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Preclinical challenges for developing long acting intravitreal medicines

Sahar Awwad[1,2]*, Christin Henein[1,2], Nkiruka Ibeanu,[1] Peng T. Khaw[2] and Steve Brocchini[1,2]

[1] UCL School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, United Kingdom
[2] National Institute for Health Research (NIHR) Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London EC1V 9EL, United Kingdom

*Corresponding author: s.awwad@ucl.ac.uk

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1. Abstract

The majority of blinding conditions arise due to chronic pathologies in the retina. During the last two decades, antibody-based medicines administered by intravitreal injection directly into the back of the eye have revolutionised the treatment of chronic retinal diseases characterised by uncontrolled blood vessel growth, e.g. wet age-related macular degeneration (wAMD), diabetic retinopathy (DR) and choroidal neovascularisation. Although intravitreal injections have become a commonly performed ophthalmic procedure that provides a reproducible dose to maximise drug exposure in the back of the eye, there is a need to minimise the frequency and cumulative number of intravitreal injections. Developing longer-acting intraocular therapies is one key strategy that is being pursued.

Pharmaceutical preclinical development of intraocular medicines is heavily reliant on the use of animal models to determine ocular tolerability, pharmacokinetics, biodistribution and drug stability. Animal eyes are different from human eyes, such as the anatomy, organisation of vitreous macromolecular structure, aqueous outflow and immune response; all which impacts the ability to translate preclinical data into a clinical product. The development of longer acting protein formulations using animals is also limited because animals reject human proteins. Preclinical strategies also do not account for differences in the vitreous due to ageing and whether a vitrectomy has been performed. Intraocular formulations must reside and clear from the vitreous body, so there is a need for the formulation scientist to have knowledge about vitreous structure and physiology to facilitate preclinical development strategies.

Preclinical pharmaceutical development paradigms used to create therapies for other routes of administration (e.g. oral and intravenous) are grounded on the use of preclinical *in vitro* models. Analogous pharmaceutical strategies with appropriately designed *in vitro* models that can account for intraocular mass transfer to estimate pharmacokinetic profiles can be used to develop *in vitro-in vivo* correlations (IVIVCs) to accelerate the preclinical optimisation of long acting intraocular formulations. Data can then inform preclinical *in vivo* and clinical studies. With the now widespread use of intravitreal injections, it has also important early in preclinical studies to ensure there is a viable regulatory pathway for new therapies. Knowledge of these factors will help in the development of long acting intravitreal medicines, which is rapidly evolving into a distinct pharmaceutical discipline.

2. Introduction

Blinding diseases have increased globally due to the rise in population growth and ageing. Diseases in the back of the eye/posterior segment, such as age-related macular degeneration (AMD), diabetic retinopathy (DR) and glaucoma, are leading causes of blindness worldwide.[1,2] Approximately 10 million people over the age of 60 are affected by AMD, which exists in two forms; wet (wAMD) and dry (dAMD). Projected growth of wAMD, glaucoma and diabetic populations are expected to increase rapidly over the next 20 years due to the ageing population.[3] Given the rise in obesity, a major risk factor for diabetes, the rate of growth in the diabetic population in the UK is estimated to be between 1-4% annually.[4–7] The number of people with diabetes in China and India alone is expected to be 200 million in the next 2 decades.[8]

The intravitreal injection of antibody-based drugs that target vascular endothelial growth factor (VEGF) has profoundly revolutionised ophthalmic medical practice for treating conditions confined to the back of the eye.[9] At the turn of the 21st century, intravitreal injections were not routinely administered. There has been near exponential growth[10] of intravitreal injections since 2007 after the introduction of the anti-VEGF drugs, pegaptanib sodium (Macugen®, Bausch & Lomb) in 2004 and ranibizumab (Lucentis®, Genentech) in 2006.[11] Globally, there are now approximately 20 million intravitreal injections annually that target intraocular VEGF.[12] The cause of blindness attributed to wAMD has been reduced by 50-72% since the introduction of anti-VEGF medicines.[13]

The need for repeated intravitreal administration is understandably difficult for patients, while posing potential risks for harmful effects to ocular tissues.[14] While the eye is thought to be broadly a privileged organ, inflammation and immune-mediated reactions can occur when the eye ball is breached by injections.[15] Frequent injections increase the risk of bleeding, infection, non-pathogenic inflammation (e.g. endophthalmitis) and retinal detachment, all which may lead to complete vision loss.[14] Reduced compliance after the first year of treatment has also become evident,[16–18] especially for patients that have not previously been part of a clinical trial.[19,20] There have been efforts to use antibodies systemically to treat ocular inflammatory conditions such as uveitis,[21] however, systemic administration requires high doses for most drugs to be present intraocularly, resulting in the exposure of non-target tissues and dose limiting side effects.[2] Systemically administered drugs, such as tablets, must overcome the blood-retina barrier (BRB), and this usually results in low intraocular drug concentrations, due to an inability to penetrate the BRB.

As important as intravitreally injected anti-VEGF therapies have become, with the ageing population there are also provision and economic burdens on healthcare providers that would be considerably eased if the cumulative number of intravitreal injections could decrease over the treatment period. Several anti-VEGF intravitreal injection schedule regimens have been described to try to decrease the cumulative number of injections over time.[16] Combination regimens have also been described that could comprise a small number of initial loading intravitreal injections of an anti-VEGF medicine followed by the dosing of another anti-VEGF drug topically with eyedrops or with tablets by oral route. This strategy would be based on maintaining a clinically beneficial low intraocular level of VEGF to reduce the number of maintenance intravitreal injections over time.[22] Low, reproducible vitreal dosing and systemic toxicities are potential limitations for these strategies.

The development of longer acting intraocular therapies is another important strategy to reduce the number of intravitreal injections needed to treat chronic blinding conditions. Compared to the situation at the turn of the 21st century when there were no effective anti-VEGF treatments, the regulatory pathway now is much more challenging as any new anti-VEGF therapy must demonstrate at least non-inferiority with current medicines. Increasing the duration of action of existing anti-VEGF actives as they come off-patent is therefore a worthwhile strategy, although new actives are being developed.

Of paramount importance is the need to develop dosage forms that avoid intraocular toxicity such as inflammation and tissue damage.[15] The complexity of ocular anatomy and the physiology of the retina (e.g. the retinal pigment epithelium, RPE),[23] the nature of intraocular mass transfer[24] and the overriding need to maintain ocular tolerability[3] present challenges not present with other routes for drug administration. Many of the challenges are uniquely associated with the eye as they relate to determining intraocular pharmacokinetics and maintenance of drug stability during the development of intraocular formulations that cannot suitably be translated to the clinic using animals alone.

The clinical use of anti-VEGF therapies has raced ahead of the preclinical pharmaceutical development sciences used to develop dosage forms for other routes of drug administration. Existing pharmaceutical preclinical *in vitro* models are widely used to aid in the development of dosage forms destined for other routes of administration such as oral, pulmonary and subcutaneous.[25–28] Many of these *in vitro* models are described in national pharmacopeia. Preclinical *in vitro* models generally approximate one or two elements of a biological system of interest to determine relevant physicochemical and materials factors that can be correlated to optimise

formulation candidates. Simulated biological fluids are also often used with some models. Pharmaceutical preclinical models are frequently used as a basis to develop *in vitro–in vivo* correlations (IVIVCs) and extrapolations (IVIVEs).[29–33] Some of the same pharmaceutical models (e.g. dissolution) are also used to ensure batch-to-batch quality control (QC) during manufacturing.[34,35]

This review describes the challenges for determining intraocular pharmacokinetics during preclinical studies. Optimising intraocular pharmacokinetics relies on the fact that intravitreally injected formulations will reside and clear from the vitreous body, so there is a need for the formulation scientist to have knowledge about vitreous structure and intraocular mass transfer, which are also described in this review.

3. Clinical need for longer acting therapies

Intravitreally administered antibody-based medicines targeting VEGF[16] that are established in the clinic are aflibercept (Eylea®, Regeneron Pharmaceuticals) and ranibizumab (licensed) and bevacizumab (Avastin®, Genentech, unlicensed). Ranibizumab is a monoclonal antibody fragment (Fab) that binds to $VEGF_{165}$ to block it from interacting with the VEGF receptor 2 (VEGFR-2). Ranibizumab displays better clinical efficacy than pegaptanib sodium, which binds to the heparin-binding site on $VEGF_{165}$. Aflibercept is an antibody fragment crystallizable region (Fc) fusion protein that was registered for intravitreal administration in 2011. Aflibercept is given at approximately 2 times higher molar dose compared to ranibizumab. It binds to two variants of VEGF and has been reported to have a higher affinity than ranibizumab. Aflibercept is administered approximately every 8 weeks, whereas ranibizumab is administered approximately every 4 weeks.[16]

These antibody-based medicines are large molecular weight drugs that diffuse slowly through the viscous vitreous body because of their size to clear via the anterior chamber of the eye.[36,37] Smaller, lower molecular weight molecules (e.g. steroids) diffuse much more rapidly through the vitreous body displaying clearance times of hours to days rather than months. The introduction of antibody medicines with monthly or every other monthly dosing was instrumental in more widely establishing intravitreal injections as an increasingly used route of administration. However, with the chronic nature of many retinal diseases in our ageing population, it has become clear that there remains a need to further decrease the frequency needed for dosing by intravitreal injections.

There does not appear to be any difference in visual improvement between aflibercept and ranibizumab after a 12 month study of visual acuity.[38] A supplementary

Biologics License Application (BLA) has recently been granted for aflibercept for a three-month dosing interval based on data from VIEW1 and VIEW2 trials.[39] In addition to wAMD, these medicines are used to treat other conditions such as retinal vein occlusion (RVO), diabetic macular edema (DME), and diabetic retinopathy. Conbercept is another Fc fusion protein and has been approved in China and may enter phase III evaluation in Europe/USA.[40] The monoclonal antibody, bevacizumab, binds to VEGF and is a widely used, unlicensed formulation to treat wAMD. Bevacizumab has demonstrated efficacy in comparison with ranibizumab for intravitreal use from the IVAN and CATT trials.[41] Ranibizumab and aflibercept are more costly per intravitreal dose compared to bevacizumab.[42–44]

Brolucizumab (Beovu®, Novartis) is a long acting protein therapeutic that has recently received Food and Drug Administration (FDA) approval in the US for the treatment of wAMD.[45] Other longer acting protein therapies that are in clinical trials include abicipar pegol (Allergan) and ForSight VISION4's port delivery system (PDS) for ranibizumab (Genentech).[22,46] Phase IIII studies (HAWK and HARRIER) showed 12 week treatment interval with brolucizumab, a humanised single chain variable antibody fragment of 28 kDa. A 6.0 mg intravitreally injected dose of brolucizumab has been shown to be non-inferior in the mean change in best-corrected visual acuity from baseline to an 8 week treatment interval with aflibercept for wAMD.[47,48] Brolucizumab has recently been registered for clinical use and displays a small number of adverse reactions,[49,50] which are gaining attention to ensure the incidence is comparable to other antibody based medicines.[51] Abicipar pegol is a PEGylated conjugate of a type of protein known as a Designed Ankyrin Repeat Protein (DARPin).[22] The CEDAR and SEQUOIA trials showed abicipar pegol at 8 or 12 week dosing to be non-inferior to Lucentis® 4 week dosing for wAMD.[52] The PDS allows a continuous delivery of ranibizumab to the vitreous[53] from a non-biodegradable system[54] and is currently in Phase III clinical trials.[55] Phase I studies showed patient tolerance to the PDS including implant functionality and change from baseline in best-corrected visual acuity (BCVA).[53] Phase II LADDER studies concluded that patients did not require a refill of ranibizumab for 6 months.[53]

Low molecular weight drugs are more amendable than therapeutic proteins to be formulated into implants that can display prolonged action. Steroids are poorly soluble and inhibit inflammation, which facilitates their development into long acting ophthalmic implants. Long-acting steroid implants that are clinically available include Ozurdex® (Allergan) and Iluvien® (Alimera Sciences Inc.).[23] Ozurdex® (dexamethasone in poly(lactic-co-glycolic acid) (PLGA)) has been used to treat macular edema secondary to diabetic maculopathy, uveitis and RVO with peak

effectiveness at 3 months.[56,57] Previous trials of intravitreal corticosteroids to treat DME have shown that levels of inflammatory cytokines are reduced, therefore, corticosteroids represent an alternative therapeutic option for patients with chronic DME. Iluvien® (fluocinolone acetonide in polyimide) is a slow-release and non-bioerodible intravitreal implant that releases fluocinolone acetonide at a concentration of approximately 0.2 µg/day into the vitreous up to 36 months.[58,59]

Formulation strategies reported in literature to extend the duration of action of drug therapies include microparticles and microspheres,[60,61] hydrogels,[61–71] protein conjugation with poly(ethylene glycol) (PEG),[54,64,72,73] implants,[74,75] liposomes[76] and affinity-based drug delivery systems (e.g. targeting ocular tissues).[77–80] However, considerable care is required for many of these strategies due to the sensitive nature of protein therapeutics and the need to ensure there is no ocular toxicity.[15]

Therapeutic proteins are much different from low molecular weight drugs such as steroids. It is much more difficult to develop particulate depot formulations of protein therapeutics because of its propensity to aggregate. Stress factors, such as changes in temperature and pH, mechanical and freeze/thaw stress, can lead to protein aggregation, conformational destabilisation or protein unfolding.[81,82] Humanised proteins can also cause an immune response in some humans. Humanised and fully human monoclonal antibodies, and other therapeutic proteins are less immunogenic in human beings than are non-human proteins (e.g. murine antibodies and non-endogenous protein scaffolds). Repeated Eylea® injections in wAMD, RVO and DME resulted in ADA production in a few patients; however, there were no differences in efficacy or safety between patients with or without immunoreactivity.[83] The potential for immunogenicity with Eylea® is low as the molecule contains only human sequences.[84] It is crucial to evaluate the clinical consequences of ADAs in terms of efficacy and patient safety.[85,86]

The avoidance of ocular toxicity for any formulation is paramount. Ocular tolerability of formulations for the eye raises considerable concerns about the influence of intraocular formulations on ocular inflammatory and immune responses. Tolerability[87,88] is an important factor to avoid complications, such as a rise in intraocular pressure (IOP), ocular inflammation, retinal detachment and endophthalmitis, which can occur following intravitreal anti-VEGF injections.[89] Outbreaks of endophthalmitis (a serious complication resulting in blindness) in 2011 and 2012 were reported from the use of bevacizumab that was compounded for intravitreal injection.[90,91] A cohort study in the US showed that compounded bevacizumab did not alter the risk of endophthalmitis compared to ranibizumab,[92,93] but it is clear that bevacizumab compounding is not regulated. Laws and policy

regarding compounding varies between countries (outside of the US and EU); which raises considerable concerns of about potential sterility. Sterile endophthalmitis has also been observed on rare occasions with the use of ranibizumab and aflibercept.[94] Part of these reactions may possibly be due to the lubricants in the syringes; stressing the importance of ensuring all components of the drug delivery are carefully controlled. However, it is now better recognised that many aspects of ocular tolerability can determined during preclinical development.[15]

4. Relevant intraocular differences between humans and animals

4.1. Anatomical and physiological differences between animal models

Animal models are widely used during preclinical drug development to estimate efficacy, pharmacodynamics, pharmacokinetics and biodistribution properties.[95] A range of opinions exist about the suitability of animals for determining ocular pharmacokinetics and biodistribution.[96,97] Problems include difference in time for tissue analysis and cross-contamination of ocular tissue after euthanasia.[98] Different routes of drug administration can affect the ocular and systemic pharmacokinetics including differences in drug distribution amongst dead, anaesthetised, and conscious animal tissue.[96,98]

Rabbits are most often used for ocular pharmacokinetics studies[3,97,99] although other animals are also used, e.g. rodents and minipigs.[100] Non-human primates are generally only used during late preclinical studies. There are differences between each of the animal models[96,101–103] and along with differences with the human eye that should be considered[97] e.g. anatomical, vitreous volumes, vitreous diffusional pathlengths and aqueous outflow (**Table 1**). Differences in vitreous diffusional path length and volume between species can result in inaccurate data translation to a human.[96,104] For example, the half-life of ranibizumab is about 2.8-2.9 days[105,106] and 2.6[103] days in rabbit (~1.0-1.5 mL vitreous) and monkey (~3.0-4.0 mL) eyes respectively. Though monkey eyes have similar vitreous volume (~4.0-4.5 mL) and viscosity to human eyes, the half-life of ranibizumab is 7.2-9.0[107,108] days in human vitreous (**Table 2**). Similarly, the half-life of bevacizumab is 2.8 days[109] in monkey eyes and between 6.7-11.7 days[110–112] in human eyes. **Table 2** highlights the differences in drug pharmacokinetic profiles of some common intravitreal medicines i.e. aflibercept, ranibizumab, bevacizumab and triamcinolone acetonide in different animal models. Intraocular half-lives of intravitreal medicines can further be reduced because of vitrectomy or a medical condition such as retinal detachment.[113] Efforts have been made to understand quantitative pharmacokinetics in rabbits[97] and rodents such as mice[114] to aid in animal to human translation.

Table 1. Differences in vitreous volume and aqueous outflow between species.

Species	Vitreous volume (mL)	Aqueous outflow (µL/min)
Human	4.0-4.5	2.5-3.0[115] 2.8 (20-30 years)[116] 2.4 (>60 years)[116]
Cat	2.4-2.7	5.0-5.9[117,118]
Dog	1.7	5.2[119]
Horse	26.15	N/A[119]
Monkey	3.0-4.0	2.8[120]
Mouse	5.6×10^{-5}	0.18[121]
Pig	3.5	3.7[122]
Rabbit	1.0-1.5	2.0[123]
Rat	$1.3\text{-}5.4 \times 10^{-2}$	0.35[124]

Aqueous outflow from the ciliary body near the lens is the main cause of mass transfer in the eye.[115,116,125–127] Aqueous outflow nourishes and removes metabolites from the avascular lens and cornea. The majority of aqueous flow passes the anterior hyaloid membrane and flows into the anterior chamber to exit the eye via trabecular and uveoscleral pathways. Upon intravitreal injection, therapeutic proteins such as antibody-based drugs will diffuse throughout the vitreous. Therapeutic proteins are large molecular weight and charged molecules, which are not permeable into cell membranes, so they will not be taken up by the retina and cleared by retinal-choroid-sclera (RCS) pathway.[96,128–130] Instead proteins will clear anteriorly via the aqueous outflow pathway.[105,112,131–133] Intraocular clearance of permeable low molecular weight drugs will occur both pathways, anteriorly via aqueous outflow and by permeation of the RCS. The wide surface area of retina for elimination dominates the anterior bottleneck between the lens and the ciliary body.[104]

A concentration gradient from the front (i.e. aqueous humour) into the back (i.e. vitreous humour) will be developed for drugs that diffuse into the posterior segment.[134] The concentration gradient is shallow and rapidly reversed as the drug concentration in the aqueous humour falls.[134] The diffusional drive is inversely proportional to the square root of time. Therefore, small concentrations in the posterior segment will be present if a depot is placed in the anterior globe.[96] The short diffusional path length in rabbits will reduce the half-life by 1.7× and 2.0× for posterior and anterior clearance respectively when compared to humans.[96]

Table 2. Differences in pharmacokinetic profiles of common intravitreal medicines between species

Drug	*In vivo* model	Dose (mg)	Half-life (days)
Large molecular weight drugs (e.g. proteins)			
Aflibercept	Human	2.0	11.00[135]
	Monkey	2.0	2.20[136]
	Monkey*	2.0	1.50[136]
	Monkey	2.0	2.44[137]
	Rabbit	0.3	3.63[138]
	Rabbit	1.2	3.92[139]
	Rabbit	2.0	4.58[140]
Bevacizumab	Human	1.5	7.85[110]
	Human	3.0	11.69[110]
	Human	1.25	6.70[111]
	Human	1.5	9.82[112]
	Monkey	1.25	2.80[109]
	Rabbit	1.25	4.32[105]
	Rabbit	1.25	~5.7 (0.82 weeks)[141]
	Rabbit	1.25	6.61[142]
	Rabbit	1.25	7.06[143]
	Rabbit*	1.25	6.99[143]
Ranibizumab	Human	0.5	7.19[108]
	Human	0.5	9.00[107]
	Monkey	0.5	2.63[103]
	Monkey	2.0	3.95[103]
	Rabbit	0.5	2.88[105]
	Rabbit	0.625	2.90[106]
Small molecular weight drugs (e.g. steroids)			
Triamcinolone acetonide	Human	4.0	15.4[144]
	Human	4.0	18.6[145]
	Human*	4.0	3.2[145]
	Human	20.0	29.6[146]
	Rabbit	0.3	2.89[147]
	Rabbit*	0.3	1.57[147]

Note: * after vitrectomy

4.2. Vitreous composition and structure

The vitreous is transparent and is located between the lens and the retina with a weight of approximately 4.0 g (average adult human vitreous). Other properties include a pH range of 7.0- 7.4 (neutral), a refractive index of 1.3345-1.3348 and a density of 1.0053-1.0089 g/cm^3.[148,149] The vitreous helps in the maintenance and metabolism of the eye tissues,[150] protecting the eye from trauma, inhibiting angiogenesis, coordinating eye growth[151] and regulates the shape of the eye during development.[152]

The structure of the vitreous is based on a three-compartment complex: collagen-glycoaminosglycan (GAG)-hyaluronic acid (HA)-GAG-collagen, which was first described by Balazs.[153] There is a random meshwork of fine collagen fibrils of approximately 10 nm with HA dissolved and entrapped within the collagen spaces.[154]

The vitreous consists mostly of water (98-99%) and other components include macromolecules such as GAGs e.g. HA, proteoglycans, glycoproteins (e.g. opticin), collagens and non-collagenous structural proteins (e.g. fibrillin).[151,154–158] Immunohistochemical studies have shown non-collagenous molecules such as laminin, fibronectin, proteoglycans and glycoconjugates to be localised on the inner limiting membrane (ILM), which is the boundary between the retina and vitreous.[159] Fibrillar proteins help maintain the shape of the vitreous including its flexibility, strength and resistance to traction forces. Charged carbohydrates, such as GAGs, attract water and counterions, and swell to resist compressive forces.[152] Each component has a role in the functioning of the eye and the vitreous (**Tables 3** and **4).** While there are many different collagens (**Figure 1**) and macromolecules in the vitreous, the major components of the vitreous body are HA and type II collagen (**Table 5**) with the presence of some soluble proteins (e.g. albumin, **Table 5**).

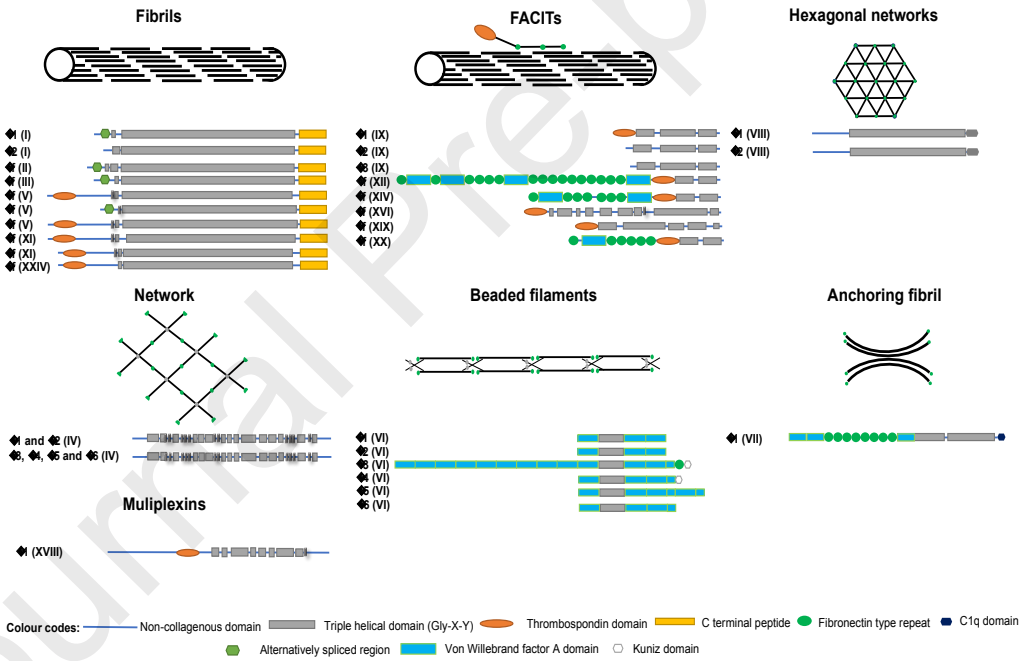

Figure 1. Types of collagen that are present in the eye. The most predominant collagen in the vitreous are types II (FACIT) and IX (FACIT) with traces of type V (fibril), VI (beaded filaments), XI (fibril) and XVIII (membrane collagen). Image adapted from Karsdal 2016.[160]

Table 3. Collagen distribution in human eye (The vitreous is highlighted in green).

Type	Family	Gene	Location	Functions
I	Fibril-forming	COL1A1 & 2	Choroid, cornea (Bowman's layer and stroma), ciliary body (BM of pigmented and non-pigmented epithelium, vascular BM, stroma and ciliary muscle), iris (stroma, vascular BM and dilator muscle BM), lamina cribrosa, optic nerve head (central retinal artery and cribriform) retina, sclera and trabecular meshwork (corneoscleral meshwork, core of trabecular beams, BM of trabecular beams and cribriform layer).[161–165]	Expression of type I in early stages of wound healing.
II	Fibril-forming	COL2A1	Near developing ciliary body, iris, RPE, retina (inner and outer plexiforms), sclera and vitreous[151,163,166–171]	Maintains structure of vitreous
III	Fibril-forming	COL3A1	Choroid, ciliary body (BM of pigmented and non-pigmented epithelium, stroma and ciliary muscle), cornea (Bowman's membrane and stroma), iris (stroma), optic nerve head (central retinal artery, prelaminar and cribriform) retina, sclera, trabecular meshwork (corneoscleral meshwork, core of trabecular beams, basement membrane of trabecular beams and cribriform layer).[161,162,165,172–175]	Expression of type III in early stages of wound healing. Provides tensile strength.
IV	Network	COL4A1, 2, 3, 4, 5 & 6	Choroid, ciliary body (BM of pigmented and non-pigmented epithelium, vascular BM, stroma and ciliary muscle), conjunctiva, cornea (Basement membrane and Descemet's membrane), developing lens capsule (ciliary zonules), iris (vascular basement membrane and dilator muscle basement membrane), lamina cribrosa, optic nerve head (prelaminar, cribriform) retinal microvessels and capillaries, trabecular meshwork (corneoscleral meshwork, basement membrane of trabecular beams and cribriform layer).[162,165,174,176–178]	Forms a 3D scaffold for cell adhesion. Due to its negative charge, it possesses a protective function against enzymatic breakdown.
V	Fibril-forming	COL5A1, 2 & 3	Basement membranes, choroid, cornea (Bowman's membrane), lamina cribosa, retina, sclera and vitreous[172,175,179–181]	Modulates cellular activities (adhesion, differentiation, migration, synthesis), tissue remodelling and provides tensile strength.
VI	Beaded filament forming	COL6A1, 2, 3 & 5	Ciliary body (vascular BM and ciliary muscle), cornea (Basement membrane and Descemet's membrane), retina (ILM), sclera, trabecular meshwork (core of trabecular beams,	Role in (posterior) vitroretinal attachment and mechanism of (posterior) retinal detachment.

			basement membrane of trabecular beams), vitreous.[162,163,172,180,182,183]	Stabilises interfibrillar matrix in cornea and sclera. Helps organise and maintain the supramolecular structure of the vitreous gel.
VII	Anchoring	COL7A1	Conjunctiva and cornea (Descemet's membrane and epithelium).[176,184]	Role in (posterior) vitroretinal attachment and mechanism of (posterior) retinal detachment.
VIII	Network forming collagen	COL8A1 & 2	Cornea (Descemet's membrane), choroid, optic nerve head (central retinal artery and cribriform), sclera and trabecular meshwork (corneoscleral meshwork and cribriform layer).[177,184]	Important for endothelial cell phenotype.
IX	FACIT	COL9A1 & 2	Ciliary body, cornea, ILM, iris, lens, sclera, retina and vitreous.[183,185]	Interacts with type II collagen and maintains the structure of the vitreous.
XI	Fibril-forming	COL12A1	Central core of major collagen fibrils in vitreous.[163,186,187]	Adds support to the vitreous structure.
XII	FACIT	COL13A1	Cornea (Bowman's membrane), limbus and sclera.[180,181,188]	Maintains corneal structure, transparency and lamellae organisation.
XIII	MACIT	COL14A1	Ciliary body, cornea (stroma), lens epithelium, optic nerve head (ganglion cell area) and trabecular meshwork.[189]	Important for axons and myelinated oligodendrocytes.
XIV	FACIT	COL15A1	Embryonic cornea.[190]	Helps maintain the interaction of fibrillar collagens with other matrix constituents.
XVI	FACIT	COL17A1	Ciliary muscle.[190]	Helps maintain the interaction between other ECM proteins.
XVII	MACIT	COL18A1	Cornea (vascular BM), ILM, lens and RPE.[191]	Transmembrane component of cell-tissue interface.
XVIII	Multiplexins	COL19A1	Cornea (vascular BM), ILM, lens and RPE.[163,191]	Helps maintain anchoring between the vitreous fibrillar collagens and the ILM; and helps inhibit angiogenesis
XIX	FACIT	COL20A1	BM.[191]	Provides structural support.
XX	FACIT	COL21A1	Embryonic corneal epithelium.[192]	Interacts with collagen I.

XXIII	MACIT	COL24A1	Retina.[193,194]	Transmembrane collagen seen in cell culture.
XXIV	Fibril-forming	COL25A1	Cornea and retina.[195]	Regulates type I collagen fibrillogenesis.

Abbreviations: BM: Basement membrane, ECM: extracellular matrix; FACIT: fibril associated collagen with interrupted triple helices; ILM: inner limiting membrane, MACIT: membrane-associated collagens with interrupted triple helices; RPE: retinal pigment epithelium.

Table 4. Distribution of proteoglycans and macromolecules in the human eye (The vitreous is highlighted in green).

Name and types	Location	Functions
Amino acids		
Ala, Arg, Asp, Cys, Glu, Gly, His, Hydroxy Lys, Hydroxy Pro, Iso Leu, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Tryptophan Val	Cornea and intraocular fluids.[165,196–198]	Influx transporters and neurotransmitters.
Sulphated GAGs		
(i) Chondroitin sulfate (Hyalectans)		
Aggrecan	Retina and sclera.[199,200]	Helps in retinal development and maintenance with multiple effects on neurite outgrowth and normal astrocyte differentiation
Biglycan	Choroid, retina, sclera and trabecular meshwork.[199]	Possesses a neurotropic factor for retinal cells and regulators of their differentiation
Decorin	Choroid, cornea, retina, sclera and trabecular meshwork.[199,201]	Possesses a neurotropic factor for retinal cells and regulators of their differentiation, and regulates fibrillogenesis.
Neurocan	Developing neural retina, ECM and optic nerve.[202,203]	Helps in the developmental processes of the CNS.
Versican	Bruch's membrane, retina and vitreous.[199,204]	Maintains vitreous structure by linking HA to other components; and helps in retinal development and maintenance
(ii) Dermatan sulfate		

	Choroid, cornea (sclera), optic nerve head and retina.[199,205–207]	Role in wound repair and fibrosis.
(iii) Heparan sulfate (Basement membrane PGs)		
Agrin	BM, ILM and vitreous.[191,208]	Helps in the development of the nerve tract and optical pathway
(iv) Keraten sulfate		
Fibromodulin	BM.[209]	Regulates fibrillogenesis by binding to type I and II collagen. Maintains collagen architecture
Keratocan	Cornea and sclera.[210]	Maintains corneal transparency (it is a major corneal proteoglycan).
Lumican	Choroid, cornea (Bruch's membrane) and sclera[209,211]	Maintains corneal transparency and possesses biomechanical properties of the sclera, regulates collagen fibrillogenesis and has pro-inflammatory effects; and maintains structure of Bruch's membrane and collagen architecture.
Mimecan	Cornea and sclera[199,212]	Maintains corneal transparency.
Perlecan	BM and trabecular meshwork[213]	Maintains endothelial barrier function. Stimulates endothelial growth and re-generation.
Syndecan-1	Trabecular meshwork[214]	Assists in cell matrix adhesion and cell growth.
Non-sulphated glycosaminoglycan (GAG)		
Hyaluronic acid (HA)	Vitreous, ECM and anterior chamber (and throughout the body)[215,216]	Helps in the swelling of the vitreous and interacts with type II collagen to maintain the structure of the vitreous [151,152].
Others		
Cartilage oligomeric matrix protein (COMP)	Vitreous.[151,168]	-
Elastin	Cornea, lamina cribrosa and trabecular meshwork[162,217–219]	Provides connective tissue with additional resistance and recovery from deformation at low stress levels.

		Maintains corneal stability and curvature.
Integrin	BM and cornea[184]	Aids in the attachment of cells to the ECM and helps in cell signal transduction.
Kalinin	BM.[176,184,220,221]	Helps in keratinocyte attachment.
Nidogen-1 (Entactin)	BM and cornea.[222,223]	Interacts with other molecules in the BM, and connects networks formed by collagen and laminin together. Helps in cell interactions in the ECM.
Opticin (vitrican)	Choroid, ciliary body, cornea, ECM, iris, posterior capsule of lens, retina and vitreous[185]	Important in collagen fibril assembly. Prevents bundle aggregation of adjacent collagen fibrils. He;ps in the development of NPE
Prolargin	BM, ECM and retina.[199]	Interacts with heparin sulfate
SPACRCAN	Retina (IPM),[224]	Interacts with HA and stabilises HA
VIT 1	Vitreous.[225]	Collagen binding macromolecule, maintain vitreous gel structure

Abbreviations: Ala: alanine; Arg: arginine; Asp: asparate; BM: basement membrane; CNS: central nervous system; Cys: cysteine; ECM: extracellular matrix; GAGs: glycosaminoglycans; Glu: glutamate; Gly: glycine HA: hyaluronic acid; His: histidine; ICAM: intercellular adhesion molecule; IPM: interphotoreceptor matrix; Iso: isoleucine; Leu: leucine; Lys: lysine; MMP: matrix metalloproteinase; Met: methionine; NPE: non-pigmented epithelium; Phe: phenylalanine; Pro: proline; PG: proteoglycan; Ser: serine; Thr: threonine; Tyr: tyrosine; Val: valine; VIT: vitrin.

Table 5. Vitreous concentrations of hyaluronic acid (HA), type II collagen and other soluble proteins.

Component	Species and age	Concentration (µg/mL)
Hyaluronic acid (HA)	Human	65-400[216,226]
	Bovine	430-555[227]
	Owl monkey	100-180[216]
	Rhesus monkey	300-900[216]
	Porcine	160[168]
	Rabbit	14-52[216]
Soluble proteins	Human (10-50 years)	500-600[228]
	Human (50-80 years)	700-900[228]
	Human (>80 years)	1000[228]
Type II collagen	Human	300[168]
	Human (15-20 years)	50[228]
	Human (70-90 years)	100[228]
	Bovine	60[168]

4.2.1. Hyaluronic acid (HA)

HA is a key component in the vitreous and is a non-sulfated GAG. HA is a highly hydrated polyanion and a high molecular weight polysaccharide that consists of a repeating disaccharide of β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine.[229–231] Deprotonation of the glucuronic acid at physiological pH is the source of negative charge.[232] Space-filling networks are formed that possess lubricant properties and acts as an osmotic buffer.[151] HA can have loops, folds, or turns regardless of its stiff coil configuration.[151] HA and type II collagen are primarily responsible for the structure of the vitreous.[233] Type II collagen fibrils are separated by HA, which prevents aggregation of collagen fibrils.[234] It is suggested that the sulfated GAG functions as a 'glue' between hyaluronate and collagen.[235]

The hydrodynamic size of HA and its molecular weight also varies between species and can alter the viscosity of the vitreous (**section 4.3**). The molecular weight of HA is 2-4 million and 1.7 million Da[236] in human and bovine vitreous respectively, where the latter is more polydispersed in molecular weight.[168] Porcine vitreous has also been reported to have a lower molecular weight and concentration of HA (including lower concentrations of total protein and collagen) than human vitreous.[205,237] HA is also reported to not to be uniformly distributed throughout the vitreous. Low and high HA concentrations are present in the front (anterior) and back (posterior) part of the vitreous respectively[168] and it differs between species (**Table 5**). In a comparative study, uronic acid per millilitre (analysed by carbozole assay) present in sheep (124 µg) and goat (128 µg) vitreous were higher than human (96 µg) and porcine (76 µg).[237] Goats and sheep have a higher amount of HA and chondroitin sulfate compared to humans and porcine vitreous.[237]

Research has been conducted where a long-acting therapy could bind to a tissue in the body to extend its duration of action.[238,239] The same concept has been reported for therapies that bind to the vitreous.[77–80,240] Natural human proteins have been reported to bind to HA with modest affinity. These include TNF-stimulating gene-6 (TSG-66), receptor for HA

mediated mobility (RHAMM), cell surface glycoprotein (CD44) and lymphatic vessel endocytic receptor (LYVE-1).[54] The intravitreal half-life of single-chain variable fragments (scFvs), Fabs, Fc-Traps and antibodies (IgGs) was extended by the incorporation of a hyaluronan-binding peptide (HABP) to increase the intraocular half-life.[77] The half-life of the HABP modified proteins increased 3-4 fold in rabbit and monkey eyes when compared to proteins not modified with HABP.[77] It was thought that a reduced dosing frequency could be achieved by the incorporation of HABP to bind to vitreous HA without requiring the need for loading doses (e.g. 3 monthly loading doses are frequently used for current anti-VEGF therapies).[77] Further research is ongoing to determine the effect of vitreous binding on drug exposure in the retina and its safety profile.[16,54]

4.2.2. Type II collagen

Several collagen subtypes are present in the vitreous in a unique structural arrangement. Collagen belongs to the subfamily of fibrillar macromolecules comprising of three polypeptide chains α1(II) that form a compact triple-helical rod-like building blocks of collagen fibrils. Type II collagen accounts for 70-80% of the collagen that is present in the vitreous;[172] and is covalently cross-linked in a “quarter staggered” array to form the major bulk of the heterotypic (mixed) collagen fibrils.[151] Human type II collagen contains approximately 120 repeat units of Gly-Pro-X (X being any amino acid).[77] Type II collagen molecules are secreted into the extracellular environment to form a soluble precursor, pro-collagen II, which is processed by an N-proteinase and a C- proteinase. These enzymes cleave N- and C-peptides in pro-collagen to decrease collagen solubility with the formation of fibrils.[152,168]

Other types of collagen (e.g. type V, XI and IX) and non-collagenous molecules (e.g. opticin, laminin and fibronectin) are responsible for stabilising type II collagen in the vitreous,[241] and are altered during ageing (**section 4.3**). The different collagen types are characterised by considerable complexity and diversity in their structure, assembly and function. Type II collagen (and XI) contributes to the fibrillar matrix of articular cartilage, where its stability and tensile strength provides structure to tissue. Type IV collagen is an abundant collagen found in the ILM and is a basement membrane collagen. Compared to the other collagen types, type IV collagen is a more flexible triple helix that is often assembled into meshwork structure.[242] The triple helix structure of type II collagen is similar in size and biochemical properties to that of type I collagen. However, type II collagen has a higher content of hydroxyl-lysine, glucosyl and galactosyl residues leading to a greater number of interactions with proteoglycans. Another collagen component found in the vitreous is type XI collagen. Although the α3-chain of type XI collagen is encoded by the same gene as α1-chain of type II collagen, the amount of glycosylation and hydroxylation differs more from α1(II).[242]

The average concentration of type II collagen in the vitreous is 50-100 μg/mL[228] (**Table 5**), though some have reported slightly higher concentrations (150-300 ug/mL).[77,168] Type II collagen is reported to be higher in the anterior part of the vitreous body (up to 30×) than the cortical part of the vitreous.[167] Balasz initially reported that new type II collagen in the vitreous is not synthesised after birth.[243] Similar trends have also been reported for bovine eys.[168] However, another study has reported that a higher concentration of type II collagen was seen in age groups between 70-90 years (100 μg/mL) as compared to 15-20 years (50 μg/mL) human eyes (**Table 5**).[228] Differences in collagen concentration can either be due to the area sampled or the age of the animals used for the studies.[244]

4.3. Vitreous age-related changes

Intravitreally administered anti-VEGF medicines often display interpatient variation. Some of the reasons for the variation are thought to be lens status, prior vitrectomy, baseline visual acuity, genetic factors, and type and duration of underlying disease. Variation is also possible due to ageing and other pathological processes that can disrupt the vitreous structure (**Figure 2**). The vitreous undergoes progressive liquefaction with age,[151] where the HA-collagen complex often dissolves within the central vitreous body.[233] There is a reduction in vitreous volume and the entire vitreous shape can collapse and lose its texture during degeneration.[235] There is an increase in vitreous liquefaction and a decrease in gel volume post the age of 40 years. More than half the vitreous becomes liquefied by the age of 80-90 years.[151] There is a progressive increase in fluid-filled areas (i.e. synchisis) and an increase in optically dense structures (i.e. syneresis). In both situations, symptoms known as ‘mouches volantes’ or floaters are reported.

Figure 2. Dark-field slit microscopy of age-related vitreous changes in fresh and unfixed whole human eyes. Vitreous body shown attached to anterior segment. Vitreous body of **(A)** 11 year old showing homogenous structure and minimal light scatter, **(B)** 56 year old showing aggregation of visible collagen fibres and **(C)** 88 year old showing thickened central body of collagen fibres, pockets of liquid vitreous called lacunae (white arrow). Image from Milston et al, 2016 with permission.[245]

The prevalence of posterior vitreous detachment (PVD) is reported to increase with the ageing population, which is seen in 24% of patients between 50-59 years of age and 87% of patients between 80-89 years of age.[246] PVD separates the adhesion between the retina

and vitreous cortex (which is attached to the basement membrane of the inner limiting lamina, ILL),[187] and can result in intraocular haemorrhage, retinal detachment/tears and cystoid macular oedema.[247] PVD is mostly associated with senescence and large amounts of liquefied spaces.[155] Progressive liquefaction does not occur in an uniform manner within the vitreous. Instead, there are pockets formed in the central vitreous where significant enlargement and coalesce are observed.[152]

Type IX collagen molecules display an antiparallel pattern along the surface of type II collagen fibrils (**Figure 3**).[242] Type II collagen is shielded by type IX collagen, which avoids the fusion of type II collagen. The shield provided by type IX collagen is lost during the ageing process resulting in the fusion of type II collagen with adjacent fibrils on contact and further vitreous liquefaction. Other vitreoretinal disease processes can be modulated from the increased surface exposure of type II collagen and age-related loss of type IX collagen.[187] Vitreal collagen fibrils are coated with macromolecules (e.g. opticin). These macromolecules and HA can either allow for short-range interactions or prevent collagen fibrils from aggregating with one another. The decreased spacing between the collagen fibres and the collagen breakdown may influence the ageing changes and can lead to its uneven distribution within the vitreous.[151] In addition, there is an increase in protein content that contributes to the ILM thickening and increases the size and aggregation of the collagen molecules in the vitreous base in ageing patients.[248] Eye movements/saccades can enhance liquefaction by bringing the type II collagen fibrils together.[187]

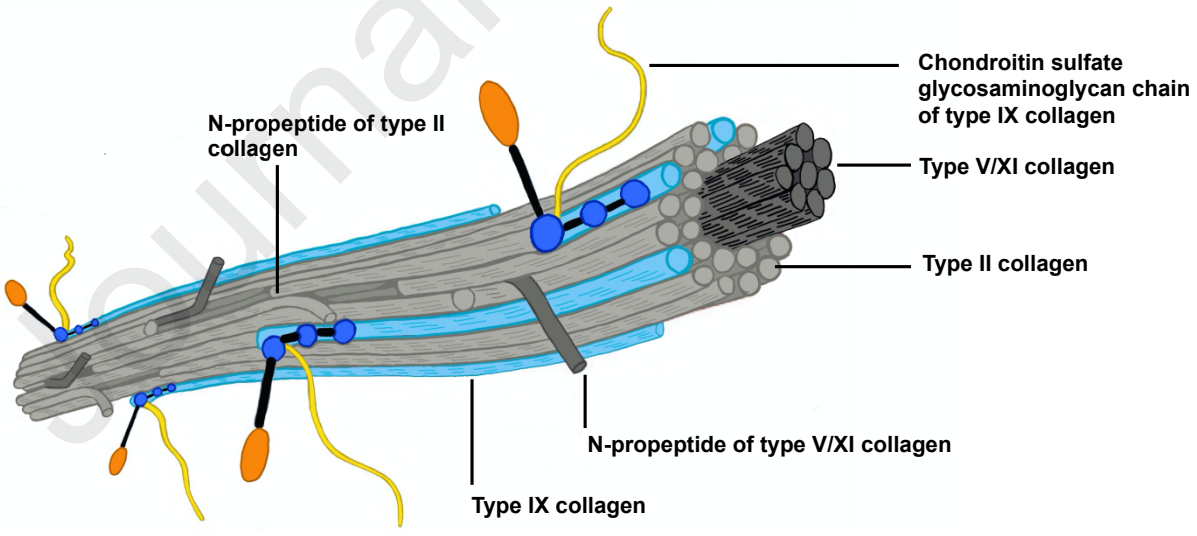

Figure 3. Structure of major heterotypic collagen fibrils of the vitreous. Image adapted from Bishop, 1996.[168]

The selection of an appropriate animal model to replicate human disease conditions should take into account species vitreous variability and age-related differences as it

modulates the diffusion of various molecules (diffusion is inversely proportional to the viscosity of the media according to Stoke-Einstein equation).[249] In a study reported by Shafaie et al,[249] calculated diffusion coefficients of fluorescein sodium was higher in human eyes than bovine, porcine and ovine eyes with the animal species displaying respective viscosity values (at 0.1 Hz) of 0.9 ± 0.51 Pa.s, 1.6 ± 1.32 Pa.s, 12.3 ± 0.94 Pa.s.[249] Vitreous degeneration will increase vitreous diffusivity and diminish the drug concentration gradient across the medium. A loss in the vitreous barrier function is noted with an increase in vitreous stirring caused by convective flow in the vitreal liquefied areas. Enhanced convective flow is seen in a liquefied vitreous.[250] An unequal distribution of protein-bound drugs and free-concentration in equilibrium are noted when proteins accumulate in the remnants of the vitreous.[96] Drug efficacy is still overestimated despite numerous reports of vitreous liquefaction with age and disease; as pre-clinical intravitreal drug development is conducted using young laboratory animals that have an intact vitreous structure.[250] Any model used to study differences in ocular pharmacokinetic properties in an ageing vitreous should consider the effect of liquefaction.[251–254]

4.4. Effect of vitrectomy on intravitreal pharmacokinetics

Vitrectomy is the surgical removal of vitreous. Vitrectomy reduces the macular thickness and relieves the tractional forces exerted by a degenerating vitreous on the retina (**Figure 4**). There are over 500,000 vitrectomy surgeries performed each year, with 150,000 in US alone.[255,256] Vitrectomy has been reported to improve visual acuity in diffuse non-tractional DME patients.[257] A number of drugs i.e. triamcinolone acetonide[145] (TA) (**Table 2**), amphotericin B,[258] amikacin,[259] vancomycin[260,261] and 5-fluorouracil (5-FU)[262] have been reported to show faster clearance rates in vitrectomised eyes. Differences in the clearance rates in vitrectomised and non-vitrectomised eyes would be expected to influence the clinical effectiveness of a drug therapy.

A study reported the half-life of ranibizumab to be 2.81, 2.13 and 1.79 days in rabbit eyes that were non- vitrectomised, vitrectomised and lensectomised respectively.[263] In another study, TA showed a half-life of 1.57 days and 2.89 days in vitrectomised and non-vitrectomised rabbit eyes respectively.[147] TA was seen to decrease 1.5× faster in vitrectomised eyes.[147] Intravitreal injected TA condensed/accumulated into the small spaces in the gel-like spaces in the vitreous of the non-vitrectomised eyes and due to its high water insolubility its clearance was slower. Increased intravitreal circulation is expected in vitrectomised eyes as a drug would distribute evenly throughout the vitreous.[147]

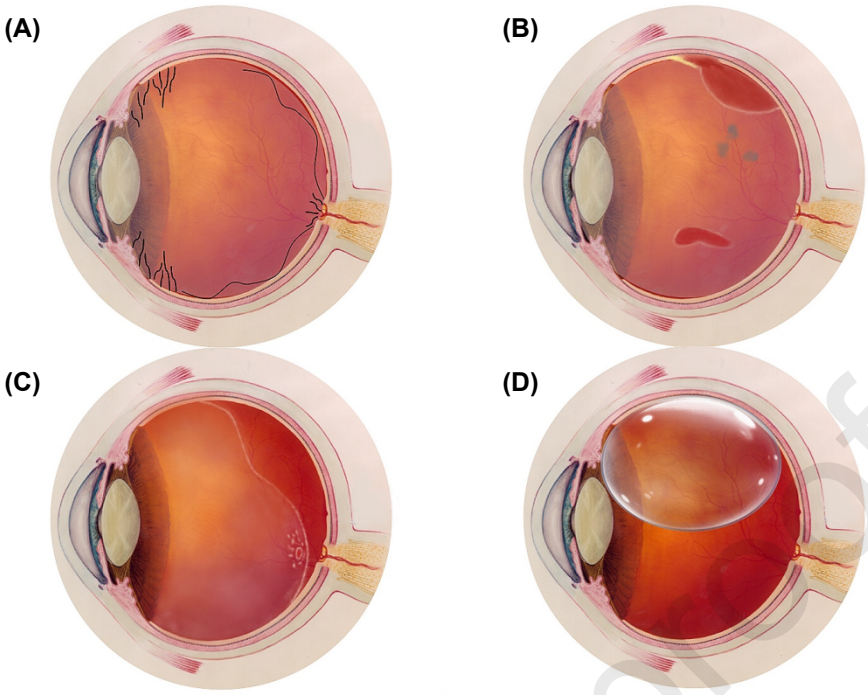

Figure 4. (A) Areas of high vitreous fibrillar density and adhesion (black lines), **(B)** vitreous changes seen in myopia, **(C)** vitrectomised eye and **(D)** gas or silicone oil bubble in posterior cavity. Image adapted from National Eye Institute, National Institutes of Health.[264]

VEGF concentration was investigated in vitrectomised macaque eyes after the intravitreal injection of bevacizumab.[265] Aqueous humour samples indicated a shorter half-life in vitrectomised eyes, a decrease of 54% compared to non-vitrectomised eyes. VEGF concentration was below the lower limit of detection after bevacizumab injection and remained below that threshold for about 2 weeks until its returned to detectable levels between 2-4 weeks.[265] Most of the pivotal studies of anti-VEGF drugs were conducted in non-vitrectomised eyes.[266–268] Hence there remains uncertainty regarding the clinical effectiveness of intravitreal drugs in vitrectomised eyes due to more rapid clearance than in non-vitrectomised eyes. In most cases, vitrectomy is conducted due to complicated situations or advanced retinal diseases such as submacular haemorrhage, CNV or cataract extraction.[269] Therefore, it is important to take these situations into account. Patients with DME may have undergone macular laser prior to receiving intravitreal anti-VEGF, which may reduce drug effectiveness irrespective of vitreous status.[270,271] Better-designed studies with controlled confounding baseline characteristics are warranted to address this question.

4.5. Melanin

Melanin is present in some intraocular tissues (e.g. uvea and RPE).[197] Melanin is a polyanionic biopolymer and the most common light-absorbing pigment. Dark eumelanin and yellow pheomelanin are the most common melanin forms found in the human body.[3] The RPE is dense with eumelanin; and the iris and choroidal melanocytes are packed with a combination

of eumelanin and pheomelanin.[3,272] Melanosomes are organelles; and the site of synthesis, storage and transport of melanin. They are found in the iris, choroid, RPE and other parts of the body (e.g. hair and skin).[273] Melanin within melanosomes of RPE cells also absorbs light scatter and is likely to aid in protection against photooxidation.[274]

Drugs can also bind to melanin (similarly to HA and collagen) affecting their pharmacologic and pharmacokinetic properties.[275] Melanin is an important pigment (approximately 6-8 mg of melanin in human choroid-RPE)[276] where drugs can bind to form a reservoir hence prolonging the residence time.[24,277] A number of small molecules (e.g. β-blockers, celecoxib and chloroquine) injected intravitreally are reported to bind to melanin,[273,278–280] however, protein binding to melanin has not yet been reported. Drug binding to melanin is also known to modulate drug response and distribution in pigmented tissues, with greater differences in total and free drug concentrations reported in pigmented than in non-pigmented cells.[281] Drugs like chloroquine have a high binding affinity to melanin,[282] however, the drug-melanin complex may result in retinal toxicity if taken in large enough doses and for prolonged period of time.[283] Melanin toxicity can be a result of high drug concentrations in the RPE cells or by the alteration of melanin granules after drug-melanin binding.[284]

The amount of melanin differs between different ocular tissues, pigmentation strains or phenotypes and animal models.[275] Durairaj et al reported the distribution and difference in concentration of melanin in human, minipigs, rabbit, monkey and dog models.[275] A longer duration of action of topical atropine and pilocarpine was seen in pigmented animals than albino animals.[285,286] The higher concentrations of pilocarpine needed to cause pupil constriction in a darker iris versus a blue iris with less melanin is an example of this possible sequestration of drugs in melanin. There should be careful consideration in selecting animal models and interpreting ocular pharmacokinetics for administered drugs that binds to melanin. More research is needed in understanding the pigment binding of drugs and permeation across the RPE.

5. The need for experimental preclinical *in vitro* pharmaceutical models

It is generally not possible to determine intraocular pharmacokinetics in humans because of the risks for adverse events with repeated vitreous sampling[97,100] and difficulty in obtaining direct samples.[287] However, when intraocular pharmacokinetics properties are sought, they can be determined by obtaining samples from either the anterior chamber or in some cases from the plasma. In the case of animals during preclinical studies, samples are often obtained from the vitreous, often by sacrificing a few animal species at each time point of interest. One-time sampling involves inserting a 20G needle through the outer canthus until the tip of the needle is inside the globe where suction is then applied to extract the entire vitreous humour.[288] Curve-fitting software (e.g. Kinetica® or Phoenix WinNonlin®) are used to determine

ocular pharmacokinetic parameters.[97] It is crucial to have enough data points to be fitted to compare it to the observed mean values and individual points.[97,99]

Techniques such as microsampling and microdialysis strategies have also been developed in an effort to obtain real time data over time from individual animals.[289,290] Microsampling consists of obtaining smaller samples (10-15 µL) of the vitreous using a syringe (200 µL), and avoids large volume withdrawal of the vitreous and other ocular fluids.[291] Microdialysis has been reported to provide statistically robust data and reduces the number of animal models.[290] The technique involves the use of an intraocular probe consisting of a cannular and a semi-permeable membrane to determine drug concentrations in the vitreous. Free drug molecules diffuse across the membrane into saline flowing through the cannula at a known rate. Drug concentration is measured continuously to help quantify intraocular concentration of the drug. The effects of anaesthesia and the breakdown of blood aqueous barrier (BAB) can also be monitored. A number of microdialysis studies using the rabbit as a model have been described.[104,290,292–294]

5.1. Production of anti-drug antibodies in animals

Although obtaining pharmacokinetic data from animals is widely conducted, the administration of a human protein therapeutic to an animal will often result in intraocular inflammation and the production of anti-drug antibodies (ADAs) in the animal. The eye is not a perfectly immunologically privileged organ[15] and after intravitreal injection, intraocular immune-mediated and inflammatory reactions can occur.[15] ADAs can accelerate the clearance of an administered protein candidate[295] and ADA generation[296] in animal models is not predictive of immunogenicity in humans.[95,297]

Rabbits, for example, are known to give robust immune and humoral responses to foreign proteins, limiting their use in preclinical studies.[87] Independent studies by Genentech, Roche, Novartis and Allergan confirmed the rapid development of ADAs in rabbit species following intravitreal injection of antibodies and antibody mimetics.[87] These studies indicated that challenges from intravitreal injection of biologic drugs preclude the use of rabbits for safety evaluation. Serum and plasma ADAs were observed relatively quickly in rabbits, along with intraocular inflammation after injection of the initial drug dose. ADAs were also seen and persisted in the vitreous, limiting drug exposure due to their presence in the vitreous compartment.[87] Another study[298] to determine the pharmacokinetics of a long-acting formulation of a dimeric anti-VEGF molecule (consisting of two anti-VEGF antibodies attached to a human IgG_1 Fc region) noted the formation of ADAs in ocular rabbit samples, which interfered with study parameters and resulted in the subsequent use of primates to reduce the observed impact of immunogenicity in rabbits.[298]

These observations point to the limitations and thus difficulties in the applicability of rabbit and indeed other animal models in preclinical analysis of biologics for the eye.[87] Animal models are less likely to determine production of neo-epitopes and neutralising antibodies on modified proteins due to different antibody response mechanisms.[95,299] Serum or plasma samples can help detect ADAs[86] including specific immune response in the vitreous.[300] A comprehensive toxicological study must be performed before translation to phase I clinical trials.

5.2. In vitro models as preclinical tools to develop intraocular therapies

Pharmaceutical *in vitro* models have long been used to accelerate preclinical research. Many *in vitro* models have been developed[301] to determine correlations of relevant physicochemical and materials factors to optimise formulation and dosage form design. These *in vitro* preclinical models are generally designed to measure one or two relevant factors related to the mode of administration of a dosage form of interest. Simulated biological fluids are also often used for studies related to drug dissolution, release and stability. Preclinical development using *in vitro* preclinical models can be optimised if IVIVCs have been established, and can help to validate the use of dissolution models to establish dissolution specifications.[302] The FDA has developed a regulatory guidance for some dosage forms, for example, to validate the use of dissolution models to establish dissolution specifications,[302] and to reduce the number clinical bioequivalence studies to support biowaivers and bioequivalence criteria.[302,303]

Ocular models for topical or anterior segment delivery are more established than models for the posterior segment. For example, *in vitro* models using isolated corneal epithelial cells from rabbits[301] mimic the corneal barrier for use to develop of topically administered medicines such as eyedrops. However, limitations exists that involve the lack of complexity of natural eyes and functions such as aqueous humour and tear fluid;[304] and low availability of cornea donors.[305] There are also many preclinical cell culture models[130] and *ex vivo* preclinical models that use isolated tissues,[306] which are outside the scope of this review.

Determining the intraocular drug release and clearance profiles are crucial parameters to optimise and to ensure consistent *in vivo* performance. Since the widespread use of intraocular medicines is so recent, there are no *in vitro* preclinical models described in the pharmacopeia specifically designed to determine intraocular pharmacokinetics of ocular drugs.

5.2.1. Adaptation of existing pharmaceutical models

The types of apparatus described in the pharmacopeia that can be used for the evaluation of long acting intravitreal formulations are those that have been designed to study drug release from extended release dosage forms for oral administration, namely Apparatus IV (flow-

through cell) and Apparatus VII (reciprocating holder). Apparatus IV consists of a reservoir containing the release medium and a flow through of 1.5 mL/min.[307] A water bath helps to control the cell temperature (37 ± 0.5°C). Two different cells provide approximate volumes of 8.0 and 19.0 mL with the bottom cone filled with small glass beads and one bead at the apex to protect the inlet tube.[308] Apparatus IV has many advantages over more widely used dissolution apparatus as it can operate under different conditions, i.e. an open or closed system. Medium is delivered fresh and drug elute is removed in an open system while a constant volume, different flow rates and temperatures are used in a closed system.[309] For intravitreal formulations in particular, the ability to control flow rates, temperature, and the option to test in an open system (thus maintaining sink volume) allows for dissolution testing to model drug clearance in the eye. However, ocular dimensions and orientation, appropriate vitreous media and compartmentalisation are not accurately modelled, which are important parameters to consider for the development of longer lasting ocular formulations.[251]

Originally used to test transdermal formulations,[310] Apparatus VII is also used for the dissolution testing of oral extended release tablets and medical devices such as drug eluting stents. The Apparatus VII has also been adapted for testing of intravitreal products, in particular Ozurdex®. The Apparatus VII consists of volumetrically calibrated solution containers with an assembled motor/drive. The temperature is maintained by immersing the solution containers in a water bath.[311] The use of Apparatus VII for testing of intravitreal implants is due to the capability to test dissolution in volumes of 3.0-30.0 mL with automated replacement of dissolution media. The Apparatus VII is thought to model the volumes within the posterior compartment of the eye (<5.0 mL) better than other dissolution apparatus currently available while also providing the sink conditions for dissolution testing. However, similarly to Apparatus IV, Apparatus VII does not effectively model the eye with regards to flow rate, viscosity and compartmentalisation. Diffusion control in the posterior cavity is missing.[251]

5.2.2. Importance of flow in an *in vitro* model

Mass exchange within the eye is dominated by aqueous flow, which is secreted at 1.5–3.0 µL/min from the ciliary body into the vitreous. Different flow rates are seen in the morning (3.0 µL/min), afternoon (2.4 µL/min) and night (1.5 µL/min).[312,313] Therapeutic proteins such as antibodies are charged, high molecular weight molecules and will predominantly clear through the anterior route with little protein clearing through the RCS route.[105,112,131–133,314] Proteins are large molecules, therefore, they have longer half-lives (i.e. days) and slower diffusivity in the vitreous than most low molecular weight drugs in the soluble phase (i.e. hours).[36] Low molecular weight drugs often display greater retinal permeability that also allows RCS clearance.[251] Steroidal implants achieve prolonged exposure in the posterior segment for a

low molecular weight drug. Extensive research and efforts are still required to increase the duration of action of intravitreal administered protein-based medicines.

Currently, there is little information reported in research for the development of an *in vitro* model that accounts for aqueous flow.[315] This concept becomes extremely crucial for the development of long acting protein formulations that predominantly exit via the anterior route. Drugs administered by intravitreal injection are cleared posteriorly through the retina or from the aqueous outflow via the hyaloid membrane through the anterior chamber. A number of studies to evaluate intraocular flow have been conducted in rabbits.[316] Duke-Elder[317] was the first to describe fluid turnover in the rabbit vitreous. Fluid was reported to originate from the capillaries of the ciliary body and pars plana and then travel posteriorly through the vitreous and exit near to the optic nerve head. The slow stream of fluid was a result of 'simple diffusion'.[317,318]

Fowlks[318] then studied the migration of India ink and nitro blue tetrazolium chloride in the posterior segment in rabbits. When an injection was made close to the retina or pars plana, it followed a 'meridional' flow pattern.[318] This has been reported to be 'simplistic' for poorly soluble suspensions where aggregates may be present from the initial dispersion phase.[319] For example, a study reported drug aggregates of triamcinolone in the posterior chamber of the rabbit eye up to 15 days post-injection.[320] Clearance across the RCS pathway by permeation is a rapid process because of the relatively large surface area of the retina compared to the anterior hyaloid between the lens and ciliary body. The concentration of HA in the vitreous is also reported to be larger near the retina and as a result might be responsible to the radial or 'meridional' component of flow near the retinal surface of the vitreous.[318]

Convection is a result of steady permeating flow through the vitreous due to the pressure drop between the anterior hyaloid membrane and the retinal surfaces and/or by active transport through the RPE.[321] Drugs with high diffusivity experience almost negligible effects on its absorption from convection. Whereas, a more dominant effect for drug with low diffusivity coefficients, such as macromolecules, is seen with convection.[322,323] Significant differences in drug distribution for both large and small molecules are seen with the effects of geometry, diffusion and convection.[324] Convection effects has been reported to have a bigger influence to mixing than saccades.[24,325–328] A study[329] reported the differences in transport mechanism between human and neonatal mouse eyes.[329] The transport mechanism in the eyes of a mouse was predominantly diffusive. Whereas, both types of mechanisms is possible with the eyes of a human depending on the injected drug, i.e. balanced between convection and diffusion for small molecules and convection for large molecules.[329]

A few ophthalmic *in vitro* models have recently been described. The EyeMos model is a single compartment, non-flow model used to study eye movements/saccades and convection.[325,330,331] Researchers from Genentech have developed a 3-compartment model to

determine permeability into the blood circulation from posterior cavity that fits data to the rabbit.[314] They have also described a static/no flow *in vitro* model (ExVit) designed to estimate protein stability after intravitreal injection, which is a crucial parameter to consider during the development of long lasting formulations.[332] The PK-Eye™ is a two-compartment, innovative, aqueous outflow model that estimates the clearance of biologics and their formulations[62,64,65,251,333,334] and can be used to establish IVIVCs for small molecules and implants.[333,334] The PK-Eye™ can help in the efficient screening and optimisation of formulations while reducing the use of animal models during the testing of suboptimal preclinical candidates.[65]

5.3. Drug distribution after intravitreal injection

Computer modelling studies have been extensively conducted to understand how a drug is distributed within the vitreous after drug administration.[328] Two common methods of computational modelling of drug distribution recognised by the FDA include Compartmental Modelling (CM) and Finite Element Analysis (FEA).[335,336] CM is a cruder method, where the globe is divided into large segments and rules applied to each one; whereas FEA can map very specific local variations in greater detail. Various research with computational modelling has been reported to model drug transport following intravitreal injection.[323,327,337–340] Others have reported the effect of drug distribution from intraocular or periocular implant[329,338,341] and for the development of IVIVCs.[342,343] Most studies describe the distribution of injected materials in the vitreous both theoretically and experimentally, but there has been much less described to correlate with real-time data on the distribution and elimination of drugs released from implants or ocular formulations.

There are several features about intravitreal injections that affect drug distribution and kinetics such as needle depth/size, location, angle, and injection speed/volume.[328,344] Some guidelines do exist for intravitreal injection strategy,[345] however, these features vary between different practices. Poiseuille's law describes the liquid flow resistance in a tube in relation to the radius of the tube.[346] Fluid resistance, or pressure, is proportional to the fourth power of the diameter and linearly related to length.[346] Therefore, smaller needle bore size will have a higher post-injection IOP due to less drug volume reflux from the injection site.[347] Different methods of fluid injection into the vitreous through the pars plana can result in a difference in the amount of injected fluid reflux from the needle,[348] which can reduce drug bioavailability.[349] Intravitreal injection procedures can result in a scleral wound with higher chances of retinal detachment and cystoid macular oedema with vitreous incarceration.[348]

The shape of the injected bolus volume also affects drug peak concentrations.[328] Most computational models consider a spherical/cylindrical shape as an accurate representation of a drug solution in the vitreous humour when developing the diffusive-convective transport

equation.[25,323,329,337,338,341] However, the shape of the injected bolus can alter depending on the viscosity of the vitreous or the type of vitreous substitute. For example, one study[328] reported the shape of injected Coomassie blue dye in different vitreous substitutes (HA, silicone oil and water).[328] The dye displayed a spherical shape at slower injection rates in silicone oil, whereas the dye showed an ellipsoidal distribution in HA.[328]

There will be significant concentration gradients within the vitreous due to the localised initial distribution of the drug and mass transfer; and changing the variables of an intravitreal injection or implant may affect the concentration gradients.[337] Different drug concentrations in the lens, vitreous and retina can be achieved with varying the position of an implant. In one study,[350] the effects of implant (cylindrical and disc) location on various parts of the posterior segment using a finite model was discussed. Drug concentrations in the vitreous was not significantly altered when the implant was located in the front and rear parts of the posterior segment.[350] Implant placement becomes more important for a specific (and sensitive) target like the macula. For example, higher drug concentrations can be achieved with an implant placement in the sub-retinal space instead of sub-Tenon. However, placing the implant closer to the macula (i.e. sub-Tenon) resulted in higher drug concentrations.[350] In general, subretinal administration is less accessible and less feasible.

5.4. Replacement, reduction and refinement of animal use in intravitreal drug development

The over-use of animal models has been criticised for its ethical and financial implications. Animals are used for all aspects of developing medicines for the eye. For example, over 20,000 rabbits were used for Draize eye irritancy tests in the UK in 2004 with more than 3 million animals were used for experimentation by 2011.[351,352] Researchers may face an austere environment of regulatory and welfare organisations of animals to conduct pharmacokinetic studies in animals. It is difficult to use what is observed in animals to predict efficacy and pharmacokinetics in humans. Animals are generally sacrificed at each time point for pharmacokinetic sampling.[301] These types of experiments are routinely conducted throughout preclinical development, however, there is a recognised need to reduce animal experimentation because much more is known about the limits of animals for translation[15] and about ocular pharmacokinetics, which can be modelled preclinically.[37] It has also become increasingly recognised that immunogenic animal responses (such as those seen with rabbits) may impact not only toxicologic and pharmacokinetic tests but also animal welfare, potentially leading to suspension or termination of such studies.[87,88]

According to the Ipsos report, the public's attitude towards the acceptability of the use of animals in all types of research was evaluated.[353,354] The results suggest that the general acceptance of using animals for all types of research was restricted to the situation of no alternatives. The percentage of people against animal testing from 2014 to 2018 was

comparable to those who gave a favourable response to an animal study.[354] Public acceptability is based on the purpose of the research.[354] There are many directives/policies aimed at decreasing animal model numbers needed during preclinical drug development.[355] In 2009, single dose acute toxicity studies were removed from the international pharmaceutical guidelines ICH M3, saving up to 100 rodents used per drug study.[356,357] Cross company sharing efforts and an addendum to ICH S6 has resulted in the number of non-human primates used in a typical monoclonal antibody development programme being reduced from 144 to 64.[358] Furthermore, cytokine release syndrome can be determined with well-powered and dynamic *in vitro* cytokine release assays.[359,360]

The concept of the 3Rs emphasised by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has begun to gain more traction since the centre's establishment in 2004.[207] It was first proposed in 1959 by Rex Burch and William Russell, and it involves the potential to refine animal use to avoid pain and distress to research animals, replace animals with non-animal systems and to reduce the number of animals required for studies in research.[361] The NC3Rs has opened the discussion amongst scientists for the adaptation of the way animals are used in research, pointing specifically to the idea that animal models are poor predictors of both human disease and safety.[362] Thus, this offers the opportunity to deliver more reproducible and cost effective results by the practice of better science through 3Rs technologies.[363] As a result of this work, many countries now have regulations and policies in place requiring the consideration of the 3Rs before animal approval for use in research or testing.[361]

From an economic standpoint, preclinical studies are generally costly in terms of effort, time and finances.[364] Typical costs in conducting animal research for ocular studies cover animal purchase, technician fees, sanitation supplies, medical and surgical supplies, equipment usage, space cost and animal husbandry costs.[363] High costs, ethical considerations and scientific limitations have thus begun to hinder the use of animal models for pharmacokinetic evaluation of drugs for ocular therapy.

6. Regulatory considerations

The average per-clinical study costs across all clinical phases is estimated to be $49.8 million (USD) for ophthalmology.[365] Since the first anti-VEGF intravitreal therapy (Macugen®), 26% of all FDA approved ophthalmic drugs were licensed for intravitreal administration (**Table 6**).[366] Despite this and the need for intravitreal therapies for retinal diseases, there is a lack of specific International Committee Harmonisation (ICH) or FDA Nonclinical Regulatory Guidance on intravitreal drugs. FDA requirements for marketing approval can be sought from submitted reviews for approved products. **Table 6** lists New Drug Applications (NDA) and BLAs made for intravitreal drugs to treat retinal diseases. All manufacturing processes must

be controlled and validated to ensure pure and potent drugs are produced reproducibly. Sterility, stability and shelf-life assurance must be provided with ongoing audit of manufacture and sterilisation processes to ensure quality is maintained. Regulatory compliance is necessary to ensure patient safety but also there is also a need for efficient preclinical studies to support innovation, and new therapies reaching patients. Preclinical modelling of candidate drugs may expedite drug development and reduce costs by narrowing down the number of potential drug candidates or formulations for subsequent studies.

For most intravitreal protein-based biologics (e.g. ranibizumab and aflibercept), pharmacodynamic studies include binding affinity to VEGF isoforms by surface plasmon resonance (SPR) analysis and changes in vascular permeability following intradermal administration to male hairless guinea pigs.[367,368] According to the BLA filed for ranibizumab and aflibercept, distribution pharmacokinetic studies were conducted in normal New Zealand White rabbits and pigmented rabbits respectively.[367,368] The vitreous, aqueous humour and serum, and retinal tissue following intravitreal, subconjunctival and intracameral administration were analysed.[367] Pharmacokinetic modelling was then performed in rabbits and cynomolgus monkeys following intravitreal and intravenous administration using enzyme-linked immunosorbent assay (ELISA).[367,368] Toxicology studies included 4,13,16 and 26-week repeat dose intravitreal toxicity studies in cynomolgus monkeys and local tolerance testing in rabbits. Measurements included clinical observations, IOP, electroretinography (ERG), fluorescein angiography and ocular photography, histopathology, vitreal sampling for toxico-kinetics and serum antibody analysis. Cross-reactivity of biologic with cryosections of normal human tissue was conducted with immunohistochemistry. Haemolytic potential, blood compatibility and vitreal fluid compatibility testing assessed the suitability of the drug product and formulation.[367,368]

Phase I and II studies include open-label studies single-dose escalation studies and repeated dosing regimen studies. Multiple double masked randomised controlled phase III studies are conducted to determine non-inferiority or superiority compared to controls. Primary endpoints were the group who lost fewer than 15 letters in visual acuity one year compared to baseline. Clinical studies also collect data on systemic and ocular adverse events. It has been reported that the systemic delivery of anti-VEGF antibodies can lead to potential adverse effects such as hypertension, myocardial infarction, gastrointestinal perforations, stroke, thromboembolic events and kidney diseases.[369] Some intravitreal anti-VEGF medicines have been reported to show detectable levels in the systemic circulation that may significantly suppress systemic VEGF levels.[370–372] Long term follow-up post-market approval studies need to be monitored for these events.[373]

Table 6. New Drug Application (NDA) and Biologic License Application (BLA) made for intravitreal drugs to treat retinal diseases

Product	Application Number	Date of Approval	Indication	Route of Administration	Pivotal Clinical Studies
Abicipar pegol	BLA filing	Pending	wAMD	3-monthly IVT	CEDAR, SEQUOIA
Avastin® (bevacizumab)	BLA 125085	2004	(off label ocular use) Metastatic colorectal carcinoma	4-weekly IVT	CATT, IVAN, GEFAL, MANTA, LUCAS
Beovu® (brolucizumab)	BLA 761125	2019	Wet AMD	3-monthly IVT	HAWK, HARRIER
Eylea® (aflibercept)	BLA 125387	2011	wAMD, DME and RVO	8 or 12-weekly IVT	VIEW 1/ 2, VISTA/VIVID
Iluvien® (fluocinolone acetonide)	NDA 201923	2014	DME	Implant 36 months	FAME
Jetrea® (ocriplasmin)	BLA 125422	2012	Sympto-matic VMA	Single dose IVT	TG-MV-006/ TG-MV-007
Lucentis® (ranibizumab)	BLA 125156	2006	wAMD, DME and RVO	4-weekly IVT	ANCHOR/MARINA, RISE/RIDE/RESTORE
Macugen® (pegaptanib sodium)	NDA 21756	2004	wAMD	6-weekly IVT	EOP1003 /1004
Ozurdex® (dexamethasone)	NDA 22315	2009	DME	3 monthly implant	MEAD
Retisert® (fluocinolone acetonide)	NDA 021737	2005	Chronic non-infectious uveitis	IVT implant	BLP 415-001, BLP 415-004, MUST
Vitrasert® (ganciclovir)	NDA 020569	1996	CMV Retinitis	IVT implant	-

Abbreviations: wAMD: exudative or wet age-related macular degeneration; BLA: biologics license agreement; DME: diabetic macular edema; IVT: intravitreal; NDA: new drug application; RVO: retinal vein occlusion, VMA: vitreomacular adhesion.

Special considerations need to be made for intravitreal implants. Biodegradable components of intravitreal implants must be shown to be biocompatible and can be demonstrated in chronic intravitreal ocular toxicity studies in appropriate species.[374] PLGA is the biodegradable component of Ozurdex®. PLGA polymers have been used safely for up to 28 years in a number of commercial medicinal products such as absorbable sutures Vicryl.[373]

Polyvinyl alcohol (PVA) is another polymer used in ophthalmology with a long track record in medical applications. Iluvien® implant comprises of a PVA matrix inside a polyimide tube with a reported release rate of 0.25 µg/day of fluocinolone acetonide for a 36-month period. Nonclinical toxicological program includes determining the ocular toxicity and pharmacokinetics of such materials for 24 months in rabbits. An additional 9 months is required for ocular toxicity in rabbits with forced degradation in an accelerated ageing chamber.[375] Additional biocompatibility studies of the polyimide tubing are required.[375] Applicators of intravitreal implants must be easy to operate and safely insert, and easy to dispense the implant.

There are risks with the use of off-label compounded intravitreal drugs. The safety, effectiveness and quality of compounded drugs are not evaluated; hence they are not approved by a regulatory agency. Issues such as sterility assurance, reformulation and change in route of administration can potentially pose a risk to patients. Kenalog® is a common off-label intravitreal injection that is used in clinic.[376] Due to safety reports of sterile endophthalmitis and larger aggregate size inducing retinal cytotoxicity, a preservative-free formulation is available.[377] Furthermore, the preservative is potentially toxic, and without dilution or washing this may lead to retinal damage or death. Triescence™ (Alcon Inc.) is a preservative-free triamcinolone acetonide preparation used in compounding pharmacies.[146,378,379]

Studies have shown that compounding bevacizumab to treat wAMD causes protein aggregation in the formulation,[380] and ocular inflammation,[381–384]infection[382,385] and formation of floaters.[386–388] The repackaging process may introduce silicone oil microdroplets into the formulation.[380,389] Compounding, refilling, mechanical shock and multiple freeze-thaw cycles carries risk for aggregation, particle generation and formation of silicone oil microdroplets.[389,390] Sufficient padding and use of shock indicators in shipping containers can reduce the chances of mechanical shock.[390] FDA report specific and careful comments on the preparation and handling of bevacizumab syringes. Discrepancies in the report have been noted as the number of syringes filled and vials used per batch was not always documented on compounding and repackaging.[90,391] Bevacizumab can be repackaged into multiple single dose syringes but would not be accepted if bevacizumab is mixed, repackaged or diluted.[392]

Recently, the FDA has announced safety alerts on compounded intravitreal injections containing vancomycin after cases of haemorrhagic occlusive retinal vasculitis (HORV, a rare and sight threatening condition) were reported.[393] It is unclear if these adverse events were caused directly from vancomycin or the compounding with triamcinolone and moxifloxacin for intravitreal use. A higher risk of HORV is possible with the general prophylactic use of intravitreal vancomycin, whether used alone or in a compounded drug combination (with

presence of other multiple active ingredients).[393] Hence, the safety and efficacy of intravitreal vancomycin still requires to be achieved with well-controlled trials.[393]

According to the FDA guidance "Nonclinical Safety Evaluation of Reformulated Drug Products and Products Intended for Administration by an Alternate Route" (2015), a change in route of administration or formulation of a pre-existing drug should prompt a re-evaluation of toxicity.[394] Evidence from the literature can be used, however, establishing a clinical bridge would need to be made. Additional nonclinical studies to confirm safety of change in route of administration especially if duration of use is markedly different or different excipients are used.[394] Toxicity studies (ocular and systemic) in two species are required for new formulations that have not been previously administered by a certain ocular route.[394] Ocular toxicity can be evaluated with fundoscopy, electroretinography and histopathology, slit lamp biomicroscopy and tonometry.[394] Complete clinical formulation groups and vehicle control are required for accurate ocular studies. It is important to investigate the systemic exposure and ocular tissue distribution. Although histological evaluation may be limited to locally exposed tissues if systemic exposure by a new route of administration is equivalent to or less than the approved route. Durations of the toxicity studies should follow the recommendations outlined in ICH M3(R2) or ICH S9.[358]

7. Conclusions

The widespread use of intravitreally injected protein-based medicines has fundamentally changed how retinal diseases are now treated. A critical unmet need is to minimise the number of intravitreal injections that patients need over time to treat progressive chronic conditions. Maximising the duration of action of new intraocular drugs poses preclinical challenges. New dosage forms must first above all requirements maintain formulation stability and avoid protein aggregation to reduce the cases of ocular tolerability. Developing long-acting intravitreal therapies requires knowledge of intraocular mass transfer processes, the varying nature of the vitreous and the limitations of animal models. The review has highlighted the difference in human and animal models in terms of its anatomical features (e.g. vitreal path length), aqueous outflow, immune response and organisation of vitreous macromolecular structure. It is crucial to consider these factors and the effects of ageing, changes in the vitreous structure, differences in inter-species viscosity and effects of vitrectomy on drug diffusion and distribution.

Evaluating human ocular pharmacokinetics is expected to remain prohibitively invasive, so efforts to develop appropriate preclinical models to mimic intraocular mass transfer and clearance mechanisms will be important to accelerate preclinical development and may also become a viable surrogate for modelling human intraocular pharmacokinetics. Regulatory considerations for the next generation of intravitreal medicines will be based on

criteria and clinical data from the first generation of antibody-based intraocular medicines. Knowledge of the regulatory landscape will be important for effective preclinical development.

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