strategy with particular attention.[152] The degradation rate can be tuned by the crosslink density and the nature of degradable linkers (by chemical and/or enzymatic hydrolysis) in the polymer chains[183] (Fig. 4). Formulation scientists exploit these parameters to design hydrogels with predictable and controlled protein release profiles, as recently described in a review by Chang *et al.*[20]

3.3. Intravitreal hydrogels based on natural polymers as matrices for the controlled release of therapeutic proteins

Natural polymers display multiple advantages and have been used to develop drug delivery systems.[187,188] These advantages are related to the fact that natural polymers often display good cell adhesion properties, and most of them are biodegradable. Specifically, polysaccharide-based hydrogels have often been used for the development of ocular drug delivery systems and formulations. The most frequently used polysaccharides are hyaluronic acid, alginate, semisynthetic chitosan and dextran.[189–194] Although natural polymers display many advantages, they also have several drawbacks related to the possible risk of infections and immunogenicity due to complexity in their purifications from natural sources.[160] However, both natural and synthetic polymers allow tailoring of hydrogel properties by changing the polymer architecture and composition (Fig. 5), initial water content and crosslink density, as well as their degradation mechanism and kinetics which will reflect on their release profiles.

3.3.1. Hyaluronic acid-based hydrogels for protein delivery

Hyaluronic acid (HA) is a negatively charged, naturally occurring polysaccharide with molecular weights up to 10^7 Da, which is abundantly present in the human vitreous body. Importantly, HA can presently be produced via biotechnological routes reducing the risks associated with the use of animal sources for this polymer.[195] HA, also known as hyaluronan, is a glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and *N*-acetylglucosamine. HA-based materials have been investigated for different biomedical [196] and pharmaceutical applications.[191,197–199] Often, the carboxylic acids or primary hydroxyl groups are chemically modified to obtain crosslinking functionalities.[199–202] In the field of ophthalmic protein delivery, a few intraocular HA-based gels have been developed including enzymatically crosslinked HA − tyramine conjugates, vinyl sulfone functionalized hyaluronic acid (HA − VS) crosslinked with thiolated dextran (Dex-SH) using a thiol-ene reaction, and HA-tetrazine crosslinked by catalyst-free inverse-demand Diels − Alder reaction with PEG-bisnorbornene (Table 3). Protein release from HA hydrogels can be sustained up to a few months, depending on the protein net charge and size, hydrogel crosslink density and degradation kinetics. As mentioned, HA is negatively charged, and cationic proteins (pI > 7.4) can be retained in the polymer network by electrostatic interactions.[163] In addition, protein release can also be controlled by enzymatic degradation of HA hydrogels by hyaluronidases,[203] which are present in most human tissues, including the vitreous.[25]

An injectable transparent *in-situ* forming hydrogel based on HA-VS/Dex-SH was formulated for intravitreal delivery of bevacizumab.[165] The controlled release performance of this *in situ* forming hydrogel formulation was evaluated *in vivo* in a rabbit eye model, and the formulation released bevacizumab (pI ~8.3).[204] After vitreous aspiration from the rabbit eye at different time points, the concentration of released protein was measured by using an enzyme-linked immunosorbent assay (ELISA). Specifically, the assay determines the amount of bevacizumab that can still bind to VEGF after being released in the vitreous. Six months after intravitreal injection of the hydrogel formulation (40 μl) into a rabbit eye, it was shown that the gel maintained intravitreal protein concentration above the therapeutic level (> 50 ng/ml).[165] Egbu *et al.* reported the sustained release of infliximab (INF; MW:149.100Da pI:8.25) from two gel systems based on crosslinked HA for the treatment of intraocular inflammation. Tyramine-substituted HA (HA-Tyr, 4.8 kDa) was enzymatically crosslinked in the presence of INF to form a drug-loaded gel (HA-Tyr, INF). A second gel was prepared by the polymerization and crosslinking of NIPAM with poly(ethylene glycol) diacrylate (PEGDA) in the presence of unmodified HA (50 kDa) and INF to form a thermosensitive PEGDA-

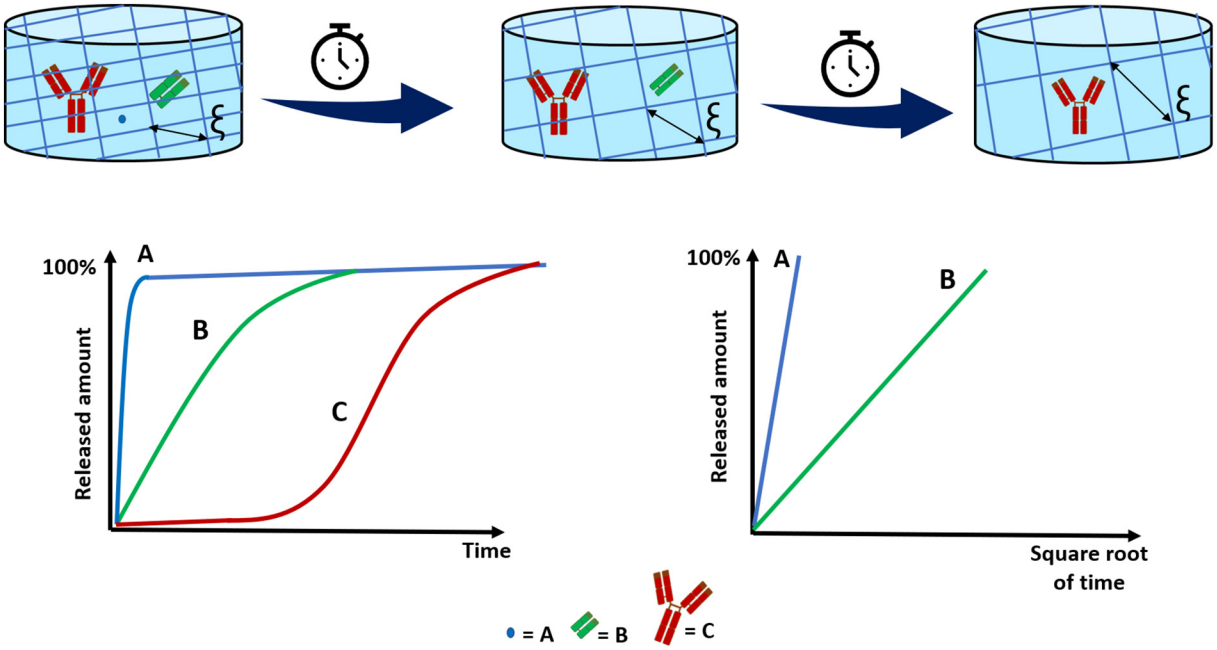

Fig. 4. Schematic representation of the correlation between the release rate of protein therapeutics and the mesh size (ξ) of the hydrogel network, as illustrated by the three hydrogels. The curves A, B and C show different release profiles depending on the drug molecular weight and changes in hydrogel pore size over time due to swelling and degradation. Low molecular weight drugs are generally released from hydrogels by Fickian diffusion because their size is much smaller than the hydrogel mesh-size. On the other hand, the release rate of larger molecules such as pharmaceutical proteins is determined by degradation and swelling rate of the hydrogel when the protein is entrapped in the hydrogels matrix, meaning that their hydrodynamic size is larger than the hydrogel mesh size.[185,186] The blue A curve represents a fast first-order release of a loaded small drug, whereas the green B curve indicates sustained first-order release of a loaded therapeutic with a relatively low molecular weight (e.g. a Fab fragment). The release follows first-order kinetics since the released amount is proportional to the square root of time, meaning that the size of the loaded therapeutics is smaller than the pore size of the hydrogel and that the hydrogel is dimensionally stable during the release time. The red C curve represents delayed-release kinetics of a loaded therapeutic (e.g. a full IgG type antibody). Here the protein is initially entrapped in the pores of the hydrogel and only releases when the crosslink density decreases (and thus pore size increases) over time due to degradation.[174,183]

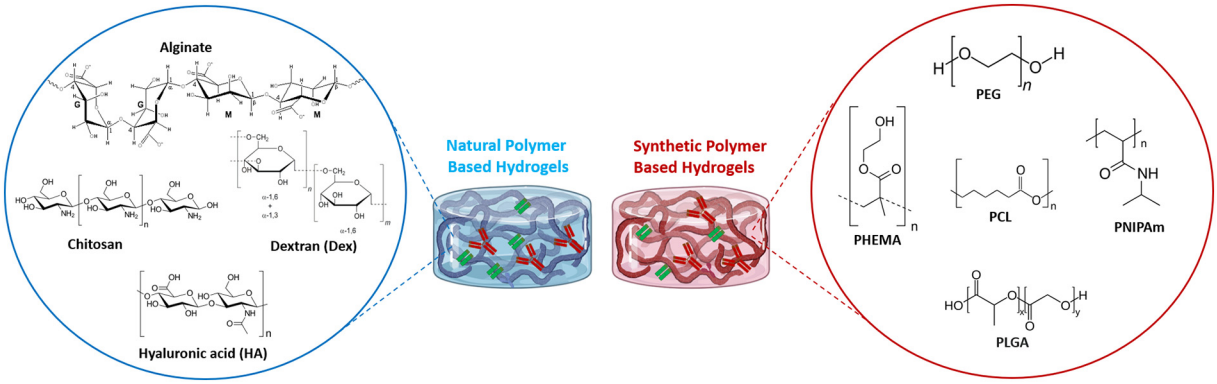

Fig. 5. Schematic representation of hydrogel design for protein delivery to the back of the eye using natural synthetic polymers.

pNIPAAM/HA semi-interpenetrating polymer network. *In vitro* release of INF from these hydrogels was studied by using a two-compartment *in vitro* outflow model of the human eye, called the PK-Eye. This model was previously developed by Awwad *et al.* to estimate the clearance of ocular drugs by the anterior aqueous outflow pathway. [205] The model is made of an anterior and posterior cavity, which are separated by a cellulose membrane. This model previously showed clearance values for the studied drugs (ranibizumab, bevacizumab, triamcinolone acetonide) that were comparable to that observed in humans.[205] Egbu *et al.* showed that the two hydrogel formulations had a controlled release of INF, with the slowest release rate being ~25% of the loaded protein in 9 days.[169] However, important issues among which the *in vitro* hydrogel cytotoxicity, *in vivo* biocompatibility, possible interference of protein functional groups in the crosslinking reaction and protein activity after release from the hydrogels were not addressed in this study. Tetrazine-modified HA and norbornene-modified poly (ethene glycol) were used to obtain an HA-Tz/PEG-bisnorbornene hydrogel for *in-situ* encapsulation of a Fab fragment (Fab1) as a model protein.[172] In this hydrogel system, a catalyst-free inverse-demand Diels – Alder reaction between tetrazine and norbornene groups was used to exploit the bio-orthogonal nature and protein friendly conditions of this crosslinking method (Fig. 6). At room temperature, the gelation of the soluble hydrogel precursors occurred within 3 min.[172] Protein release kinetics showed that the hydrogels formed from 20 and 50mg/mL HA showed a sustained release of Fab1 with a daily release rate > 1μg for 7 and 27 days, respectively (Fig. 6A-B). Importantly, more than 95% of the loaded Fab1 was released from the hydrogels suggesting that the protein was physically entrapped and had not undergone covalent bond formation with the hydrogel matrix.[172] The integrity of the released protein was analyzed by size-exclusion

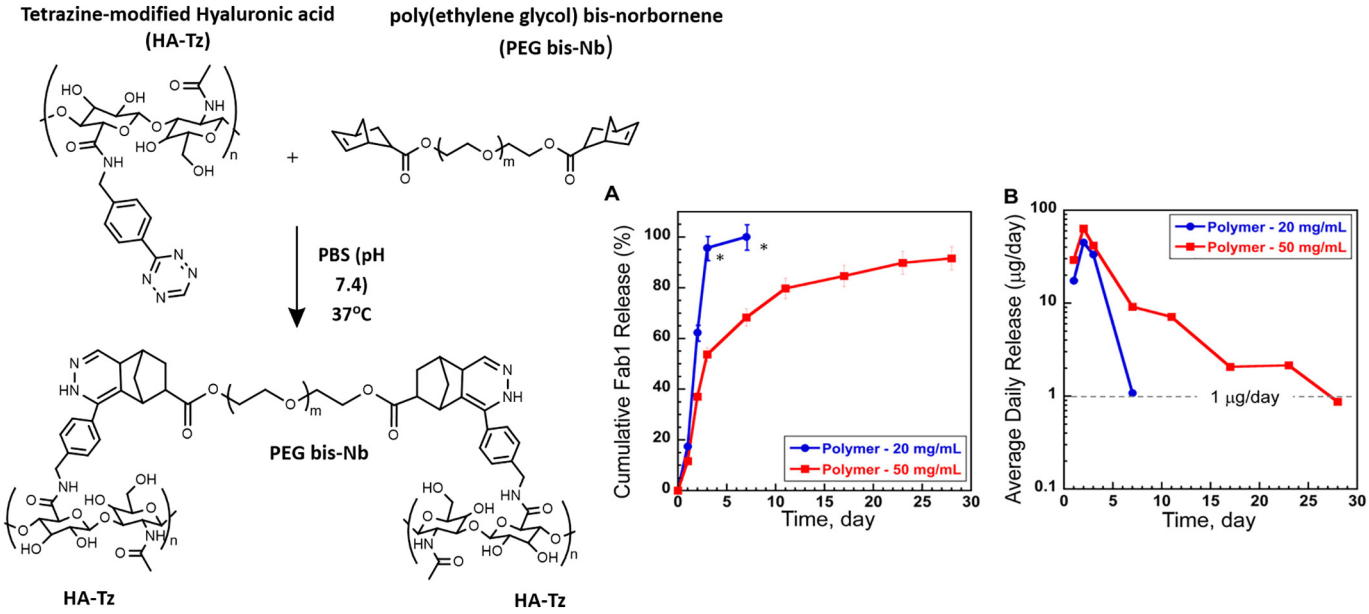

Fig. 6. Schematic representation of the reaction between tetrazine-modified hyaluronic acid and poly(ethylene glycol) bis-norbornene at physiological conditions reported by Famili et al.[172] to form an *in situ* hydrogel network. A) Release of a model Fab1 protein from an *in-situ* formed gel based on HA-TZ, PEG-bisnorbornene hydrogel in PBS at 37°C. Both high (50mg/ml) and low (20mg/ml) starting polymer concentrations resulted in hydrogels, which showed a quantitative release of Fab1, suggesting that the protein has not undergone unwanted reaction with the functional groups present in the hydrogel building blocks. B) Hydrogels formed from 20 and 50mg/mL HA showed a sustained release of the loaded Fab1 protein > 1 μg/day for 7 and 27 days, respectively. Data points represent mean ± standard deviation of n = 3 replicates. Adapted and reprinted with permission from [172] Copyright © 2017 American Chemical Society.

chromatography (SEC), ion-exchange chromatography (IEC) and by liquid chromatography-mass spectrometry (LC-MS). The proteins maintained their structure during their residence time within the hydrogel network at 37°C and did not carry fragments of hydrogel precursors. Although the authors had not investigated this system for intraocular protein delivery, the *in vitro* release data are encouraging and suggest that intraocular release of ranibizumab may be achievable as well with this system. Furthermore, additional *in vitro, in vivo* studies, degradability studies and protein bioactivity after been released from the gel are necessary to ascertain whether this delivery system is indeed safe and suitable for intraocular use.

3.3.2. *Intravitreal hydrogels based on chitosan*

Both natural (alginate, dextran, hyaluronic acid and gelatin) and semi-synthetic polymers (chitosan) have been successfully used to prepare different drug delivery systems for ophthalmological applications.[206]

These polymers are primarily used in the preparation of nano- or micro technology-based drug carriers but also for the development of in situ gels for the delivery of low molecular weight drugs such as corticosteroids, antibiotics, antimetabolites and siRNA mainly for topical therapy.[207–215] In contrast, fewer studies are showing the potential use of these natural polymer based materials for ocular delivery of pharmaceutical proteins.[216–218]

Chitosan is a cationic polysaccharide that is prepared from the natural polymer chitin after partial or complete deacetylation. In ophthalmic applications, chitosan-based nanocarriers and in situ forming gels have been extensively investigated for the delivery of low molecular weight drugs mostly to prolong the release of topically administered drugs.[219–221] There are some promising examples of chitosan-based drug delivery strategies to deliver pharmaceutical proteins, using nano- or micro particles and hydrogels.[222,223] The in vitro sustained release of bevacizumab and ranibizumab from chitosan-PLGA nano- or micro- particles have been studied by Pandit et al.[224] and Elsaid et al.[225] for posterior eye therapy. The studied chitosan PLGA nano- or micro- particles release 15% of loaded bevacizumab within 72h of the study, while ranibizumab showed a biphasic release profile with an initial 20% burst release followed by a second dose after ~135 days. Although chitosan is not soluble in water at a pH of 7.4, chitosan can be dissolved in acidic solution, and because of the charged amino groups, it is able to interact spontaneously with anionic polymers to form a hydrogel network.[226–228] Chitosan hydrogel systems are being studied for the delivery of small drugs such as ciprofloxacin [229], chloramphenicol [230], and latanoprost [231]) for ocular applications.[232–234]

Xu et al. studied the sustained release of bevacizumab from an alginate-chitosan hydrogel.[223] Specifically, the hydrogel network was formed by glycol chitosan crosslinked by oxidized alginate. The hydrogel completely released the protein within three days. This release rate is too fast for long term ocular treatments; therefore, more extended release profiles should be investigated. The in vitro and in vivo toxicity in relevant models were not reported in this study.

Carboxymethyl chitosan is a water-soluble polysaccharide with good biocompatibility used to prepare hydrogels for pharmaceutical applications. Li-Qun Yang *et al.* described O-carboxymethyl chitosan (CMCS) *in-situ* forming hydrogels for intraocular delivery of 5-fluorouracil (5-FU) or bevacizumab using genipin as the water-soluble crosslinker.[177] After subconjunctival injection, the effect of this drug-loaded CMCS hydrogel in modulating wound healing and reducing postoperative IOP values following glaucoma filtration surgery on rabbits was evaluated. As expected, based on the molecular weight of the drugs, it was shown that that bevacizumab was released slower (20% after 53h, by anomalous transport) from the hydrogel than 5FU (8h, by Fickian diffusion). *In vivo* evaluation showed that the CMCS hydrogels were compatible with the cornea and gradually biodegraded in the rabbit's eye. However, the mechanism that governs the degradation was not studied. Drug-loaded CMCS hydrogels effectively delayed subconjunctival scar formation after glaucoma filtration surgery and controlled postoperative IOP.[177] However, it is essential to consider that genipin can readily react with primary amines present in lysine residues of proteins that could potentially alter the structure of the protein and its function.[235] This hydrogel system has shown to

increase the therapeutic efficacy of glaucoma filtration surgery, and in future studies, it would be interesting to investigate their potential use as long term protein release depot for ocular applications.

3.4. Intravitreal hydrogels based on synthetic polymers as matrices for the controlled release of therapeutic proteins

Hydrogels based on synthetic polymers have been used as release systems of bioactives into the eye. Poly(ethylene glycol) (PEG), poly(2-hydroxyethyl methacrylate) (pHEMA), poly(vinyl alcohol) (PVA), polyacrylamide (PAM), poly(D,L-lactide-*co*-glycolide) (PLGA), poly(ε-caprolactone) (PCL) are the most commonly used synthetic polymers to prepare ocular drug delivery systems.[206,236,237] PEG is a frequently used synthetic polymer for biomedical and pharmaceutical applications, also in ophthalmology. It is a water-soluble polymer that can be eliminated from the circulation by the kidneys up to a molecular weight of 50 kDa.[238] Since 1990, several PEG-based pharmaceutical products have been approved by the FDA for clinical use.[236,239,240] Jing Yu *et al.* used an *in-situ* covalently crosslinked PEG hydrogel for the intraocular delivery of Avastin® (bevacizumab).[241] The PEG hydrogels were formed via a thiol-maleimide reaction between 4-arm PEG-Mal and 4-arm PEG-SH at physiological conditions. Variation of polymer concentrations yielded PEG hydrogels with differences in gelation time, the extent of swelling, pore size and mechanical properties. These PEG hydrogels and their leachables were non-toxic to L-929 cells after seven days of cell culture. Importantly, L-929 cells on the surface of this PEG hydrogel showed a different morphology and did not proliferate because of poor cell adhesion. Since the system is aimed to function as a long-term intravitreal reservoir, the fact that cells do not adhere to the hydrogel surface might be beneficial to prevent undesired cell adhesion and proliferation on the hydrogel depot. The PEG hydrogel formulation showed *in vitro* (PBS buffer of pH 7.4 and at 37 °C) release of 25% encapsulated bevacizumab within 1 day and subsequently released 70% of the loaded protein for the subsequent 14 days. Further, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that no fragments with molecular weights smaller than that of the native protein were detected. However, the authors did not show whether the activity of the released protein was retained.[241] Besides the examples given above, many other *in-situ* forming PEG hydrogels have been investigated to deliver both pharmaceutical proteins and low-molecular-weight drugs with promising results for ophthalmic application. [173,241–244] Imperiale et al. summarized the use of synthetic and natural polymers for ophthalmic drug delivery.[206] Synthetic and natural polymers can be engineered to stimuli-responsive hydrogels, meaning that they jellify or release drugs from their network in response to external stimuli (for example, changes in temperature, oxidation, light intensity, pH, ionic strength). These hydrogels are very attractive for ophthalmic applications as they can be formed in situ in the ocular tissues. However, the majority of currently developed stimuli-sensitive hydrogels are focused on the topical delivery of small molecular drugs. [209,245–248] While, in protein delivery, light-activated gels (section 3.4.1) and temperature-sensitive hydrogels (described in section 3.4.2), are the most commonly developed systems for intraocular medications.

3.4.1. Photosensitive synthetic hydrogels:

Light-activated in situ forming gels are an attractive concept for intraocular delivery mainly because light can travel to the posterior segment from the anterior segment, causing the formation of an in situ hydrogel network. Thus, after injection or deposition of hydrogel precursors in the eye, light can be used together with a photoinitiator to photocrosslink the gel in situ. Furthermore, the release of therapeutic proteins from the hydrogel depot can also be photo-modulated, as described in section 3.5. Tyagi et al. reported about a light-activated, in situ forming gel based on polycaprolactone dimethacrylate and hydroxyethyl methacrylate.[249] The gel network was formed in the presence of a photoinitiator 2-dimethoxy-2-phenylacetophenone (DMPA) and 365 nm UV light. After 10 minutes of crosslinking, the gel network was able to sustain the delivery of bevacizumab up to 4 months in vitro after an initial 20% burst release. In vivo studies showed sustained release in the suprachoroidal space for at least 60 days in a rat model. Williams et al. investigated the extended-release of bevacizumab by embedding protein containing microparticles into hydrogel matrices (based on photocurable PEG).[250] The in vitro release profile from this system showed a zero-order release of active bevacizumab over 90 days.

Although photosensitive hydrogels may represent an interesting delivery strategy for ocular application, several drawbacks could limit the clinical translation. The use of photoinitiators that form free radicals upon exposure to light could potentially harm ocular tissues or the encapsulated protein cargo. Long crosslinking times may also be an issue as the use of specific laser light and wavelengths may not be well tolerated by the eye.

3.4.2. Temperature-sensitive synthetic hydrogels

Due to the fast *in-situ* gelation properties, temperature-sensitive hydrogels have been frequently investigated as systems that slowly release therapeutic proteins to the back of the eye. After the administration of the formulation, the change from room to body temperature triggers its gelation. These systems are characterized by a phase transition at a certain temperature in aqueous solutions, which is known as "lower critical solution temperature (LCST) behavior."[251] Thermoresponsive polymers most commonly used in the pharmaceutical field for the formulation of injectable hydrogels are characterized by an LCST between room and body temperature. Formulations based on this type of polymers can be used as injectable release systems since they are liquid at room temperature and jellify rapidly at body temperature after administration. Therapeutics can be easily loaded in these gels by dissolving/dispersing them in the hydrogel precursor solution. After crosslinking, the encapsulated drugs will be released in a controlled manner with kinetics depending amongst others on the size of the therapeutic agent, hydrogel pore size and swelling/degradation. Temperature-sensitive polymers can be either synthetic or natural origin. However, in this review, we focus on synthetic thermosensitive polymers since they have been predominantly used to deliver and release proteins to the retina. The most frequently investigated thermosensitive polymers are poly-(*N*-isopropylacrylamide) (PNIPAM), poloxamers (Pluronics®), poloxamines (Tetronics®), poly(methacrylamide hydroxylpropyl lactate) (pHPMAm-lac), poly(2-ethyl-2-oxazoline)-b-poly(ε-caprolactone)-b-poly(2-ethyl-2-oxazoline) (PEOz-PCL-PEOz), and poly (DL-lactic acid-co-glycolic acid) (PLGA)–polyethylene glycol (PEG)–PLGA (PLGA-PEG-PLGA). These polymers can be derivatized with functional groups that allow chemical crosslinking for further stabilization of the hydrogel network. Hydrogels obtained by covalent crosslinking of thermosensitive polymers swell below the LCST but collapse (shrink) above the LCST.[251] Xie *et al.* reported the use of a thermosensitive PLGA-PEG-PLGA hydrogel for sustained release of Avastin® to treat posterior segment diseases.[252] The sol-gel transition of the system occurred at 26°C, as demonstrated by rheological studies. However, the Avastin® loaded PLGA-PEG-PLGA hydrogel showed lower gelation temperatures of 22-24°C depending on the concentration of the protein. The authors suggest that hydrophobic regions of the protein might interact with hydrophobic polymer segments of PLGA-PEG-PLGA, resulting in a lower gel formation temperature. The authors also mentioned that a similar result was reported by Park *et al.*[253] It should be remarked that these hydrophobic interactions might cause undesired protein unfolding resulting in loss of its biological activity. Furthermore, although a fast thermal transition is observed at these temperatures (22-26°C), the LCST temperature close to room temperature might cause unwanted gelation prior to the intravitreal injection. The authors claimed that the system slowly releases the protein up to 14 days *in vitro*. However, reported data showed a 10-13% release

within 8 hours, followed by marginal release (< 2.5% of the loaded amount of protein) for two weeks.[252] Nevertheless, *in vivo* pharmacokinetic studies (formulations were injected in rat eyes) showed that the PLGA-PEG-PLGA hydrogel could extend the presence of Avastin® in the vitreous humor and retina for 4 weeks (35 ± 14 ng/mL) compared with bolus injection (no Avastin® was detected in the vitreous humor after 4 weeks).[252]

PNIPAm gained substantial attention for biomedical and pharmaceutical applications after being described by Heskins and Guillet as a temperature-dependent phase transition polymer in aqueous solution.[254–256] This phase transition (LCST) occurs at 32°C and is suitable for the design of *in-situ* gelling systems.[257,258] PNIPAM has isopropyl ($CH(CH_3)_2$) and amide (CONH) moieties in its structure. Below the LCST, the polymer is soluble in water because of hydration of the amide bonds, whereas above this temperature, the hydrophobic characteristics of the isopropyl groups dominate the properties in water, resulting in polymer precipitation.[256] The incorporation of hydrophilic monomers in the polymer chains results in an increased LSCT, while more hydrophobic units decrease the LCST. [259] Because of its attractive characteristics, PNIPAm based hydrogels have been studied as drug delivery systems for various applications, including ocular therapy.[173,260–263] However, PNIPAM based hydrogels can be very fragile and unstable, resulting in the relatively fast release of the loaded drugs.[260,264] Furthermore, at high concentrations, both physically and chemically crosslinked PNIPAM-based hydrogels often show a common phenomenon known as syneresis due to the shrinking of gel size with the expulsion of water from the network.[265] This dehydration of the network will mediate the rapid expulsion and release of proteins and/or cause undesired gel-protein interactions.[260] There are different ways to enhance the mechanical stability of PNIPAM hydrogels, as reviewed by Haq *et al.*[264] For instance, an interpenetrating polymer network can be formed by the incorporation of another polymer in the hydrogel matrix to obtain a multicomponent polymeric system.[266–268] PNIPAM can be co-polymerized with different monomers, e.g. acrylic acid (AA),[269] N, N-dimethylacrylamide (DMA)[270] to prevent syneresis.[271,272] Alternatively, PNIPAM copolymerized with functional monomers can be covalently crosslinked with other complementary polymers by, e.g. azide/alkene click chemistry, Michael addition, photopolymerization and thiol-ene reactions to form stable hydrogels. [273–275] It should be mentioned that pNIPAM and its copolymers with hydrophilic and/or hydrophobic monomers are generally not biodegradable, which represents a major limitation for its use as a drug delivery system for the eye. Nevertheless, several PNIPAM copolymers have been engineered to obtain networks that are degradable under physiological conditions. For example, PNIPAM copolymers with hydrolyzable side groups can, in time, result in the formation of soluble polymer chains, which can be eliminated by the kidneys when the molecular weight is sufficiently low.[7,276] Neradovic et al. developed an AB block copolymer of PEG (A block) and a random copolymer of NIPAM and *N*-(2-hydroxypropyl) methacrylamide lactate (HPMAm-lactate) (B block).[277] Interestingly, these block copolymers formed micelles at 37 °C which is above the LCST of the B block, but after hydrolysis of the lactate side groups, the cloud point (CP) increased above 37°C resulting in the dissolution of the polymers. Since then, other copolymers of NIPAM with hydrolytically labile monomers have been developed.[278–282] Vernon *et al.* reported on the hydrolysis-dependent degradation of NIPAM copolymerized with dimethyl-γ-butyrolactone acrylate (DBA). As shown in Fig. 7, after hydrolysis of DBA, the overall polymer becomes more hydrophilic due to the formation of hydroxyl and carboxyl groups that will then promote polymer dissolution.[280,281] The suitability of NIPAM and DBA copolymers for injectable hydrogels for biomedical applications was demonstrated for example by Vo et al. and Boere et al.[282,283]

Imai *et al.* reported on a synthetic subconjunctival implanted hydrogel for sustained release of insulin to rescue retinal neurons from apoptosis in diabetic rats.[171] The hydrogel was synthesized by UV photopolymerization of *N*-isopropylacrylamide and a dextran macromer containing oligolactate-(2-hydroxyethyl methacrylate) units. FITC-insulin was dissolved in *N, N*-dimethylformamide (DMF) together with hydrogel precursors and upon UV polymerization in a Teflon mold, FITC-insulin–loaded hydrogels were obtained. This protein was released from this gel *in vitro* for least five months by a combination of degradation and diffusion, depending on the hydrogel composition.[171] Interestingly, FITC-insulin released from the hydrogel after subconjunctival injection could be detected on the target site (euthanized retina model) by confocal imaging. *In vivo,* biosafety studies showed normal retinal histology after two months post blank hydrogel implantation in Sprague-Dawley rats. The developed poly(NIPAm-Dex-lactate HEMA) hydrogels showed the capacity to release biologically active insulin for 1 month to the retina via subconjunctival implantation with the potential to minimize DR without the risk of hypoglycemia. Although the authors showed that there was no toxicity in the animal model, possible residual of DMF present after preparation and purification of the hydrogel may pose a risk for toxicity in the eye.[171] Derwent *et al.* developed thermoresponsive hydrogels as drug delivery systems for ocular applications.[173] Poly(ethylene glycol) diacrylate (PEG-DA) was polymerized with NIPAM to obtain a non-degradable hydrogel, which was used to encapsulate bovine serum albumin (BSA) and immunoglobulin G (IgG) as model proteins. The crosslinked PNIPAm − PEG-DA hydrogel showed a phase transition at 32°C and was injectable using a 27-30 gauge needle below this temperature. This injection was possible because, at room temperature, the formulation is liquid but rapidly gelated within 1 minute at 37°C. The release kinetics of the loaded proteins could be tuned by varying the ratio of PEG-DA/NIPAM and, therefore, the crosslink density. The different hydrogels showed a rapid release within 48 hours of the loaded proteins, which might be due to the initial volume collapse of the hydrogel in response to temperature. Thereafter, the loaded proteins were released for three weeks. *In vivo* tests confirmed hydrogel formation in the vitreous of a rat model after intravitreal injections of a FITC-labeled hydrogel formulation. A fluorescein angiography image of the FITC-hydrogel showed the localized formation of the gel in vitreous, and no adverse effects on the retina were observed in histological studies.[173] Furthermore, a test on retinal function after the injection of PEG-DA crosslinked PNIPAm hydrogel showed a limited transient effect on retinal function without any long-term adverse effects.[166] However, the clinical translation of this formulation is unlikely because the gel system does not degrade under physiological conditions. Wang *et al.* developed a thermosensitive and biodegradable hydrogel based on a triblock copolymer of poly(2-ethyl-2-oxazoline)-b-poly(caprolactone)-b-poly(2-ethyl-2-oxazoline)(PEOz-PCL-PEOz) which was used as an intraocular controlled release system for bevacizumab (Fig. 8).[284] The aqueous solution of the polymer showed a reversible sol (room temperature) −gel (physiological temperature) phase transition to yield a hydrogel. The protein was loaded in the hydrogel by the dissolution of bevacizumab with the PEOz-PCL-PEOz triblock copolymer at low temperature, and subsequently, the temperature of the obtained solution was increased to 37°C. The formed hydrogel was incubated in balanced salt solution as the standard saline for intraocular use (BSS) at 37 °C, and the released protein was detected by enzyme immunoassay (ELISA). During the first 11 days, a diffusion-controlled release was observed, while from day 12 to 20, the release was controlled by a combination of diffusion and erosion. Overall, 80% of the loaded bevacizumab was released from the hydrogel network in 20 days (Fig. 8A), and the released protein was able to bind to VEGF, demonstrating that the biological activity of the protein was preserved. However, longer-term release profiles should be aimed as currently soluble bevacizumab is typically administered once a month. The hydrogel hydrolytic degradation was conducted at 37°C in BSS and analyzed by scanning electron microscope. Initially, the hydrogel surface morphology was smooth and became more and more porous over time due to hydrogel erosion. After ten days of degradation, pores of 10 − 20 μm in size were

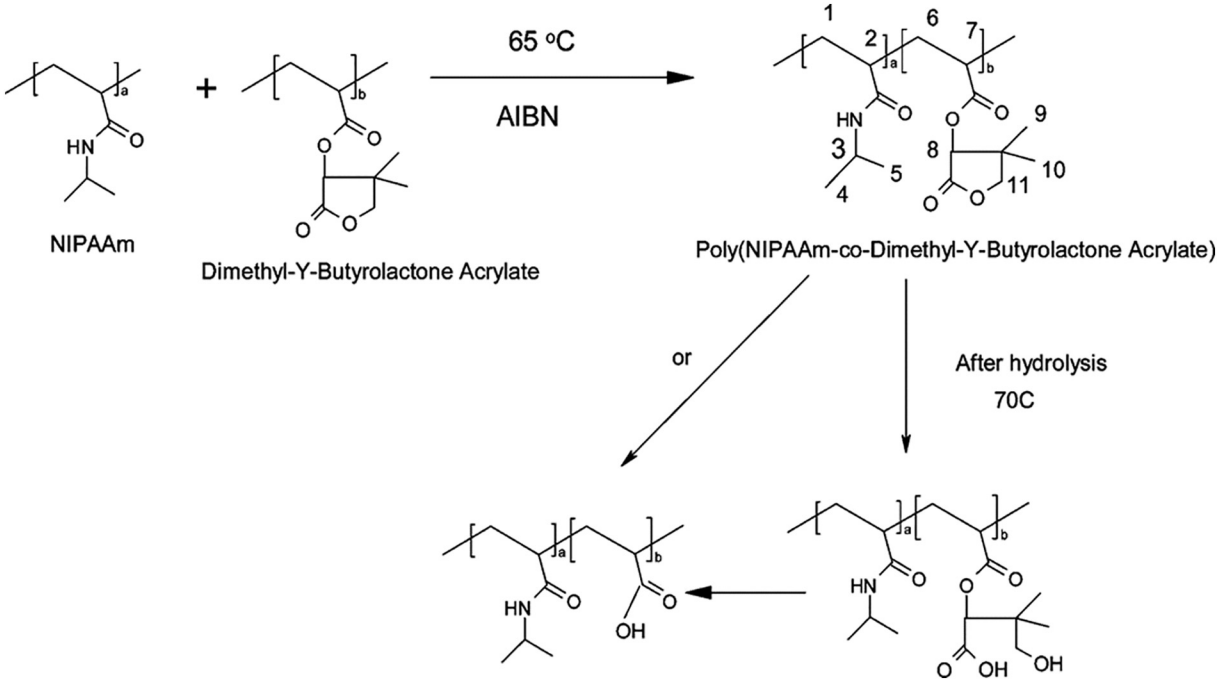

Fig. 7. Synthesis and hydrolysis of poly(NIPAAM-co-dimethyl-γ-butyrolactone). Adapted and reprinted with permission from [280] Copyright © 2007, American Chemical Society.

seen, and channels were observed through which the protein could have been released. *In vivo* studies demonstrated that the hydrogel was formed in a localized position in the vitreous after intravitreal injection. Histomorphology and electrophysiology were studied on rabbit neuroretina, and normal functions were preserved two months after injection and thus showing the safety of this system in the rabbit eye. Gregoritza *et al.* investigated the controlled release of bevacizumab from a degradable thermoresponsive hydrogel crosslinked by Diels-alder chemistry (see Fig. 9).[285] The hydrogel was prepared by reacting maleimide modified four, and eight-armed poloxamines with furyl modified four and eight-armed poloxamines. This thermal gel showed a rapid sol-gel transition at 37°C due to its thermosensitivity, which was stabilized by the Diels-Alder click reaction between the maleimide and furyl groups. The obtained gels released > 90% of the loaded protein over a period of 7, 21 and 115 days, depending on the used ratios between four- and eight-armed polymers at a fixed polymer total concentration. Furthermore, binding studies of the released antibody at 7 and 30 days demonstrated that the structural integrity of the released protein was almost fully preserved. The hydrogel might be beneficial for the treatment of ocular neovascularization.

Rauck *et al.* investigated the *in vivo* release of bevacizumab from poly(ethylene glycol)-poly-(serinol hexamethylene urethane) thermal hydrogels (ESHU) after intravitreal injection in a rabbit eye.[286] Rheological studies on ESHU solution in phosphate-buffered saline showed a phase transition at 32°C with a maximum elastic modulus at 37°C as previously described by Park *et al.*[287] Rauck and co-workers analyzed samples obtained from the anterior chamber paracentesis to quantify the concentrations of released protein using ELISA.[286] Compared to bolus bevacizumab injections, ESHU hydrogels were able to sustain therapeutically active bevacizumab intravitreal concentrations for nine weeks, which is ~4-5 times longer than observed after the injection of the free protein (~2 weeks).[286]

3.5. Hybrid-delivery hydrogel technologies

Besides the dispersion/dissolution of a proteinaceous drug in a hydrogel matrix, sustained release can also be obtained by incorporating drug-loaded polymeric nanoparticles, microparticles, micelles and liposomes in a hydrogel network, which is named hybrid-delivery technology.[134,217,288] As previously discussed in section 2.2.3, drug delivery particles with size < 500nm generally diffuse rapidly in the vitreous and are rapidly cleared from the posterior segment, especially if neutrally or negatively charged. Therefore once loaded in hydrogels, drug-loaded particles are immobile and thus retained at the site of administration. Rong *et al.* recently evaluated the use of insulin-loaded chitosan nanoparticles/PLGA-PEG-PLGA hydrogel (ICNPH) for a neuroprotective effect in a diabetic retinopathy rat model.[167] The primary objective of the study was to provide evidence for its potential clinical application for DR. After subconjunctival injection of ICNPH in diabetic rats, retinal histological and functional changes were evaluated at different time points. The authors showed that the system was able to protect retinal function, reduced cell apoptosis and decreased VEGF expressions compared to controls.[167] Another intraocular hybrid nanoparticles/hydrogel delivery technology was studied by Basuki *et al.* [170], who described the use of polymer-coated gold nanoparticles (AuNPs) loaded in an agarose hydrogel as a depot for intraocular delivery of both model proteins (BSA, IgG) and therapeutic proteins (bevacizumab, ranibizumab, conbercept). The system was engineered to release the proteins to the retina by a visible light-trigger with an "on-off" release mechanism for non-invasive repetitive dosing. The exposure of visible light (400-500 nm, 508 mW/cm^2, 10 min) caused a localized temperature increase of the hydrogel depot (up to 50 °C) due to the conversion of photons to thermal energy by gold nanostructures. As a result, the agarose matrix underwent a reversible reduction of the storage modulus (G') that resulted in the triggered release of the preloaded proteins. This reduction of G' is associated with lower crosslink density, increased swelling and thus larger mesh size of the network. The authors showed the *in vitro* "on-off" release of the loaded proteins from a 2% agarose 0.01% polymer-coated gold nanoparticles (AuNPs) agarose hydrogel (AuNPs hydrogel), and the rates were generally comparable.[170] The system showed photo-thermal modulation after implantation of the depot loaded with FITC-BSA in the anterior chamber of an isolated bovine eye since high intensity of the FITC-BSA was measured in the aqueous humour after light exposure compared to suitable controls. It would be interesting to study the photo-thermal modulation of this system after intravitreal implantation, as released

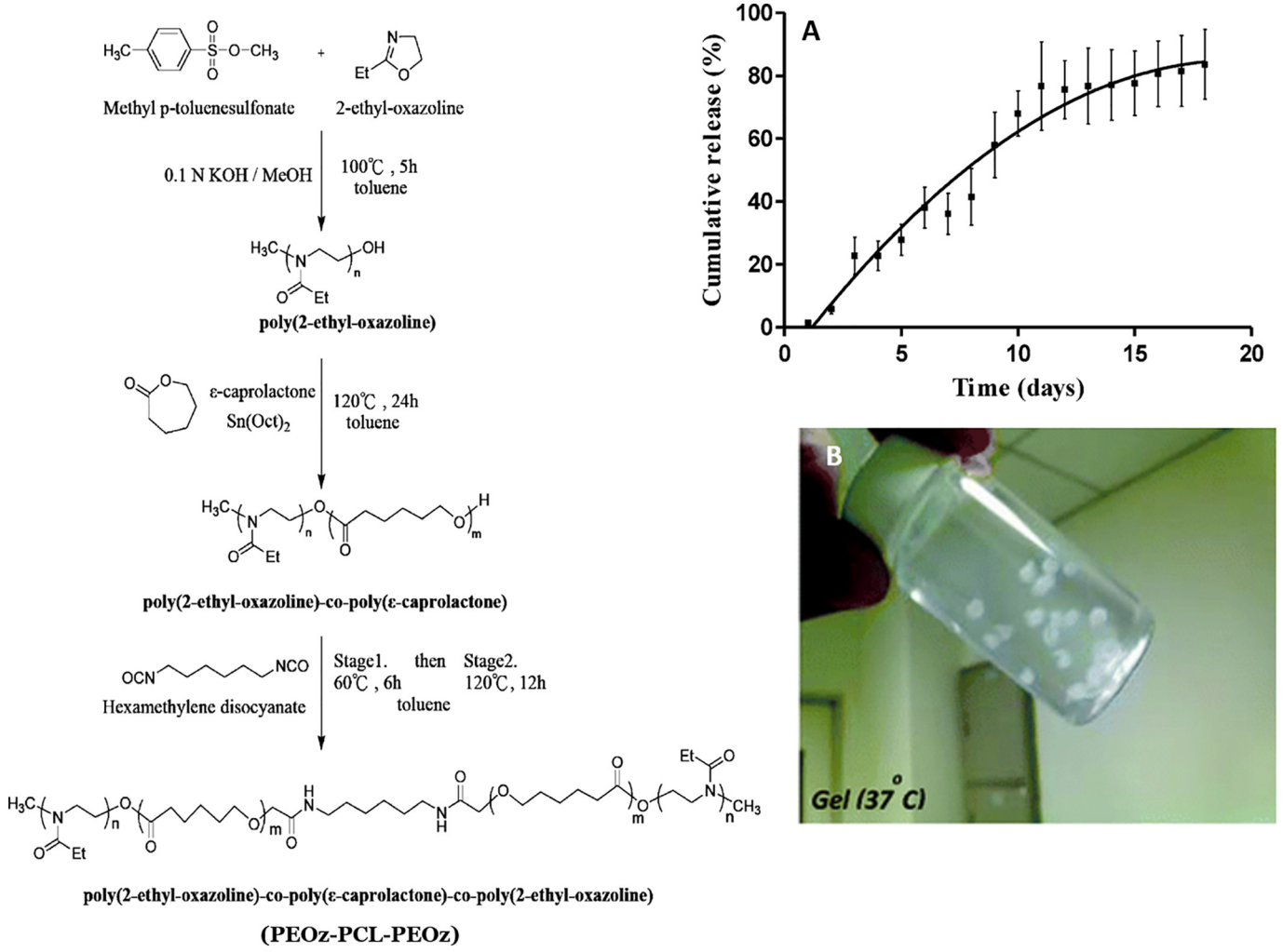

Fig. 8. Schematic representation of the synthesis method of amphiphilic PEOz-PCL-PEOz triblock copolymer. A) In vitro cumulative release of bevacizumab from 20 wt % ECE hydrogel. B) Crosslinked PEOz-PCL-PEOz hydrogel particles at 37°C in a viscous solution (B). Adapted and reprinted with permission from [284] Copyright © 2012, American Chemical Society.

protein from the hydrogel matrix can easily diffuse to the retina. The system may have good potential as an on-demand release of therapeutics for the treatment of various chronic diseases. Further, the authors did neither discuss nor show data regarding the biodegradability and elimination of the delivery system after the complete release of the loaded proteins. No toxicity was shown to L929 (mouse fibroblasts) after 2 minutes of exposure to blue light (400-500 nm) with increasing light intensity at a fixed distance. However, blue light can cause loss of mitochondrial respiratory activity and therefore result in a decrease of retinal cells viability, as shown by Godley *et al.*[289] Therefore, parameters such as blue light exposure time, light intensity and wavelength should be investigated carefully and optimized to prevent undesired toxicity to retinal tissues.

4. Current challenges for ocular protein delivery by hydrogels

Despite the many advantages of hydrogel-based protein delivery systems, several important issues have to be addressed to develop an effective intraocular drug delivery system suitable for clinical translation and application.

4.1. Protein stability

The physical and chemical stability of the encapsulated and released proteins is crucial at the different stages of drug delivery system development. Ideally, the protein should retain its complex secondary and tertiary structure and biological activity during loading, storage and release, because the loss of structural integrity can result in unwanted immunological responses.[104–106,290] Hydrogels have proven to stabilize proteins by limiting their mobility in the network structure. [291–293] However, the development of *in-situ* forming hydrogel formulations with crosslinking mechanisms that do not interfere with different functional groups in proteins (e.g. NH_2 and SH groups) is a major challenge. Some of the developed crosslinking methods make use of thiol-ene, thiol-maleimide, and Diels-Alder reactions, which can result in highly unwanted chemical immobilization of the protein molecules onto the hydrogel network.[294–296] The grafted proteins will only be released upon degradation of the network. Moreover, the released proteins may still carry fragments of the network, which in turn may compromise protein activity and might even induce immunogenicity.[106] Double-bonds, present in compounds carrying vinyl sulfone (VS), (metha)crylate, and maleimide groups are reactive towards thiols and amines present in proteins. Yu *et al.* reported that the formation of covalent bonds between bevacizumab and HA-VS of hydrogel networks results in an incomplete release of the encapsulated protein (less than 20% of the loaded protein was released).[294] The authors suggest the use of De Gennes's blob theoretical model for polymers at the semi-dilute state[297] as guidance to reduce the

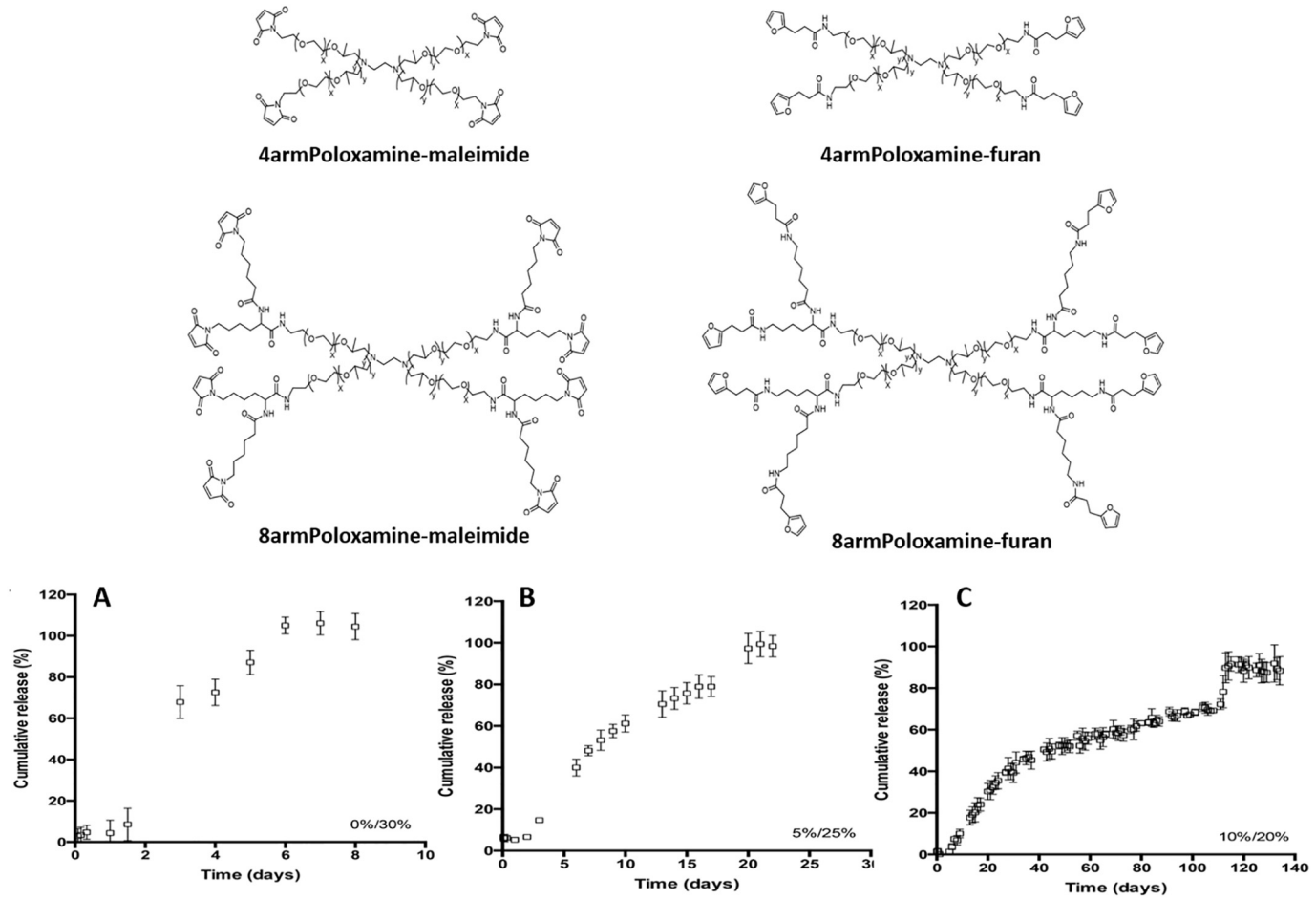

Fig. 9. Schematic representation of poloxamines with multiple maleimide or furyl groups used for hydrogel formation by Diels-Alder reaction. In vitro release of bevacizumab from 0%/30% (A), 5%/25% (B), and 10%/20% (C) 8armPoloxamine to 4armPoloxamine ratios Diels-Alder hydrogels in phosphate buffer, pH 7.4 at 37°C. Adapted and reprinted with permission from [285] Copyright © 2017 American Chemical Society.

chemical grafting of proteins to the vinylsulfone-thiol (VS-SH) hydrogel network.[294] According to this blob model, polymer chains in semi-dilute solutions can interact with each other at the entanglement points. The authors compared such polymers to the *in-situ* crosslinked hydrogel state from the blob perspective in which the VS-SH hydrogel is formed by chemically crosslinking at the entanglement points. They discussed that the covalent grafting of proteins in the hydrogel network via reaction of, e.g. SH in the protein and VS groups of the polymer is due to the mismatch between reactive groups and the entanglement points, leaving many unreacted VS and SH groups. Therefore, protein grafting to the polymer chains was reduced by using a higher degree of modification (DM) and a higher concentration of SH polymers compared to VS polymers. This strategy was used to decrease the number of unreacted VS groups to achieve extended-release profiles of approximately 60% unmodified proteins for 3 months.[294] As previously discussed Gregoritza *et al.[285]* reported that bevacizumab released from a Diels-Alder (furan-maleimide) *in-situ* forming hydrogel had the same affinity for the VEGF receptor as native bevacizumab by using ELISA assay. However, the authors did not analyze whether the released protein contained fragments of the degraded polymer network, which might, as mention before, induce unwanted immune responses. Nevertheless, the potential and unwanted protein reactions with groups present in hydrogel precursors remain one of the major hurdles faced today. Famili *et al.*[172] report the development of bio-orthogonal crosslinking chemistries that enable *in-situ* protein encapsulation without competing reactions with protein functional groups to provide sustained and complete release, as discussed in detail in section 3.3.1.

The pH of the formulation is another critical parameter to consider as both very low and high pH values can be detrimental for protein stability and activity due to irreversible conformational changes. Further, the pH, concentration and type of buffering salt can influence many degradation pathways such as deamidation, disulfide bond formation/exchange, isomerization, and fragmentation.[298–300] The optimum pH value varies largely depending on the type of protein. Generally, a weak acidic buffer is optimal for the storage of most antibodies.[298] For example, some of the presently used antibodies such as adalimumab, ranibizumab, and bevacizumab are formulated in slightly acidic buffers (pH 5.2, 5.5 and 6.2, respectively, below their isoelectric points (~8.3- 8.8)[50,301,302] for ocular treatments.[285]

4.2. Hydrogel turbidity and viscosity

Hydrogels for intraocular delivery are often designed to be thermosensitive hydrogels. However, some of these hydrogels (based on, e.g. PNIPAM, PLGA-PEG-PLGA, PEOz-PCL-PEOz), become turbid at physiological temperature resulting in non-transparent hydrogels. (Fig. 8B) This phenomenon raises the question of whether hydrogels injected in the vitreous body need to be transparent. There are no restrictions on whether a formulation or device injected into the eye has to be transparent or not.[164] Nevertheless, from the patient's viewpoint, transparency would be beneficial, and accordingly, some formulation scientists conclude that transparency is a significant asset, [165,173,217] Nevertheless, the human eye vitreous volume (~4 ml) is relatively large in comparison with the typically injected formulation

volume (50 μl). Therefore, hydrogel turbidity does not seem to represent a major obstacle when the hydrogel is injected and localized outside of the visual path (e.g. behind the iris). Derwent *et al.* reported the localized presence of a thermosensitive FITC-labeled hydrogel in an adult rat model after intravitreal injection. Laser ophthalmoscope images demonstrated that the hydrogel formed locally, and no fluorescence from any other location in the vitreous was observed, and thus the injected hydrogel might not interfere with the vision. Interestingly, after two months, the hydrogel had not moved from the site at which it was initially injected.[173] In addition, it is essential to highlight that opaque FDA approved implants are successfully used in the clinic to treat ocular diseases.[303] However, it is clear that bulky opaque materials/implants are not ideal for intravitreal drug delivery as they could prevent light from reaching the retina. Therefore, since that hydrogels are swellable materials, it is important that opaque gels do not obscure the vision by increasing in size due to swelling and/or degradation after intravitreal implantation. Moreover, small opaque materials should not be injected in the middle of the vitreous body to prevent any discomfort for the patient.

The viscosity of polymeric solutions during injection needs to be considered when developing an injectable *in-situ*, forming hydrogel as the needle diameter used for intraocular delivery is very small (~22-31G).[165,284] Generally, hydrogels developed for ocular delivery have a relatively high polymeric concentration to achieve extended-release profiles. This high concentration, in turn, may render the injectable solution very viscous and, therefore, do not allow the use of small needles. For this reason, research has been focused on hydrogels that have low initial viscosity, which can increase quickly upon a sol-gel transition at the site of injection. The sol-gel change should occur shortly after injection but not instantaneously to prevent gelation and thus clogging in the needle during the injection procedure.

4.3. Sterilization methods, safety issues, and industrial production

The sterilization methods and safety issues are parameters that need to be taken into consideration for the design and development of intraocular hydrogels that aim to receive approval by registration authorities. The sterilization process of polymeric biomaterials is often challenging, as many sterilization methods have shown to affect the properties of biodegradable polymers profoundly.[304] The sterilization processes among which radiation, chemical sterilization and exposure to high temperatures can cause unwanted polymer degradation and structural changes.[304–306] Also, undesirable effects of the applied sterilization method on the loaded proteins may be a significant issue. Therefore, preferred sterilization methods may differ depending on the polymer/hydrogel composition and drug properties. Importantly, aseptic production technologies and sterile filtration represent an attractive method to pharmaceutically produce sterile hydrogel products. Saher *et al.* used thermosensitive (poloxamer 407 and 188) and ion-activated Gelrite polymers (phytagel) to formulate an ocular *in-situ* forming gel. The authors concluded that for this system, sterile filtration via a 0.22-μm membrane filter was a more effective sterilization method than gamma sterilization.[307]

Hydrogel degradability is a crucial issue to consider when developing intraocular hydrogel formulations. As previously discussed, hydrogels can be engineered to degrade by enzymatic hydrolysis, photolytic cleavage, *via* ester hydrolysis or a combination of these mechanisms with different degradation rates, depending on the desired drug release rate and specific application. Hydrogels are generally designed to degrade into non-toxic soluble products, which can be either be metabolized and eliminated from the body. It should be mentioned that, so far, there are very limited studies describing the long term *in vivo* hydrogel degradation in vitreous. However, during material design, it is essential to scrutinize the elimination pathways of molecules used or generated during hydrogel chemical crosslinking or degradation (such as unreacted monomer, initiator, reactive groups and crosslinkers), as such molecules could cause local or systemic toxicity. For example, photo-initiators, such as 2,2-dimethoxy-2-phenyl-acetophenone (mentioned in section 3.4.1), can potentially be cytotoxic and they produce potentially harmful free radicals.[308] Unreacted functional groups (e.g. maleimide, thiols) and polymer fragments can leak out of the hydrogel network during swelling and degradation to the surrounding tissues triggering undesired side reactions. [309] To overcome these issues, research should focus on crosslinking strategies that do not require toxic catalysts and do not generate toxic by-products (e.g. copper-free strain-promoted azide-alkyne cycloaddition[201], inverse-electron demand Diels-alder[310] and Staudinger ligation). [295,311] Ideally, hydrogel network degradation should generate biologically inert molecules or polymer fragments that can be quickly eliminated from the eye and body. (e.g. small and hydrophilic fragments with non-reactive functional groups).

In addition, more information on intravitreal clearance of molecules with different properties (such as size, charge and composition) in relevant animal models that can allow animal-to-human translation are considered valuable for the development of intravitreal dosage forms. In 2015, Del Amo et al. analyzed data found in the literature on intravitreal pharmacokinetics in rabbits and humans and concluded that the rabbit animal model could provide a useful prediction for clinical translation.[312] This study did not evaluate disease state effects, which might complement the understanding of intravitreal pharmacokinetics.

Furthermore, other safety issues that are to be considered when designing injectable *in-situ* forming hydrogels for prolonged intraocular release of biotherapeutics are IOP and hydrogel toxicity. As mentioned, hydrogels are swellable materials, and it is important that the swelling properties of the designed hydrogels do not cause IOP. Therefore, efforts should be made to systematically study the correlation between hydrogel swelling ratios and the risk of IOP, as not much information has been reported until now. The toxicity of hydrogels to ocular tissues has been tested both in animal models (rat, mouse, rabbit) and cell models (e.g. RPE cell; L-929 cells) (Table 3). Overall, no significant toxicity has been observed using these models for many hydrogel formulations. However, efforts should be made to evaluate the cytocompatibility of new polymer building blocks and formulations also on other ocular cells, especially those that are relatively in close proximity to the vitreous cavity (e.g. Ganglion cells, Muller cell, and photoreceptors). Yu *et al.* demonstrated that an HA-VS/Dex-SH hydrogel was relatively safe using *in vitro* cytocompatibility tests on ARPE19 cells and "*in vivo*" compatibility on female New Zealand White rabbit's eye. Further, IOP measurement, ophthalmoscope, full-field electroretinography (ERG), and histological studies showed that the system was compatible with the rabbit's eye after ~ 3 months of evaluation. [165] Other *in vitro* and *in vivo* safety tests of different intraocular hydrogel formulations suggest that many of them are potentially safe for intraocular applications.[177,241,284,287] Nevertheless, long term safety on the human eye has to be investigated.

Furthermore, despite the promising preclinical results and the economic potential of intravitreal hydrogels, issues in industrial scale-up and production need to be adequately tackled in the development stage. The manufacturing process and quality control should all match with the standards of GMP and Pharmacopeia. Unfortunately, during the early preclinical research, these crucial aspects are generally insufficiently studied as a result of lacking information in the literature regarding scalability and process development toward GMP manufacturing of hydrogels for ocular applications. The novelty of the approach might be the primary reason for this gap in knowledge. This challenge is even more prominent due to the complex chemistry required to design in situ forming hydrogel for intravitreal protein delivery. The production of polymeric hydrogels is generally accomplished by polymerization of different monomers (mainly hydrophilic) or by modification or functionalization of existing polymers (natural or synthetic). In some cases, multiple synthetic steps are required first to

produce the monomers or the functional linkers and then proceed with polymerization or functionalization and crosslinking. This long and complicated synthesis route might limit industrial production as it may not be cost-effective. Unfortunately, another critical challenge that needs to be addressed is the issue of batch-to-batch variation seen on the polymer synthesis (e.g. molecular weight, degree of substitution of functional groups) and hydrogel crosslinking density which could then cause unpredictable release profiles. Finally, it is essential to consider that intraocular polymer depot systems can potentially be contaminated with endotoxins during the polymer production or packaging processes. According to FDA guidelines (issued in 2015, "endotoxin testing recommendations for single-use intraocular ophthalmic devices"), the recommended endotoxin limit for ophthalmic devices is ≤ 0.2 EU/mL for the anterior and posterior segment of the eye.[313] Endotoxins cannot be easily removed after contamination, and thus the recommended limit value should be taken into account in starting materials and processes. Natural polymer materials may be more prone to endotoxin contamination when compared to synthetic polymers due to difficulties in optimal purification from natural sources.

5. Conclusion and perspectives

Intraocular drug delivery is an important challenge, considering that retinal diseases are the leading cause of visual impairment worldwide. So far, the treatments with therapeutic proteins still face significant limitations, including frequent intraocular injections, related adverse effects, relatively high clearance and high costs of the treatments. The efficiency of intraocularly injected proteins such as bevacizumab, ranibizumab, aflibercept and infliximab has been shown, but despite the clinical successes, sustaining sufficient concentrations in the target tissue for an extended period is still challenging. This review describes the ideal features of hydrogels that could solve these major limitations. In the last years, tremendous efforts have been made to develop injectable, fast gelling, biocompatible and biodegradable gels. However, challenges regarding initial gel viscosity, hydrogel turbidity, crosslinking strategies, sterilization procedures, storage conditions and long-term intraocular safety need to be addressed by formulation scientists and ophthalmologists to facilitate their clinical translation.

The next-generation of intravitreal hydrogels for improved sustained protein delivery should move towards biorthogonal crosslinking to preserve the stability of the encapsulated bioactive and also improve the safety profile of the used materials. Furthermore, the continuous down-regulation of VEGF in the eye due to the prolonged release of anti-VEGF from the hydrogel depot, might, in the long run, cause undesired side effects. Therefore, the next generation of hydrogels depot ideally should focus on disease state triggered sustained release of anti-VEGF so that the depot will release the drug only when there is an up-regulation of the growth factor. Hydrogel depots that provide co-delivery of low molecular weight drugs (e.g. anti-inflammatory corticosteroids) together with therapeutic proteins for a synergic effect might offer an attractive platform for intraocular therapy. Overall, from a clinical standpoint, injectable hydrogels have shown to be attractive tools for sustained protein delivery to the back of the eye. Their administration is similar to the current clinical procedures for intravitreal injections of anti-VEGF, but less frequent injections are potentially needed. Therefore, hydrogels may represent a useful tool to solve the unmet needs in intraocular protein delivery to the retina in the near future.

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