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Protein conjugates and fusion proteins as ocular therapeutics

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Long-acting delivery (LAD) of ocular therapeutics has potential to improve the standard of care for ocular diseases, such as age-related macular degeneration (AMD), by increasing patient compliance and reducing overall treatment burden on patients and healthcare providers. Although relatively few ocular LAD technologies are currently on the market, a variety of emergent and novel protein engineering-based technologies are being investigated in both the laboratory and clinical settings. Here, we review some of the key indications and treatments that would benefit from the development of LAD for the treatment of ocular diseases and examine the current state of LAD technologies that leverage protein-engineering approaches as well as nascent technologies with potential for future impact.

Introduction

Posterior segment ocular diseases can result in impaired vision with significant impact on quality of life. Chief among these back-of-the-eye disorders of the retina is age-related macular degeneration (AMD). AMD is an inflammatory, progressive, chronic eye disease that can lead to blindness through the degeneration of retinal pigment epithelium (RPE) and photoreceptor cells. AMD affects nearly 30 million patients worldwide, with the number of impacted individuals expected to reach 60 million by 2020 as a result of the world's aging population [1]. AMD impairs normal daily functions, such as reading, driving, and visual engagement with family and friends.

AMD exists in two forms: neovascular (wet) or nonvascular (dry). Wet AMD is characterized by excessive blood vessel growth from the choroid behind the retina with concomitant impact on visual acuity. Vision of patients afflicted with wet AMD can often be improved through treatment with the US Food and Drug Administration (FDA)-approved antivascular endothelial growth factor (VEGF) agents Macugen®, Lucentis®, or Eylea®. By contrast, no treatment options are currently available for dry AMD. Although dry AMD can manifest with less impact on visual acuity, it is more prevalent than wet AMD, representing >85% of total AMD cases, and patients with dry AMD can convert to the wet form. Geographic atrophy, an advanced form of dry AMD, involves irreversible degeneration of the retina with loss of vision; in 2018, ~1.4 million patients in the USA were afflicted with this condition.

Large-molecule therapeutic agents, such as Lucentis® and Eylea®, have had success in treating posterior segment ocular disease. Given physical barriers to retinal access, including the blood–retina barrier, effective treatment with these agents requires intravitreal (IVT) injection (a needle stick in the eye). Protein therapeutics, such as Lucentis®, an anti-VEGF antigen-binding fragment (Fab), exhibit relatively short residence times in the vitreous humor following IVT injection, with elimination half-lives in the range of 6–10 days [2–4]. As a result, frequent administration is required for a durable treatment effect. For example, effective treatment of wet AMD with Lucentis requires administration every 4–8 weeks. Such frequent IVT administration carries a high treatment burden for patients, their caregivers, and physicians. As a result, real-world clinical outcomes often are inferior to those experienced during clinical trials because of poor patient compliance with treatment regimens. Less-frequent dosing of IVT therapeutics would enhance compliance, leading to improved clinical outcomes, and would further reduce the already low risk of injection-associated complications (which include endophthalmitis, hemorrhage,

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retinal detachment, and intraocular hypertension) [5] (Table 1).

Given that a doubling of the dose only provides a one half-life (1 week) increase in exposure, a substantial increase (16-fold) in drug concentration is required to shift the treatment plan from monthly dosing to bimonthly dosing (equivalent to a 32-mg and 8-mg dose of Eylea® and Lucentis®, respectively). Increasing the dose of an IVT drug could improve treatment durability; however, this can be challenging because of several factors. The volume for IVT injection is limited to a maximum of ~100 ml, such that high concentration formulations are required to achieve a higher dose. Protein therapeutics often have poor solubility or stability properties [6], rendering them unsuitable for high concentration formulation. These formulations can be viscous, making injectability poor and presenting difficulties for manufacturing. Although excipients can be added to lower viscosity, the list of excipients acceptable for IVT injection is limited. For example, arginine is often used to lower the viscosity of antibody formulations but cannot be used in the eye because of toxicity related to nitrous oxide production [7]. As a result, strategies to increase the ocular half-life of therapeutics are expected to have a more practical opportunity of achieving significant impact on treatment durability. Alternatively, a medical device could be used to accomplish sustained delivery [8], but might require a surgical procedure for implantation. Gene delivery holds promise for the treatment of ocular diseases, but obtaining high expression of inhibitors at the correct site remains a challenge. In this review, we focus on approaches to reduce dosing frequency for injectable protein therapeutics.

LAD approaches require that the physicochemical properties of the protein component of the therapeutic (particularly the long-term stability under the conditions of the vitreous humor) make it suitable for application in a LAD system. Ranibizumab, for example, exhibits long-term stability in PBS at 37 °C (a condition that mimics the vitreous humor) that would support LAD over a period of at least 6 months (K. Rajagopal, unpublished data, 2019).

Molecules administered by IVT injection are thought to be eliminated by two pathways: (i) an anterior pathway involving passage into the aqueous humor followed by removal via normal aqueous outflow to the lymphatic system; and (ii) a posterior pathway involving migration to the back of the eye, across the retina and the blood–ocular barrier into the choroid blood supply and, ultimately, the systemic circulation. Simulations suggest that, for a typical antibody Fab fragment, ~5–15% is eliminated via the posterior pathway and the remaining 85–95% through the anterior segment [9]. Prolonged retention in the vitreous could increase the chance of exit via the posterior pathway, leading to greater retina exposure, assuming that the technology used to increase vitreous retention does not have a significant impact on retinal permeability.

Hydrodynamic size appears to be a key factor mediating the elimination rate from ocular tissue because vitreal half-life shows a linear dependence on hydrodynamic radius [8,10] (S. Crowell *et al.*, unpublished data, 2019). Increasing the overall size of a molecule in solution through covalent modification offers an attractive strategy for decreased clearance and increased residence time of biologics in the eye. Recycling via a neonatal Fc-receptor (FcRn)-dependent pathway is known to promote the long systemic half-life of both immunoglobulin G (IgG) and serum albumin, but does not appear to have a role in vitreal clearance. This conclusion is consistent with the small difference (1–3 days) in the human vitreous half-life of ranibizumab, a Fab incapable of binding FcRn, and bevacizumab, an FcRn-binding competent IgG. In rabbits, the rank order of vitreal half-life for this Fab and IgG is consistent with their relative sizes and hydrodynamic radii, suggesting that FcRn recycling does not contribute to vitreous half-life [10]. Moreover, albumin levels in vitreous humor (0.5 mg/ml), and vitreous half-life (4.3 days [11]), are considerably smaller than serum values. Finally, ablation of FcRn binding through amino acid changes on the Fc was not shown to have an effect on the vitreal half-life of an IgG in rabbit [12]. Although a protein-engineering approach to increase systemic half-life through increased affinity for FcRn is viable, it would likely be unsuccessful for increased ocular exposure. Thus, strategies using conjugates or fusion proteins are perhaps more likely to result in decreased treatment burden. Here, we review the currently approved biologics for posterior ocular diseases and the various strategies to reduce the dosing frequency.

TABLE 1

Summary of approved ocular biologics

Name	Class	Approval date	Dosing frequency
Macugen®	Aptamer	2004	Every 6 weeks
Lucentis®	Antibody Fab fragment	2006	Every 4 weeks
Eylea®	Fusion protein	2011	Every 8 weeks

Currently approved biologics for posterior eye diseases

Pegaptanib sodium (Macugen®)

Most currently approved biologics for ocular indications are against a common target, VEGF. Pegaptanib sodium has the distinction of being the first aptamer to be successfully developed as a therapeutic agent and the first approved antiangiogenic therapy for the treatment of wAMD [13]. Pegaptanib comprises a single strand of RNA with a 5′-linked 40-kDa polyethylene glycol (PEG) moiety (Fig. 1a) and is administered IVT as 0.3 mg dose every 6 weeks. The mechanism of action of pegaptanib is through the inhibition of $VEGF_{165}$ by binding to its heparin-binding site [14]. It is hypothesized that binding to the heparin-binding site does not efficiently prevent the binding of VEGF165 to its receptor (VEGFR-2)-binding site but the clinical benefits result from the inhibition of amplification of the VEGFR signaling [15]. This could explain the poor clinical efficacy compared with newer agents, which have led to a dramatic reduction in sales for Macugen® [16] (Fig. 2).

Ranibizumab (Lucentis®)

Ranibizumab is a monoclonal Fab that was approved for wAMD by the FDA in 2006 (Fig. 1b). It is administered as a 0.5-mg dose monthly in the form of an IVT injection. Unlike pegaptanib, ranibizumab binds to $VEGF_{165}$ in the receptor-binding region (residues 82–91) and directly inhibits its interaction with VEGFR-2, resulting in the therapeutic benefit [15]. In contrast to pegaptanib, the MARINA and ANCHOR clinical trials demonstrated that ranibizumab not only displayed a stabilizing effect in patients with wet AMD, but also resulted in an improvement in vision in 30% of patients [17].

Aflibercept (Eylea®)

Aflibercept is a recombinant fusion protein comprising the VEGF-binding portions from the human VEGF receptors 1 and 2 that are fused to the Fc portion of human immunoglobulin (Ig) G1. Thus, aflibercept acts as a VEGF trap and sequesters circulating VEGF, in turn preventing its interaction with VEGF-R. It was first approved for wAMD in 2011 and is also administered intravitreally as a 2-mg dose every 8 weeks. The

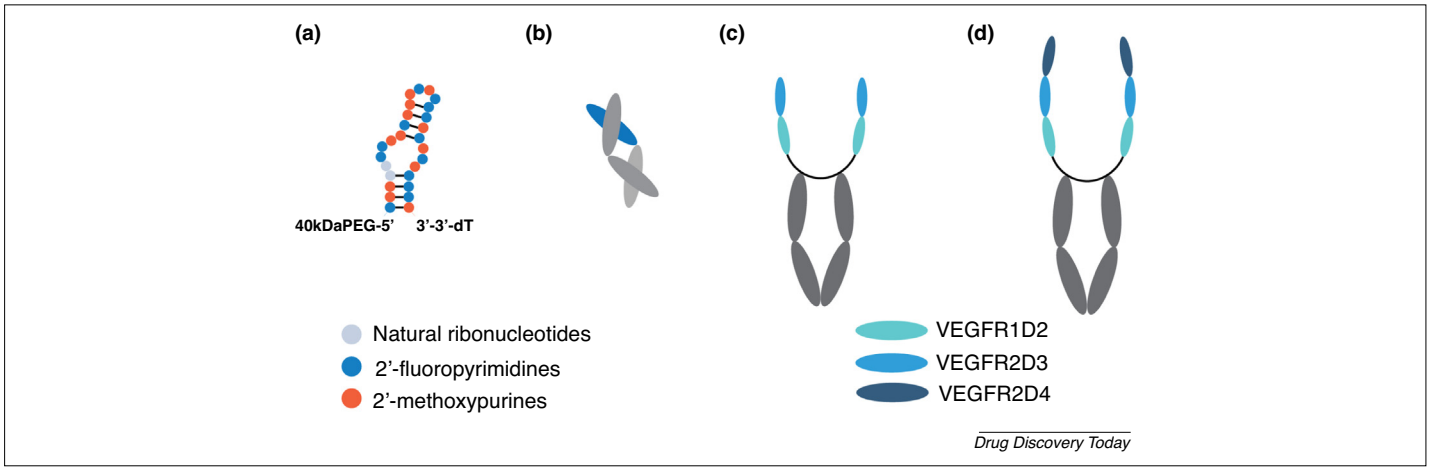

FIGURE 1

Title. **(a)** Macugen, a pegylated aptamer comprising natural and unnatural ribonucleotides. **(b)** Lucentis®, a vascular endothelial growth factor (VEGF)-binding antigen-binding fragment (Fab). **(c)** Eylea®, a fusion protein comprising the VEGF receptor (VEGFR) 1 domain 2 and VEGFR2 domain 3 fused to a Fc fragment. **(d)** Conbercept, a fusion protein similar to Eylea and containing three VEGF receptor domains.

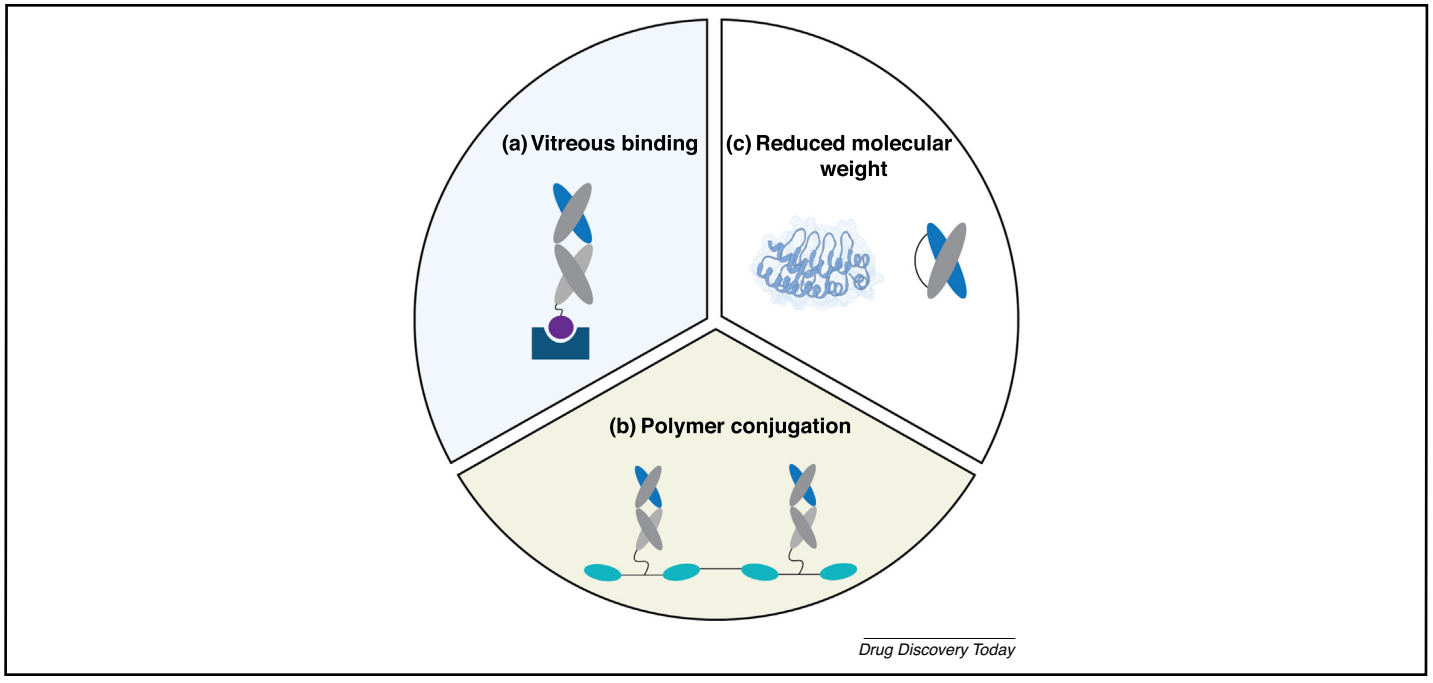

FIGURE 2

Strategies for extending ocular residence time. **(a)** Binding to vitreous humor constituents, such as hyaluronic acid (HA) or albumin; **(b)** Increase in hydrodynamic size through conjugation with polymers, such as polyethylene glycol (PEG), HA, or XTEN; and **(c)** reduced molecular weight and improved potency for higher effective dosing [(i) darpin and (ii) scFv fragment].

reduced dosing frequency of aflibercept compared with ranibizumab is hypothesized to be a result of its wider binding capacity (VEGF-A, VEGF-B, and PlGF) and higher affinity for VEGF compared with ranibizumab. However, in real-world clinical practice, this distinction does not provide any improvement in visual acuity, as shown in a recent retrospective, comparative study by Lotery *et al.*, which examined the 12-month visual acuity outcomes of patients with wAMD being treated with either ranibizumab or aflibercept [18].

Conbercept, a VEGF-trap molecule similar to aflibercept, has been approved in China for wAMD. Similar to aflibercept, conbercept is a fusion protein comprising domain 2 of VEGFR1 and domains 3 and 4 of VEGFR2 combined with the Fc portion on human IgG1 [19]. Given that there are considerable human data with conbercept demonstrating its safety and efficacy, it could be entered into Phase III testing in the USA and European Union, thus reducing time to approval.

Whereas VEGF inhibitors, such as Lucentis® and Eylea®, have shown tremendous benefit in treating wAMD, a study conducted by Holz *et al.* demonstrated that these benefits did not

translate in practice [20]. The authors observed that the patients received only 5.5 injections in the first year and 2.2 injections in the second year (compared with 8–12 injections annually). This reiterates the need for LDAD technologies that would reduce the treatment burden and improve patient outcomes. Currently, there are no approved technologies for posterior eye LDA, although several are under development, as discussed below.

Strategies for reduced dosing frequency

Polymer conjugation

Perhaps the most straightforward method of increasing ocular half-life is to increase the hydrodynamic radius (R_H) of the molecule. As discussed earlier, increasing hydrodynamic size has the effect of extending ocular residence time by slowing ocular efflux, which in turn is primarily governed by the diffusion rate in solution [10]. The only currently marketed product to exploit this approach is Macugen, in which the targeted aptamer (10 kDa molecular weight) is conjugated to a 40-kDa PEG chain. The resulting compound exhibits an ocular half-life of 10 ± 4 days in humans [21]. PEGylation strategies are not limited to large-molecule therapeutics. APL-2 is a PEGylated form of a synthetic cyclic peptide inhibitor of the C3 and C3b components of the complement pathway (Appelis Pharmaceuticals). APL-2 met its primary endpoint in the Phase II FILLY trial for geographic atrophy following monthly administration for 1 year [22].

In addition to PEG conjugation, other polymer-based strategies have been used to increase the R_H of molecules of interest. These include conjugation to long-chain polysaccharides, such as dextran and derivatives thereof [23,24], hydroxyethyl starch (HES) [25], polysialic acid [26,27], and hyaluronic acid (HA) [28,29] through several coupling chemistries. An alternative polymer-based approach that circumvents the need for chemical coupling is recombinant fusion of a protein therapeutic to a long-chain polypeptide for the sole purpose of increasing R_H. Two such technologies are the XTEN platform, which comprises a 83.5-kDa nonrepeating sequence of only Ala, Asp, Gly, Pro, Ser, and Thr residues [30], and PASylation, which comprises only Pro, Ala, and Ser residues [31]. Both of these use the rationale that these sequences of small amino acids should produce an extended polymer chain that is highly soluble, stable in vivo, and non-immunogenic. Although the XTEN technology has not been used in any clinical studies for ocular indications, it is the only of these alternative polymer conjugates to be investigated in an advanced clinical study: the Phase III VELOCITY study for XTENylated somavaratan, a drug used to treat pediatric growth hormone deficiency [32]. Finally, Phase I clinical trials are currently underway for an anti-VEGF monoclonal antibody fused to a novel phosphorylcholine-based biopolymer (KSI-301, Kodiak Sciences Inc.). This approach, similar to the others mentioned here, should increase the ocular residence time via the R_H effect and might also enhance the stability of the conjugate [33]. It remains to be determined whether increased hydrodynamic size has a significant impact on retina exposure, and whether retinal penetration is a general requirement for efficacy in the treatment of posterior segment eye disease.

DARPins

Although clearance from the vitreous is more rapid for smaller molecules, one strategy for decreased dose frequency might be to use therapeutic proteins with smaller molecular weight to increase the effective dose for a given volume. Designed ankyrin repeat proteins (DARPins) are a class of binding proteins comprising a scaffold of multiple ankyrin repeats that can be engineered for binding specificity by conventional combinatorial library approaches [34,35]. Given the small size of the ankyrin-based binding domains, scaffolds of multiple repeats of these binding domains have low molecular weight. For example, a DARPin with seven binding units has a molar mass of only 26 kDa, less than half that of a typical Fab fragment. This feature allows for smaller doses to have increased efficacy. In one study, a DARPin against the wAMD target VEGF was shown to be effective in a rabbit induced vascular leakage model at a dose tenfold less than that of Lucentis [36]. Although such DARPin-based therapeutics would be expected to clear more rapidly than a Fab, the higher density of target-binding domains could offset the differences in clearance. Furthermore, the potential exists to combine multiple specificities on a single DARPin scaffold, to afford greater clinical activity than when using single target inhibitors. Also, conjugation strategies could be used to increase the treatment durability of a DARPin. The most clinically advanced DARPin therapeutic currently in development is abicipar pegol, a PEGylated anti-VEGF DARPin. Results of the SEQUOIA and CEDAR trials showed that abicipar pegol reached its primary endpoint in both 8- and 12-week dosing regimens (www.molecularpartners.com).

Hyaluronic acid binding

The vitreous humor is a matrix comprising type II collagen and HA. Along with albumin, type II collagen is the major protein component in the eye and HA is the major carbohydrate component [37]. One attractive strategy for ocular half-life extension is engineering therapeutic proteins, such as Fabs/IgGs, to be appended with peptide or protein domains that recognize one of these constituents of the vitreous humor with sufficient affinity that the therapeutic agent will be retained in the vitreous. Recent efforts have focused on targeting HA rather than collagen, because the relative abundance of these two biopolymers would theoretically allow for ~20-fold more drug to be bound by HA (340–7000 nmol/eye of disaccharide repeat) than by type II collagen (4–7 nmol/eye of collagen monomer) [38]. Work by Ghosh *et al.* characterized members of the LINK family of natural HA-binding proteins for their suitability in long-acting ocular delivery [38]. In particular, the link domain of tumor necrosis factor (TNF)-associated gene 6 (TSG6), which exhibits an affinity for HA in the 100–1000 nM range [39], was generated as an anti-VEGF Fab fusion and tested in ocular pharmacokinetic (PK) and efficacy models. Both the ocular residence time (by rat eye PK) and the efficacy [evaluated by a monkey choroidal neovascularization (CNV) model and a rabbit retinal leakage model] of the HA-binding Fab improved three to fourfold over the unmodified Fab. Interestingly, removal of an *N*-linked glycosylation site in the TSG6 domain did not significantly impact HA affinity, but resulted in attenuated activity in the efficacy models.

Half-life extension by vitreal binding potentially offers some advantages over other approaches to LAD because an all-protein long-acting therapeutic can be directly manufactured. An all-protein therapeutic offers a higher chance of being delivered as a liquid formulation by simple IVT injection as opposed to implants or devices that require microsurgery or polymer conjugates approaches that require complex manufacturing processes. One potential pitfall of this approach is that binding HA at the posterior vitreous or within the retina might have deleterious biological effects. Although an extensive toxicology analysis of HA binders has not yet been reported, at least one set of investigators observed retinal damage in a rabbit model after administration of TSG6-Fab fusion proteins [40].

Albumin-binding peptides

An alternative approach to vitreal retention involves binding to a soluble protein component (albumin) with the goal of extending half-life by increasing the hydrodynamic size as opposed to tethering to a component of the vitreal matrix. Albumin comprises ~50% of all soluble protein in the vitreous (800 ug/ml or

18 nmol/eye) [41,42]. Fuchs and Igney tested the hypothesis that increasing effective hydrodynamic size of a trispecific Nanobody, BI-X, which has high affinity for human serum albumin (HSA; K_D = 1.4 nM) in addition to binding sites for VEGF and Ang2 [43]. In rat eye homogenates following IVT injection of either BI-X alone or BI-X mixed with a tenfold molar excess of HSA, it was demonstrated that nearly twofold more BI-X remained in the eye after 96 h when mixed with albumin. When tested in a rabbit ocular PK model, BI-X mixed with equimolar HSA exhibited an approximately threefold extension in half-life over BI-X injected alone ($t_{1/2}$ = 10 days versus 3.2 days). The concentration of albumin in the human vitreous could theoretically support a 0.5 mg dose of Nanobody, which could have implications for which targets and/or disease indications such an approach would be suitable. Given that the molecular weights of BI-X and HSA are 40 kDa and 67 kDa, respectively, the binding to albumin increases the effective size by ~2.7-fold. Thus, a similar strategy using a full-length IgG would only increase the effective size by ~0.4-fold and, as such, would be expected to produce a more modest increase in observed ocular half-life.

Reduced molecular weight for higher effective dosing

Whereas most strategies for reduced dosing frequency focus on half-life extension, another, perhaps more direct, approach is to increase the effective concentration of the therapeutic agent in a given dose volume by reducing its molecular size. Strategies using single-chain variable domains (scFv) to bind the therapeutic target while allowing for higher dosing have seen some success. Brolucizumab is an anti-VEGF scFv currently in development by Novartis. In two Phase III trials, brolucizumab achieved its primary endpoints of non-inferiority compared with aflibercept (Eylea) being dosed at 6 mg every 8 or 12 weeks [44]. Given its small molecular size (26 kDa for brolucizumab versus 97 kDa for aflibercept), a ~11-fold higher effective dose was represented by a 6-mg brolucizumab injection compared with the standard 2 mg injection of aflibercept. Any therapeutic of smaller molecular size (such as DARPins) could benefit from this strategy, provided that it is stable and nonviscous at high concentration.

Concluding remarks

The unique features of ocular clearance, including diffusion-driven PK, has fostered creative approaches for the design of biologic therapies for ocular disease. The apparent lack of FcRn-dependent recycling in the eye removes the long half-life of IgG as an advantage, such that other, lower-molecular-weight scaffolds are more strongly considered. Smaller size facilitates delivery of a higher effective molar dose and could provide better distribution to ocular tissues. Conjugates to proteins and peptides of varied polymer composition could increase treatment durability through increased half-life and stability. Alternatively, fusion proteins comprising a therapeutic and a retention domain can have improved residence time in the eye as well as a more straightforward path for manufacture. Overall, these efforts are producing exciting new treatment options that will benefit patients through improved efficacy and/or less burdensome administration.

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