Experimental Vitreous Substitution

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Experimental vitreous substitution

Henrik Barth

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Abstract

Blindness and visual disability are common following vitreoretinal pathologies such as open globe injury, proliferative diabetic retinopathy, and rhegmatogenous retinal detachment (RRD). These conditions often necessitate surgical intervention using vitrectomy with a tamponading vitreous substitute. However, current tamponades are all associated with complications such as inflammation, cataract, glaucoma, and optic nerve atrophy. Translation of new vitreous substitutes into clinical use has proven to be challenging, due to a lack of a comprehensive methodology and numerous requirements; bio-compatibility and clinical.

In this thesis, we explore several novel vitreous substitutes using newly developed methods with the ultimate goal of clinical translation.

First, in an in vitro adult rat retinal explant culture assay, polyethylene glycol, and Bio-Alcamid® gels provoked retinal degeneration, while a cross-linked hyaluronic acid hydrogel, Healaflow®, matched, and even surpassed the preservation of structure when compared with medium only. Secondly, Healaflow® used as a vitreous substitute in an in vivo rabbit vitrectomy model revealed practical usability and favorable bio-compatibility. In a third study, vitreous substitutes with disparate biocompatibility profiles (silicone oil, Healaflow®, Bio-Alcamid®, and BSS) elicited different patterns of intrinsic and extrinsic retinal inflammation in vivo. Finally, a new rabbit repeat vitrectomy RRD-model revealed excellent tamponading effect of the Healaflow® gel.

The combination of the presented in vivo and in vitro methods comprise a new paradigm in translational development of novel vitreous substitutes. Healaflow® stands out as a promising candidate for future clinical use as a vitreous substitute.

Key words: Vitreous substitute, vitreoretinal surgery, retinal detachment, retinal culture, methods, immunohistochemistry, electrophysiology, hyaluronic acid

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2018

Cover: A novel vitreous substitute, the cross-linked hyaluronic acid hydrogel Healaflow®

Front cover photo by Henrik Barth and Sven Crafoord
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To Linda,
for giving me my very own pale blue eyes
to linger on

"I draw a pair of new eyes in my mind
Reaching out, who knows what I could find" — *Anna Ternheim*

CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. **Barth H**, Crafoord S, O'Shea TM, Prichard CD, Langer R, Ghosh F. A new model for *in vitro* testing of vitreous substitutes. Graefe's archive for clinical and experimental ophthalmology. 2014.

II. **Barth H**, Crafoord S, Andréasson S, Ghosh F. A cross-linked hyaluronic acid hydrogel (Healaflow®) as a potential vitreous substitute. Graefe's archive for clinical and experimental ophthalmology. 2016.

III. **Barth H**, Crafoord S, Arnér K, Ghosh F. Inflammatory responses after vitrectomy with vitreous substitutes in a rabbit model. Submitted manuscript. 2018.

IV. **Barth H**, Crafoord S, Ghosh F. A new retinal detachment model for *in vivo* testing of vitreous substitutes with repeated pars plana vitrectomy. Manuscript. 2018.

ABSTRACT

Blindness and visual disability are common following vitreoretinal pathologies such as open globe injury, proliferative diabetic retinopathy, and rhegmatogenous retinal detachment (RRD). These conditions often necessitate surgical intervention using vitrectomy with a tamponading vitreous substitute. However, current tamponades are all associated with complications such as inflammation, cataract, glaucoma, and optic nerve atrophy. Translation of new vitreous substitutes into clinical use has proven to be challenging, due to a lack of a comprehensive methodology and numerous requirements; bio-compatibility and clinical.

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ABBREVIATIONS

BA	Bio-Alcamid®
DIV	Days *in vitro*
ERG	Electroretinogram
ERM	Epiretinal membrane
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
HA	Hyaluronic acid
H&E	Hematoxylin and eosin
HF	Healaflow®
ILM	Inner limiting membrane
INL	Inner nuclear layer
IOFB	Intraocular foreign body
IOP	Intraocular pressure
IPL	Inner plexiform layer
IS	Inner segment
MH	Full-thickness macular hole
NFL	Nerve fiber layer
OLM	Outer limiting membrane
ONL	Outer nuclear layer
ONH	Optic nerve head

OPL	Outer plexiform layer
OS	Outer segment
PDR	Proliferative diabetes retinopathy
PFCL	Perfluorocarbon liquid
PEG	Polyethylene glycol
PKC	Protein kinase C
PPV	Pars plana vitrectomy
PVR	Proliferative vitreoretinopathy
PVD	Posterior vitreous detachment
RD	Retinal detachment
RRD	Rhegmatogenous retinal detachment
RPE	Retinal pigment epithelium
SF6	Sulfur hexafluoride
SO	Silicone oil
VEGF	Vascular endothelial growth factor
VMT	Vitreomacular traction

INTRODUCTION

The anatomy and physiology of the eye

Introduction

Vision may be the most precious and important of our senses. Since long before humanity's nascent steps on the African savannah, the eyes have been highly evolutionary prioritized and protected, clearly essential for the survival of most higher organisms. Throughout our history, a loss of vision would have been catastrophic, leading to the demise of the individual. The eye is a complex organ designed to enable us to perceive light stimuli from the surroundings. It is intricately designed, with a large number of highly specialized and complex parts working together in unison. In order to work together, all components must fulfill their respective purpose—from the light transducing and refractive properties of the cornea and lens; to the detection, processing and relay of the stimuli in the retina and optic nerve.

The anterior and posterior segments

The eye is often anatomically divided into the anterior and posterior segments. The former consists of the cornea, iris, and lens; structures with the main function of focusing and transducing the light. It also includes the ciliary body and the anterior chamber, a fluid-filled space between the cornea and iris. This fluid, the aqueous humor, is produced in the ciliary body and it is vital for the maintenance of the intraocular pressure and the transport of nutrients and other soluble molecules. The posterior segment encompasses all the structures of the inner eye; the vitreous, retina, choroid, and the optic nerve. It includes the structures involved in detection, processing, and transmission of the light stimuli.

The vitreous

The vitreous is a hydrogel, filling the space behind the crystalline lens and comprising approximately 4 ml in humans; about 2/3 of the volume of the eye. It is a transparent, gel-like substance, often considered a mere space-filler. It has, however, several important and often over-looked functions. Its transparency and refractive index allows for the unhampered passage of light to the retina. The vitreous also offers structural support and an environment in which molecular transport is well-regulated. With increasing age, the vitreous liquifies, a process associated with posterior vitreous detachment; usually considered a benign phenomenon, but it is, however, associated with several retinal pathologic conditions.

The structure of the vitreous can be pictured as a viscoelastic hydrogel, with about 98–99% water, reinforced by a network of collagen and hyaluronic acid. It also contains a range of other compounds such as different salts, carbohydrates, and proteins. The vitreous has a pH in the range of 7,0–7,4 and a refractive index of 1,336 [Baino 2010]. Its outer layer, the vitreous cortex, is loosely attached to the retina in most areas, with tighter connections formed by collagen fibrils at the ora serrata and optic disc. The vitreous cortex is often referred to as the vitreous (or

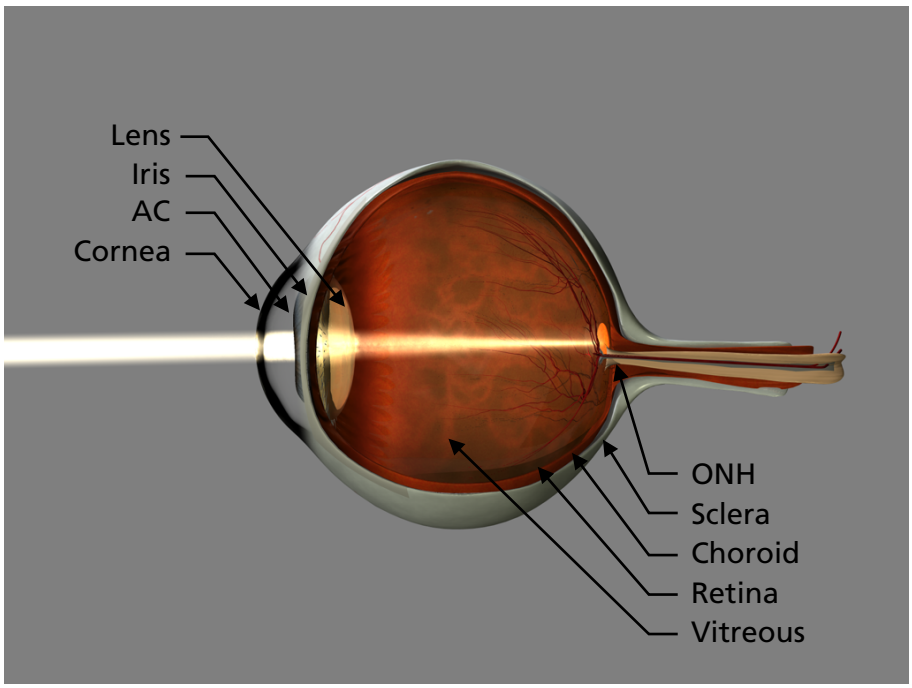

Figure 1. The path of the light through a human eye.
Abbreviations: Anterior chamber (AC), optic nerve head (ONH). Image by Fredrik Ghosh.

hyaloid) membrane, especially in clinical contexts, where its anterior and posterior surfaces are important landmarks. The vitreous is largely acellular albeit with a small number of cells present under physiological conditions, mainly in the posterior vitreous cortex. These cells, the hyalocytes, are closely related to macrophages but have distinctly different features, and contribute to the maintenance of the avascular and transparent structure of the vitreous by phagocytosis and fibrinolysis. In addition, the hyalocytes may have a role in the regulation of ocular immunity. The hyalocytes also play a part in the pathogenesis of several pathological conditions such as proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), macular pucker (also referred to as epiretinal membrane), and macular holes (MH) [Sebag 2014].

The structure of the vitreous is non-homogenous, with increased concentration of hyaluronic acid, and a corresponding higher viscosity, in the posterior part. The hydrogel allows for the diffusion of nutrients and other molecules but affects the way the solutes are transported. One instance of this kind of interplay is the upkeep of the physiological gradient of oxygen, ranging from high levels at the retinal surface to very low at the posterior surface of the crystalline lens. This gradient is significantly affected by vitrectomy [Stefánsson 2009]. It is suggested that the removal of the normal vitreous may contribute to the formation of nuclear cataract in post-vitrectomy eyes by increasing the oxygen exposure of the crystalline lens from its naturally low level, both acutely and in long-term [Barbazetto 2004, Holekamp 2005]. Furthermore, the faster diffusion and convection of oxygen, along with increased clearance of cytokines like vascular endothelial growth factor (VEGF) appears to be beneficial in diseases like diabetic macular edema and AMD. Interestingly, there seems to be a correlation between vitrectomy and development and progress of open-angle glaucoma, especially in pseudophakic eyes. It is hypothesized that this may occur due to disturbances in the physiological gradients, either of oxygen or other compounds [Chang 2006, Stefánsson 2009, Siegfried 2010].

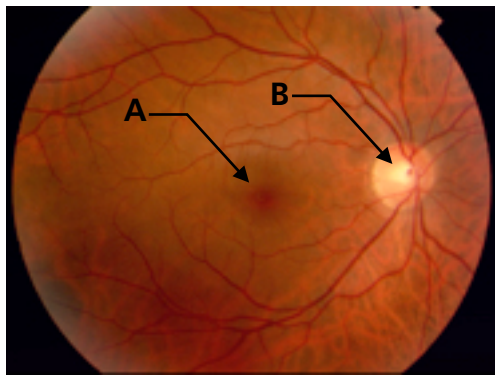

Figure 2. Photo of a healthy human retina, as seen on a clinical examination.

A) The macula lutea. B) The optic nerve head. Image courtesy of Fredrik Ghosh.

The retina

The retina is responsible for the detection of light impulses and covers the entire inner wall of the eye like a wallpaper. Its anterior boundary, the *ora serrata*, is located posterior to the ciliary body. The *macula lutea* region is situated in the posterior pole of the human eye. The central part of the macula, the fovea, is the only retinal location that allows for high-resolution vision. In addition to the detection of light, the retina converts the stimuli to electrical signals and pre-processes the information before relaying it to the visual cortex of the brain. The retina, thereby, fills several crucial roles in the process that creates the visual sensations.

The structure of the retina is highly organized, with a laminar configuration clearly evident in histological sections. Eight layers are usually recognized; the outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL). Additionally, there is a basal membrane sthat separates the retina from the vitreous space, the inner limiting membrane (ILM), which is adherent to the posterior vitreous membrane in young individuals. Bordering the retina on the other side is the retinal pigment epithelium (RPE).

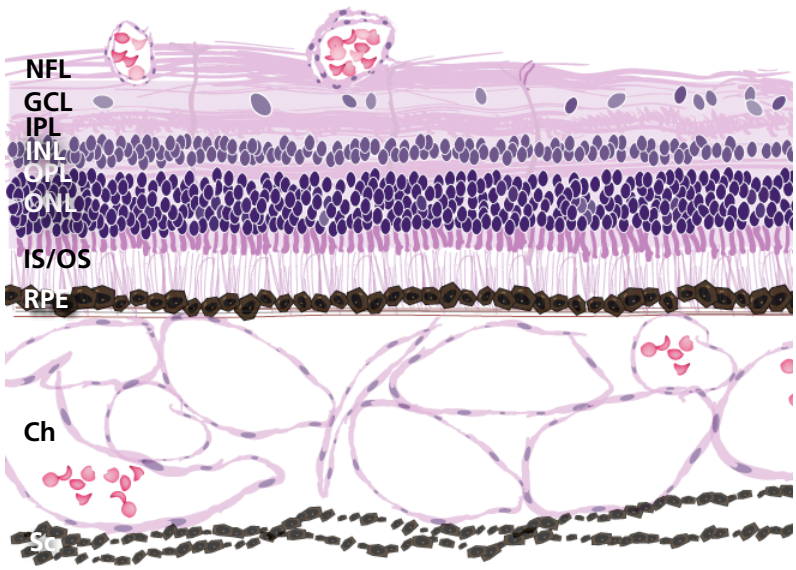

Figure 3. The laminar architecture of the retina.

From top to bottom: The nerve fiber layer (NFL) with retinal blood vessels, the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), the photoreceptor inner and outer segments (IS/OS), the retinal pigment epithelium (RPE), the choroid (Ch) with its blood vessels, and the sclera (Sc). Image by Fredrik Ghosh.

Apart from the retinal blood vessels, the cells of the retina belong to two classes: neurons and glial cells. The neuronal cells are often further subdivided into subtypes: photoreceptors, bipolar cells, ganglion cells, and the modulating cells; horizontal cells, amacrine, and interplexiform cells.

The cell bodies of the photoreceptors are located in the ONL, with outer segments (OS) specialized for phototransduction oriented towards the RPE, and processes ending in synapses in the OPL. The OS are made up of membrane discs, where light impulses are detected by photopigments (opsins). There are several different opsins, detecting different wavelengths of light. The OS are intimately, but loosely, connected to the microvilli of the RPE. The human retina is equipped with two variants of photoreceptors; cones for highly detailed color vision and light-sensitive rods for poorly lit environments. The cones are divided into three types with different opsin populations, and the human retina is therefore classified as trichromate. The cones are concentrated to the *macula lutea*, where their density is very high, while the periphery is rod-dominated with a much lower cone density. Transduced signals elicited by light stimuli are synaptically transferred to the bipolar cells in the OPL and further relayed to ganglion cells in IPL. Along this signaling path, the signal is modulated by horizontal cells, amacrine cells, and interplexiform cells, allowing for pre-processing of the visual information. The cell bodies of bipolar cells and most modulating cells are located to the INL. There are several subtypes of bipolar cells, among which the rod bipolar cells relay their signal through the AII amacrine cells to cone bipolar cells, which synapse directly with ganglion cells. The cell bodies of the ganglion cells are located in the inner part of the retina, in the GCL, with their axonal processes forming the innermost layer of the retina, the NFL.

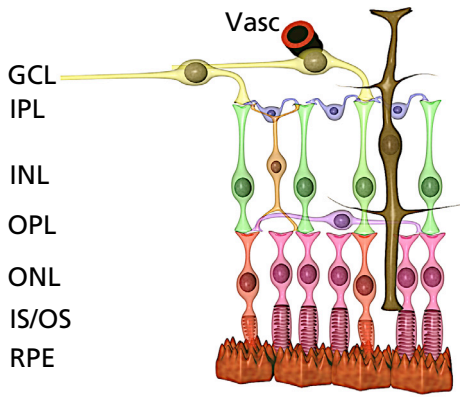

Figure 4. The cellular organization of the retina. *The cells of the neuroretina; Ganglion cells (yellow), bipolar cells (green), horizontal cells (purple), amacrine cells (blue), interplexiform cell (orange), and photoreceptors (rods in pink and cones in red). The retinal laminar architecture; the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), and the inner and outer segments (IS/OS). Also shown: the retinal pigment epithelium (RPE) and retinal vasculature (Vasc). Image by Fredrik Ghosh.*

The primary glial cells of the retina are the Müller cells which fulfill a variety of functions essential for the retinal micro-milieu, such as physical, mechanical, and metabolical support, as well as involvement in the processes of neurotransmission. The elongated Müller cells span vertically throughout the entire retina, encircling

neuronal cells and blood vessels. There are two other principal types of glial cells in the retina: the astrocytes and microglia. The microglia are the mononuclear phagocytes of the central nervous system, playing a regulatory role as well as acting as sentries activated by inflammatory responses. Astrocytes have a supportive role in the barriers surrounding blood vessels and axons.

The neurosensory retina is attached to the RPE which, in addition to retinal adhesion, also has several important metabolic and support functions such as supplying nutrients, phagocytosis of photoreceptor outer segments, contributing to the blood-retina barrier, as well as to the synthesis of inter-photoreceptor matrix. Due to its extremely high metabolic activity, the retina has the highest demand for oxygen of all human tissue, relative to its weight. The retinal blood supply, therefore, fills a crucial role. Human retina relies on retinal blood vessels as well as diffusion from the choroidal circulation; this is defined as the holangiotic (full) vascularization pattern.

The immune system of the eye

In order to protect the individual from the potentially deleterious effects of vision loss, the ocular immune system has evolved highly prioritized and specialized mechanisms to guard the eye against infections. A large part of the ocular immune system resides in the uvea: the choroid, iris, and the ciliary body. Clinically relevant intraocular inflammation is therefore often referred to as uveitis. The principal resident immune cells of the uvea are macrophages, dendritic cells, and mast cells, together with small numbers of T-cells [McMenamin 1997]. Upon activation, these cells can readily disperse through the highly vascularized tissues of the eye. The resident immune cells of the retina are the phagocytic and antigen-presenting microglia, closely related to macrophages [Yang H 2002].

Due to the potent nature of the immune system, and the risk of potentially dangerous auto-immune reactions, the ocular immunity is highly regulated through the so-called ocular immune privilege. This phenomenon was first described in the 1940s, after the discovery that foreign antigen placed in the anterior chamber of the eye provoked much fewer and milder rejection reactions compared with the rest of the body. Immune privilege has since been found in other, mostly evolutionary critical sites such as the brain and the reproductive organs. To achieve immune privilege while still maintaining adequate protection from infections, several strategies are employed; immunological ignorance, intraocular immune suppression, and peripheral tolerance to antigens from the eye. Contributing factors to achieve this are, respectively; physical barriers such as the blood-retina barrier, soluble (such as TGF-β) and cell-bound immunosuppressive factors (in Müller and RPE cells amongst others), and specialized immune-response such as the anterior-chamber associated immune deviation (ACAID) [Streilein 2003, Zhou 2010].

Vitreoretinal surgical disease

Retinal detachment

One of the most serious emergencies in any clinical ophthalmological practice is retinal detachment (RD). The symptoms are dramatic and distressing; a dark shadow successively envelops more and more of the visual field over the course of hours to days. Untreated, it can ultimately lead to blindness or severe debilitation. The most common form of this condition, rhegmatogenous retinal detachment (RRD), affects about 1 out of every 300 individuals during their lifetime [Haimann 1982]. It has a yearly incidence of about 14:100,000 in Sweden [Algvere 1999].

RRD usually starts with the seemingly innocuous but sudden emergence of floaters or other mobile opacities in the visual field, frequently accompanied by photopsia: flashes or other light sensations. These are symptoms of vitreous detachment, a condition arising from physiological, age-related degeneration of the vitreous body. This process develops with increasing age and includes posterior vitreous detachment (PVD): the complete or incomplete separation of the posterior surface of the vitreous from the internal limiting membrane (ILM) of the retina. PVD causes traction between the vitreous and retina, especially in the periphery where the adhesion between the ILM and the posterior vitreous membrane is firm. If the traction is strong enough, or if there are pre-existing lesions or weaknesses in the retina, this may result in peripheral breaks through the neuroretina. If vitreous derived fluid enters these breaks and into the sub-retinal space, the adhesion between the retina and RPE may be broken, and the retina can thus start to detach progressively.

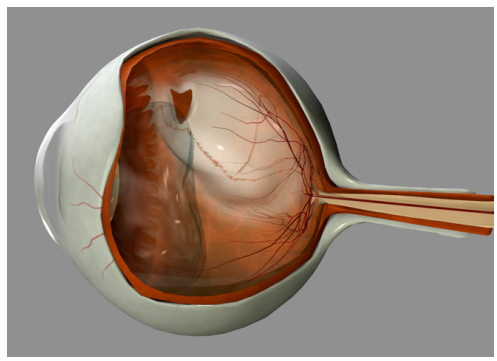

Figure 5. Rhegmatogenous retinal detachment. *The pale area represents detached retina. Note the retinal break in its superior part and the adjacent detached vitreous. Image by Fredrik Ghosh.*

Risk factors for RRD include myopia (short-sightedness), earlier RRD in the fellow eye, and genetic factors. Previous cataract surgery also increases the risk, especially procedures complicated by posterior capsule rupture. Other underlying conditions that may cause retinal detachment includes retinal lattice degeneration, blunt trauma, tumors, and uveitic disease.

The prognosis is highly dependent on early diagnosis and treatment, and the visual function can often be at least partly restored with timely treatment. The most critical factor for visual recovery is the status of the macula; a successfully treated patient with macula-on detachment can often completely regain their initial visual acuity, while a macula-off detachment usually leads to a profound, permanent loss of vision despite anatomic reattachment. The presence of fibrotic tissue, proliferative vitreoretinopathy (PVR) is another factor that negatively affects the prognosis and reattachment rate.

RRD treatment options are all surgical, aiming to decrease the vitreoretinal traction and reattach the retina. A historically common surgical method for the treatment of retinal detachment is scleral buckling, in which the vitreoretinal traction is alleviated by placing an encircling band or a piece of silicone to the exterior of the eye, pushing the sclera closer to the detached retina. The method does not per se require any surgical procedures to the interior parts of the eye, and is thus less prone to cause severe infections and cataract development than other methods that do, and is, therefore, still routinely used in some surgical centers, especially for younger patients. This surgical technique has largely been replaced by vitrectomy (see below) which more fully addresses the pathophysiology of RRD by removing the vitreous, thereby permanently treating the issue of vitreoretinal traction. These methods are often combined with cryo- or laserpexy, where the induction of scar tissue create firm adhesion between the retina and RPE.

Macular hole

Although vitreous detachment is a normal age-related condition that most people go through during their lifetime, it is related to several pathological conditions. Similar to the pathogenesis of the aforementioned retinal breaks, the pull on the retina by the posterior hyaloid membrane may cause vitreomacular traction (VMT). This syndrome arises when there is persistent traction on the fovea by adherent vitreous in the case of an incomplete PVD. This structural stress leads to deformation of the fovea and separation of the retinal layers with the emergence of intraretinal cysts, and resultant distortion and deterioration of the vision. The prevalence of VMT is about 22,5:100000. The condition may resolve spontaneously but can also progress further; defects might then form in the neuroretinal layers and eventually develop into a full-thickness macular hole (MH). The structural changes of a MH permanently damage the fovea over time, wherefore early treatment is important for the prognosis. MH is about two times more common in women than men, with an age-adjusted yearly incidence around 8:100000 in the general population. Select cases of MH and VMT might be treated with pharmacological vitreolysis, but vitrectomy with gas tamponade is still the gold standard [García-Layana 2015].

Proliferative diabetic retinopathy

Diabetes is a common disease that causes a number of severe complications and afflicts around 2,8% of the global population [Wild 2004]. One of the most feared complications of diabetes is the advanced stages of proliferative diabetic retinopathy (PDR), which can lead to blindness. According to WHO, it is the fifth most common cause of visual impairment globally, causing 4,8% of the cases of blindness [Resnikoff 2004]. Diabetic retinopathy involves pathological changes in the retinal blood vessels and impaired circulation, leading to hypoxia in the retinal tissue. As a response to the ischemia, a cascade of growth factors such as vascular endothelial growth factor (VEGF) stimulates the production of new retinal blood vessels. This proliferation creates growth of fragile, pathological blood vessels along the retina and into the vitreous space, prone to leakage and bleeding. Persistent vitreous hemorrhage can severely affect the vision and hinder the crucial retinal laser treatment. PDR can also cause severe fibrovascular proliferation, leading to tractional retinal detachment and threatening the vision. These complications are usually treated by a combination of retinal photocoagulation, anti-VEGF, and vitrectomy with a tamponade such as gas or silicone oil.

Penetrating ocular trauma

Traumatic injury to the eye is one of the leading causes of blindness and visual disability affecting young people, globally as well as in the industrialized world. Of these, open globe injury with, or without intraocular foreign bodies (IOFB) are often the most dramatic due to their sudden, unexpected and severe characteristic. Open globe injuries predominantly affect young males, with as much as 92–100% of the IOFBs treated in the USA occurring in this demographic [Loporchio 2016]. The trauma is most often work-related, and due to activities such as hammering metal on metal and power tool usage, but may also arise from a wide range of situations from assault to leisure activities.

The damage to the eye is often complex, affecting multiple structures such as the cornea, iris, lens, and the retina, all vulnerable to direct and secondary complications. Due to this, the prognosis is often dire. The first-most priorities with an open globe injury are treatment with antibiotics to prevent severe infections such as endophthalmitis, and the closure of wounds and lacerations which must be addressed as soon as possible, at the latest within the first 24–48 hours after the trauma [Kuhn 2014]. Depending on the nature of trauma and available surgical competence, vitrectomy and lens surgery is often indicated either at the primary surgery or as a secondary repair within 1–2 weeks. As a rule, IOFBs must be removed since they generally increase the risk for complications such as infections and toxic reactions.

The majority of IOFBs reside in the posterior segment and therefore require treatment with vitrectomy, often accompanied with a tamponade such as gas or silicone oil. Other complications to penetrating trauma that requires vitrectomy include traumatic retinal detachment and proliferative vitreoretinopathy.

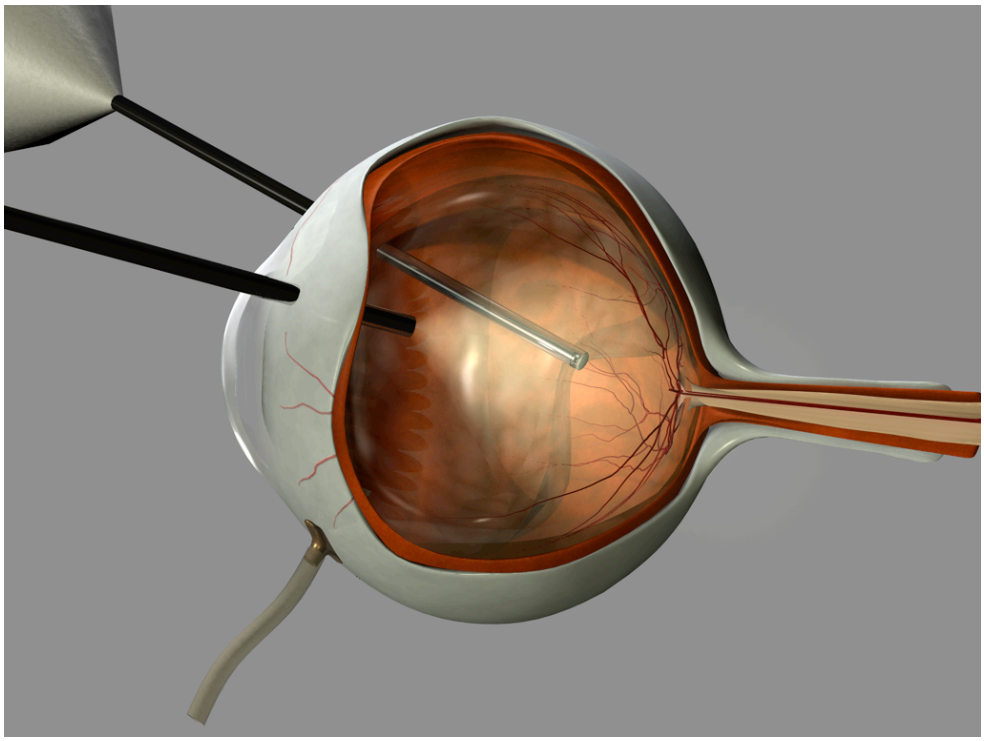

Figure 6. Vitrectomy.
Instrumentation from above: vitreous cutters at work, endoillumination probe, and infusion of balanced salt solution (BSS). Image by Fredrik Ghosh.

Vitrectomy

In order to treat vitreoretinal pathology, there is often a need for an internal surgical approach. Vitrectomy is a surgical treatment routinely used for a number of disorders of the eye in which parts of, or the majority of the vitreous is surgically removed. The cutting and removal of the vitreous gel with specialized equipment enable further surgery and manipulation of the structures of the posterior segment. Vitrectomy can be the sole treatment or used in conjunction with other procedures, such as scleral

buckling. Although mainly practiced in larger surgical centers, it is a common and crucial intervention in the treatment of a variety of conditions including rhegmatogenous retinal detachment, (RRD) severe diabetic retinopathy, penetrating ocular trauma, macular holes and epiretinal membranes (ERM), as well as complications to cataract surgery.

Vitrectomy was pioneered by Robert Machemer in 1969, originally as a means to remove vitreous hemorrhage and other opacities. Further developments of the instrumentation and techniques soon allowed the treatment of an extending range of conditions [Machemer 1995]. Modern surgical techniques utilize surgical microscopes together with specialized optical setups and intraocular illumination to offer high magnification and quality of visualization. Miniaturization of the equipment has successively allowed for decreased size of the sclerotomies. Current surgical setups are often sutureless, using transconjunctival small-gauge (25 G or 27 G) trocars.

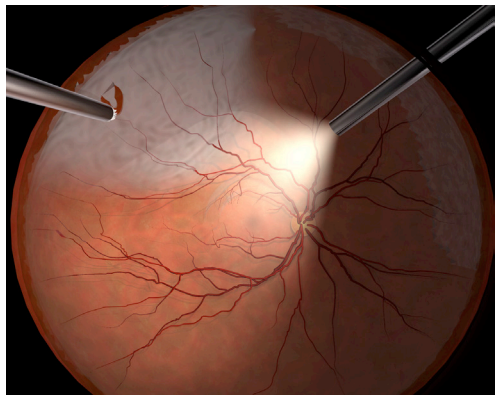

Figure 7. Vitrectomy, a surgeons view.
Vitreous cutters (left) and endoillumination probe (right). The pale area superiorly represents a retinal detachment with retinal break. Image by Fredrik Ghosh.

As mentioned above, vitrectomy is assuming an increasingly dominant position in the treatment of retinal detachments (RD). In RD surgery, the retina is reattached by active aspiration of subretinal fluid, sometimes accompanied by mechanical reattachment with a heavy fluid such as perfluorocarbons (PFCL). Endolaser probes enable internal photocoagulation in order to treat pathological retinal blood vessels such as diabetic proliferations, as well as to seal retinal breaks and increase the adhesion between the reattached retina and RPE in RD surgery. In addition to extraction of the vitreous, removal of fibrotic tissue and membranes in PVR and ERM is made possible by micro-forceps and other specialized instrumentation. At the end of the surgery, a fluid–gas exchange is commonly used to install a gas or silicone oil tamponade to seal retinal breaks.

Vitrectomy is considered a relatively safe procedure, but it is, like all surgical treatments, associated with complications. In the early post-operative period, complications such as anterior uveitis, pressure spikes, and endophthalmitis might occur, as well as RD caused by iatrogenic retinal breaks. The normal recovery after vitrectomy is well-known to elicit inflammation, wherefore topical steroids are routinely prescribed postoperatively [Ben Yahia 2016, Yasuda 2016]. This response includes

elevated levels of cytokines such as IL-6, IL-8, MCP-1, and TGF-β1 in the aqueous humor, but postoperative inflammatory reactions within the retina are still unclear [Hoerster 2013]. There are also considerable changes to the physiology of the surrounding tissue caused by the removal of the vitreous, and the consequent disruption of naturally existing gradients of oxygen and cytokines such as VEGF [Stefánsson 2009].

Vitreous substitutes

Background

During vitrectomy, the vitreous is cut and aspirated and must thus be replaced to prevent hypotony of the eye, either with saline solution or a vitreous substitute with more specific properties. A solution such as Balanced Saline Solution (BSS) is infused during surgery and is sometimes sufficient as a filler. In time, this BSS will be replaced by aqueous fluid derived from the ciliary body. In most cases, such as in RD surgery, there is, however, a need for a temporary tamponade to close retinal tears and prevent redetachment during the healing period. Due to the permanent changes to the physiology of post-vitrectomy eyes, an argument has been made for the need for a permanent vitreous substitute to alleviate potential long-term complications [Holekamp 2005]. Another potential indication for intravitreal substitution is as a vessel for the long-term administration of intravitreal drugs such as VEGF [Duvvuri 2007, Liu 2010].

Clinical use

In clinical practice, the most common vitreous substitutes are tamponading gases such as air, sulfur hexafluoride (SF6), hexafluoroethane (C2F6), and perfluoropropane (C3F8). The gases are all resorbed over a period ranging from a few days to several weeks, and there is often a requirement for strict body positioning over an extended period postoperatively, typically days to weeks. There is also a risk of complications to the gas treatment, such as raised intraocular pressure, cataract development, and impaired vision [Killey 1978, Thompson 2003]. In some complicated cases such as recurrent RDs with PVR, a longer acting tamponade is required. In these cases, silicone oil has a long history of use. Significant disadvantages such as increased intraocular pressure (IOP), inflammation, and keratopathy are common side effects which

restrict the usefulness of such agents. In addition, the vision is impaired due to the non-physiological refractive index of silicone oil. The duration of the silicone oil treatment is often limited to a few months due to the risk of long-term complications such as secondary glaucoma, proliferation of epiretinal and subretinal membranes, optic nerve atrophy, and retinal toxicity [Nakamura 1991, Asaria 2004, Wickham 2007, Papp 2007, Ghoraba 2017]. At the end of the treatment period, the oil must be surgically removed. Heavy perfluorocarbon liquids (PFCLs), such as perfluorooctane, have a high specific gravity compared to the aqueous fluid. They are sometimes used as an intraoperative tool to remove trapped subretinal fluid, and aid the reattachment of RDs.

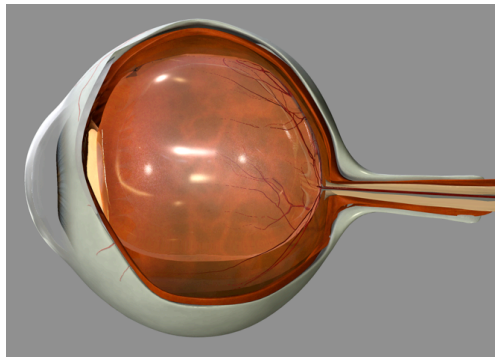

Figure 8. Vitreous substitute.
Representation of a vitreous substitute such as sulfurhexaflouride gas (SF6). Image by Fredrik Ghosh.

The perfect vitreous substitute

The seemingly simple task of devising a substitute for the vitreous has proven surprisingly difficult, which may be explained by considering the expanding knowledge of physiological functions of the normal vitreous, and the requirements needed for practical clinical use. The refractive index and the physiological properties should ideally closely mirror those of the normal vitreous, and the substance should be retained in the eye for a prolonged time or indefinitely, with no sign of toxicity or other significant side effects [Swindle 2007, Stefánsson 2009]. Additionally, the compound must be usable with a standard vitrectomy setup and therein be transferred into the eye while retaining these properties. The development of such a vitreous substitute has proven to be a daunting task, which is clearly shown by the plethora of different compounds involved in past and current research.

Past research

Research into vitreous substitution commenced as early as 1906, when Deutschmann injected animal vitreous into patients' eyes, later followed by Cutler's experiments with fresh human vitreous transplantation in the 1940s [Deutschmann 1906, Cutler 1947]. The results of these kinds of trials were not encouraging, and this line of research was therefore abandoned. In the 1950s investigations were focused on naturally occurring, and semisynthetic polymers, such as collagen and hyaluronic

acid. There were considerable problems with inflammation, increased IOP and corneal edema, as well as short retention time, hazing and fracturing of the gels after injection [Swindle 2007]. In the 1960s, Polygeline, a colloidal plasma expander with viscosity close to the human vitreous was evaluated in rabbit eyes but resulted in unacceptable inflammation [Oosterhuis 1966]. In the 1970s and early 1980s promising preclinical, and clinical tests with sodium hyaluronate (Healon®) were performed, although further clinical testing demonstrated major complications, such as persistent increased IOP [Balazs 1972, Kanski 1975, Gerke 1984, Koster 1986, Vatne 1986]. Due to these drawbacks, the interest in these molecules vaned, and the research turned more towards synthetic molecules. A number of substances such as hydroxypropyl methylcellulose (HPMC), Adcon-L hydrogel, Pluronic polyol F-127, Poly(2-hydroxyethyl acrylate) (PHEA), Poly(methyl 2-acrylamido-2-methoxyacetate) were all examined preclinically but deemed unsuitable due to short retention time and toxicity. Preformed hydrogels of Poly(1-vinyl-2-pyrrolidinone) (PVP), a blood plasma expander, were used in animal experiments, but again had short retention time and tended to be fragmented upon injection. Fragmentation was also a problem that limited the usability of injectable Poly(glyceryl methacrylate) (PGMA). In-situ polymerized silicone gels were tested in monkey long-term trials, with favorable biocompatibility, but practical issues still prevented its further development [reviewed by Swindle 2007].

Current research

Poly (vinyl alcohol) (PVA) hydrogels share important properties with the natural vitreous and have therefore been studied in several variants. Initial trials with these gels nevertheless presented several problems such as aggregation of the gel, short retention time, increased intraocular pressure, and inflammation. Further purification and cross-linking produced more promising results and seemingly acceptable biocompatibility in primates, but no follow-up studies were published [Swindle 2007]. Another relatively recently studied compound is chitosan, a derivate of the ubiquitous protein chitin found in crustacean shells, insect exoskeletons, and many other organisms. Chitosan inhibits fibroblast growth and has therefore been extensively used in surgical wound healing. It displayed optical and physical properties close to those of the natural vitreous when evaluated as a vitreous substitute in rabbit eyes, and the compound was therefore suggested as a long-term tamponade with potential PVR-limiting properties [Yang H 2008].

Acrylamide, the infamous substance of several public health scandals, is a toxic and carcinogenic compound in itself but has well-documented biocompatibility in its polymerized state. A number of experiments have been performed on different polyacrylamide derivatives, with another relatively recent preclinical study showing

promising results using an *in situ* forming hydrogel of a thiol cross-linked copolymer with good biocompatibility, physical and clinical properties [Swindle-Reilly 2009].

Several other recent studies have focused on the concept of *in situ* gelation to alleviate the issue of gel fragmentation upon injection and potentially achieve longer retention time. Over ten years ago, gellan, a derivative of an exocellular microbial hetero-polysaccharide used in the food industry, was studied *in vitro* as a potential *in situ* gelling vitreous substitute in combination with hyaluronic acid. The gel was not stable enough for further development [Suri 2006]. Ravi's group have presented several preclinical studies, including preliminary rabbit trials, evaluating different thiol-containing *in situ* forming polymers. These studies were seen as promising, but no long-term animal trials have so far been published [Swindle-Reilly 2009, Santhanam 2016]. Short- and long-term studies of two different *in situ* cross-linked poly(ethylene glycol) (PEG) hydrogels in rabbit eyes showed favorable gel-properties and biocompatibility, but follow-up studies are yet to be presented [Annaka 2011, Tao 2013]. The aforementioned substance chitosan has also been developed into an *in situ* forming hydrogel. In long-term rabbit studies, there were, contrary to earlier trials with chitosan, biocompatibility issues such as decreased rod- and cone density, and pathological electrophysiological responses [Jiang 2018].

A conceptually completely different strategy for vitreous substitution is the use of a synthetic capsule of silicone elastomer, surgically implanted through a 1,5 mm incision and filled with fluid through an adjustable tube-valve system. Animal trials with saline solution, as well as preliminary clinical trials with silicone oil, were made with some promise [Gao 2008, Lin 2016]. Perfluorohexyloctane combined with silicone oil is currently being evaluated in randomized trials for use as a long-term tamponading agent for patients with complicated, inferior retinal detachments. Early reports showed were encouraging, but mounting evidence suggests abundant side effects including intraocular inflammation, which may limit its use [Kirchhof 2002, Vote 2003, Schatz 2004, Morescalchi 2014].

Cross-linked hyaluronic acid hydrogels

In recent years, there has been a renewed interest in hyaluronic acid (HA). The substance is one of the main building blocks of the vitreous and is well-known for its biocompatibility, with a long history of extensive use in cataract surgery. As mentioned, early studies displayed multiple issues such as elevated intraocular pressure and short retention time. These problems have been attributed to enzymatic degradation by the enzyme hyaluronidase, native to the vitreous humor, and thereby fragmentation of the hydrogel which can lead to increased IOP by occluding the trabecular meshwork. To decrease the rate of degradation, the use of cross-linked hyaluronic acid hydrogels has been proposed. Cross-linking may also increase the

tamponading effect of the hydrogel, thereby making it more suitable for clinical demands. This concept was first suggested by Su 2011, who presented promising results using a hydrogel of oxidated HA cross-linked with adipic dihydrazide *in vitro* as well as in rabbit experiments. No follow-up experiments were, however, published. Later, another group published primary results with a similar hydrogel, as well as a UV cross-linked HA hydrogel, where the latter was found to be more biocompatible in a cytotoxicity assay, and well tolerated in rabbit trials [Schramm 2012]. Recently, the same group presented follow-up results including a rabbit study with reattached retina. Therein, two other, thiol cross-linked, HA gels were favorably compared to silicone oil, regarding redetachment as well as cataract development and with otherwise comparable findings [Schnichels 2017].

Inflammation

Inflammatory reactions are, as discussed above, one of the key limiting side-effects in the development of clinically relevant vitreous substitutes [Chirila 1994, Versura 2001, Swindle 2007]. Adverse effects of artificial vitreous substitutes includes inflammation evident on testing with standard cytotoxic assays such as those using cultures of endothelial- or RPE cells. In other cases the cytoxicity may be secondary to more complex events such as inflammatory reactions, necessitating testing in retinal explant or *in vivo* models [Matteucci 2007]. An example of these interactions is the foreign body reaction: a type of cellular inflammation characterized by macrophages, aggregation of giant cells, and fibrosis [Anderson]. Intravitreal biomaterials, such as experimentally injected PLGA polymer microspheres and -rods, are known to be able to elicit this kind of response [Thackaberry 2017]. Similar reactions have been reported after treatment with vitreous substitutes such as perfluoro-n-octane (PFO) [Elsing 2001, Schatz 2004], perfluorohexyloctane [Sigler 2014], and silicone oil [Parmley 1986].

Retinal cultures

Retinal cell culture models are valuable tools for detailed studies of different physiological and pathological processes of the retina. These models consist of either dissociated cells or full-thickness sheets of explanted retina. Single-cell cultures offer more detailed control of the culture environment and more straightforward methods for quantitative data regarding cell numbers and types, and the expression of specific antigen. The mechanical and enzymatic disruption process does, on the other hand, potentially damage, or alter the behavior of, the dissociated retinal cells. Using full-

thickness retinal explants, with its complete architecture and microenvironment of different cell-types, offer a higher level of physiological similarity to the normal retina [Lucas 1958, Caffé 2001, Engelsberg 2004].

Recreating the physiological environment sufficiently to allow for successful culturing puts a high demand on the culture processes. Different species and degrees of tissue maturity put different demands on these routines. One of the most critical factors is the culturing medium, which must meet a number of demands, such as the correct pH-levels and concentrations of different nutrients including salts, carbohydrates, vitamins, amino acids, and lipids. Modern culture mediums are synthetically produced, but animal serum is frequently added to optimize the culture conditions and promote cell-growth. Serum includes a number of nutritional substances, as well as a several proteins able to actively affect the retina and micro-environment, such as growth factors, enzymes, and carrier-proteins. By manipulating the culture-conditions, the impact of a wide range of different physiological and pathophysiological factors can be studied.

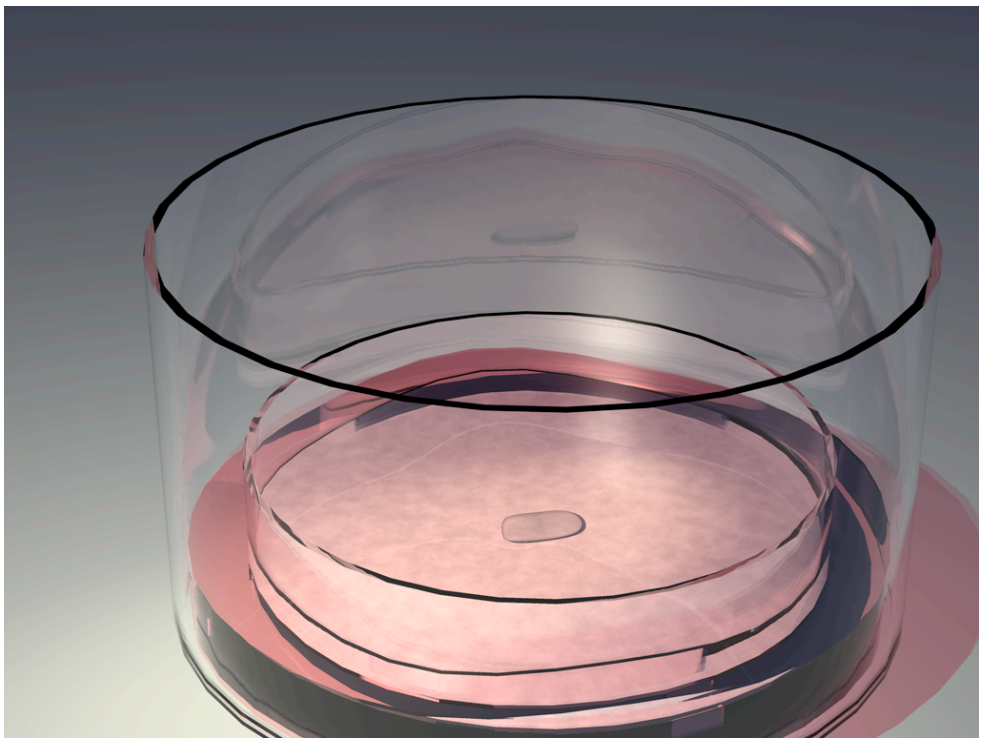

Figure 9. Retinal explant culture.

Neuroretinal explant on a 4 µm culture plate insert with the photoreceptor layer facing down. Culture medium (red) is seen surrounding the culture plate. Image by Fredrik Ghosh.

Electrophysiology

Electroretinography (ERG) is a widely used clinical method for studies of the retinal function. The basis for ERG, as well as electrophysiology in other organs, is the ionic gradients across cell-membranes in all living cells. The activity of these gradients creates externally measurable currents that reflect the physiological functions of the cells. Specifically, ERGs measure the neuronally derived electrical response to light stimuli. By altering the character of the stimuli, the response from different cell types can be elicited. Modulations of the stimuli include the wavelength, intensity, frequency, and duration of the light impulse, as well as prior dark adaption. The rod function is elicited by dim flashes after at least 20 minutes of dark adaption (scotopic conditions), whereas the mixed rod-cone function is measured during the same conditions, but with brighter stimuli. The cone response is achieved by adaption to bright light for at least 10 minutes (photopic conditions), thereby desensitizing the rods, and then stimulating the retina with bright flashes of light. Alternatively, fast flickering light can be used to separate the rod and cone responses.

In full-field ERGs, the entire retina is stimulated evenly by a diffused light source, a so-called ganzfeld sphere [Marmor 2009]. The electrophysiological responses are then measured by way of a contact lens with electrodes placed on the cornea. The shape of the detected electrical patterns can then be analyzed for abnormal or pathological responses. Full-field ERGs exhibit two major components, the negative a-wave and the positive b-wave, and reflects the responses of the photoreceptors and second order neurons respectively. Detailed analysis ERGs derived from different stimulations reveals further insight into the function of the various retinal neurons.

Animal models in vitreoretinal research

Background & history.

Animal models have been an integral part throughout the history of vitreoretinal research. In the early 20th century, pioneering studies into vitreous replacement were made directly on patients, but preceding animal studies were soon introduced—almost all of the plethora of potential vitreous substitutes evaluated throughout modern history were first tested in animals [Stenzel 1969, Shafer 1976, Denlinger 1980, Mackiewicz 2007, Su 2011]. Aside from initial testing in cell-based cytoxicity assays, these studies have almost exclusively explored the practical usability and biocompatibility in healthy, young animal eyes [Heimann 2008, Kanski 1975]. Trials

of this kind provide valuable information about the responses elicited in the living tissue and the physiological results thereof. By using macroscopic evaluation together with functional methods such as electrophysiology (ERG) and morphological studies with immunohistochemistry, a comprehensive appraisal can be made of the examined substances potential as candidates for clinical trials.

Animal studies have been extensively used in order to elucidate the pathophysiology of retinal detachments. Early models utilized mechanical dislocation of the retina. In the late 1960s, Machemer and Kroll supplemented this approach with a prior injection of intravitreal hyaluronidase to break down the vitreous before the manipulations [Machemer 1968]. The introduction of subretinal microinjections of saline solution allowed for a more controlled detachment of the retina [Marmor 1980]. Further refinements included vitrectomy combined with lens removal and, crucially, dilute viscoelastic as an adjunct in the subretinally injected fluid to prevent premature spontaneous reattachment of the retina [Anderson 1981, Anderson 1983]. Over the years, the models have involved a number of disparate species such as cat, ground squirrel, rabbit, pig, and mouse [Sakai 2001, Iribarne 2007, Sun 2007, Diederen

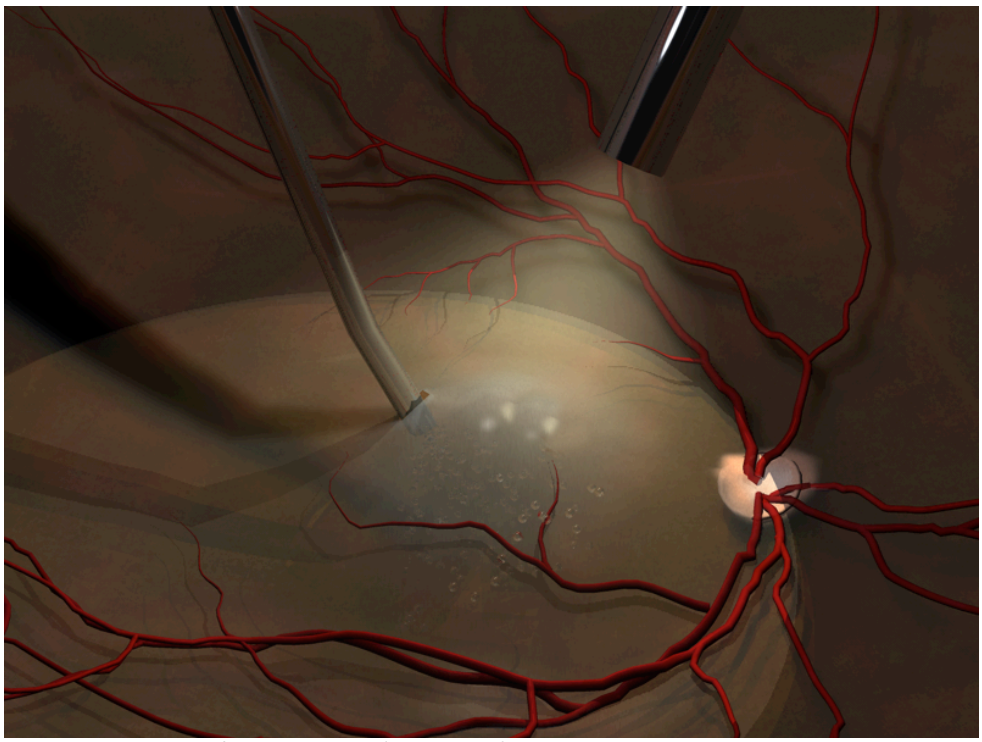

Figure 10. Creation of an experimental vitreous substitute.
Subretinal microinjection of a dilute viscoelastic solution. Endoillumination probe shown right. Image by Fredrik Ghosh.

2008, Verardo 2008, Mandal 2015]. In order to better emulate the typical clinical presentation of RRD, Jackson *et al.* further refined the methodology to include vitrectomy with PVD and retinal breaks in phakic, porcine eyes [Jackson 2003].

Only a few studies have combined these two lines of research i.e., evaluating vitreous substitutes and other surgical methods together with experimental retinal detachments. These cases most often include induction of RDs immediately before treatment with vitreous substitutes [Yamana 2000, Teruya 2009, Hirata 2013, Yamamoto 2013, Schnichels 2017]. The treatment of RDs in a secondary procedure has very rarely been studied. The few studies that do have been performed in a feline model, involving lensectomy and vitrectomy, before the creation of a RD, followed by a reattachment surgery with fluid-air exchange and injection of SF6 after an interval ranging from 1 hour up to 42 days [Anderson 1986, Lewis 2002, Lewis 2005].

The rabbit eye in surgically related research

The rabbit is one of the most common species for ophthalmological surgical research. One of the main reasons for this is the comparatively large size of the eye despite the animal's relatively small overall size. Due to this, the logistics of the trials are usually easier with more straightforward and cost-effective handling compared with larger animals, while still offering the possibility to adapt clinical surgical techniques with minor modifications.

The considerably smaller phakic rabbit eye is 16,66 mm long (anterior to posterior) compared to 24,96 mm in humans, and their equatorial diameter 18,65 mm, and 24,50 mm, respectively. The anterior segment makes up a much larger part of the rabbit eye than its human counterpart; while the anterior chamber depth is roughly the same, the crystalline lens is significantly thicker at 6,36 mm vs. 4,24 mm [Werner 2006]. These anatomical features mean that the vitreous space is considerably smaller with a volume of 1,15 ml compared to 4 ml [Del Amo 2015], making posterior segment surgery more technically demanding and with a higher risk of complications such as inadvertent touching of the lens and retina compared to clinical conditions in humans.

There are also major differences in the configuration of the blood vessels supplying the retina with oxygen and nutrients, with a less developed retinal vasculature system in rabbits eyes, the so-called merangiotic pattern, compared to the holangiotic human retina. Thus the rabbit retina mainly relies on choroidal diffusion, and only to a lesser degree on the supply from the retinal vasculature. Further, the rabbit retina is rod-rich

with comparably fewer cones [Strettoi 1995]. Instead of a true macula, the rabbit retina contains a so-called ”visual streak,” a horizontal band localized just beneath the optic nerve head (ONH), with a higher density of cones and ganglion cells compared with other parts of the retina. The rabbit retina is also dichromatic, in contrast to the trichromatic human retina. The ONH is located above the posterior pole of the eye,and has horizontally oriented bands of myelinated nerve fibers nasally and temporally, as well as accompanying retinal blood vessels. The morphology and lamination of the retina itself, however, closely resembles human counterparts and other mammals, and the electrophysiological response of the ERG is also comparable [Gjörloff 2004].

AIMS OF THE STUDY

General aim

- To develop a biocompatible synthetic vitreous substitute with suitable mechanical and biochemical properties for vitreoretinal surgery applications

Specific aims

- To develop new preclinical models for evaluating the biocompatibility, practical usability, and efficacy of vitreous substitutes *in vitro* as well as *in vivo*
- To investigate retinal morphology, function, and inflammatory responses after treatment with current and experimental vitreous substitutes *in vivo*
- To develop a new model of rhegmatogenous retinal detachment, in which novel vitreous substitutes can be tested in a manner resembling clinical conditions
- To develop and test novel vitreous substitutes in these models, with the ultimate goal of translation to clinical use as tamponades in vitreoretinal surgery

MATERIALS AND METHODS

Animals

Ethics

The animals in these studies were treated in accordance with the guidelines and requirements of the ARVO (The Association for Research in Vision and Ophthalmology) statement on the use of animals in ophthalmic and vision research. All procedures were approved by the government committee on animal experimentation at Lund University.

Species

The *in vitro* study (paper I) utilized retinal explants from female adult Sprague-Dawley rats. All *in vivo* experiments (paper II-IV) were performed with 4 month-old pigmented rabbits derived from a local breeder.

Tamponades and experimental vitreous substitutes

Healaflow®

Healaflow® (Anteis S.A., Plan Les Ouates, Switzerland) is a transparent cross-linked hydrogel consisting of 97% water and sodium hyaluronic acid (22,5 mg/ml) of non-animal origin cross-linked with BDDE (1,4-Butanediol diglycidyl ether). It is supplemented with phosphate- and NaCl-salts to maintain physiological pH (7,0) and osmolarity (305 mOsm/kg). Its estimated specific gravity is circa 1,03, and refractive index i = 1,341. The hydrogel is clinically used in glaucoma filtering surgery, where its purpose is to maintain the bleb and limit postoperative fibrosis. [Schariot 2010, Roy 2012, Bettin 2016].

PEG

Polyethylene glycol (PEG) is a well-established synthetic polymer for use in different biomedical applications. The gel used here (paper I) was custom made as a two-component cross-linked hydrogel with 20 wt.% polyethylene glycol, prepared by mixing PEGDA in phosphate buffered saline (PBS) into ETTMP-1300 in PBS. It was incubated for 20 minutes before use, allowing for some gelation. Prior experimental reports of PEG gels include use as a vehicle for drug delivery, and for sealing scleral incisions as well as retinal breaks in retinal detachment surgery. [Wathier 2006, Duvvuri 2007, Pritchard 2010]

Bio-Alcamid®

Bio-Alcamid® (Polymekon, Brindisi, Italy) is a transparent hydrogel consisting of 96% water and a meshwork of approximately 4% polyalkylimide with no free monomers. It has a pH of 6,9 and is considered to be structurally and chemically stable. The gel was developed for use in plastic and reconstructive surgery as a filler for tissue defects. When it is injected into biological tissues, a collagen capsule develops around the substance. After a period of clinical use, several reports described long-term complications such as chronic inflammation, fibrosis, and foreign body reaction. The product is now withdrawn from the market. [Claoue 2004, Lahiri 2007, Nelson 2011, Crafoord 2011]

Silicone Oil

Silicone oil, 1000 cSt (FCI S.A.S., Paris, France) is a tamponade for vitreoretinal surgery. It has been in clinical use for many years and is mainly used in complicated cases of retinal detachment and severe diabetic retinopathy where a long-term tamponade is needed. [Kanski 1973, Stappler 2011]

SF6

Sulfur hexafluoride (SF6) (Arceole®, Arcadophta, Toulouse, France) is one of the most commonly used tamponades in vitreoretinal surgery and has a long history of clinical use. It is an inert colorless gas that expands about two times when used in the human eye. It has an effective tamponading time of about one week, and a retention time of 2–3 weeks when diluted with air to the most common clinically used concentration of 20%. [Vaziri 2016, Neffendorf 2017]

Retinal explants and cultures (paper I)

Various vitreous substitutes in vitro (paper I)

After euthanization of the rats with CO_2 and subsequent decapitation, the eyes were enucleated and immediately immersed in ice-cold CO_2-independent medium (Gibco, Paisley, UK). The anterior segment and vitreous were removed, and the neuroretinas were carefully dissected from the retinal pigment epithelium (RPE). Either the half of or the entire neuroretinas were explanted and placed on culture plate inserts (Millicell Isopore-PCF 0,4 µm, 30 mm; Millipore, Billerica, ME) with the photoreceptor layer against the membrane. The explants were covered with 50–100 µl gel (Healaflow®, PEG, or Bio-Alcamid®). Thereafter, 1,2 ml Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco) supplemented with 10% fetal calf serum was added to allow for culture, and a drop of enriched medium applied directly onto the gels to ensure saturation. Additionally, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin (Sigma-Aldrich, St Louis, MO) were added to the cultures, which were kept at 37°C with 95% humidity and 5% CO2. Four groups of explants were created: culturing medium only, Healaflow®, PEG, and Bio-Alcamid®. For each group, 4 explants were cultured for 2 days, and 6 explants in each group were kept in culture for 5, and 10 days, respectively. The culture medium was changed every other day, but the gel was not changed or altered in any way during the change of medium.

Surgical procedures (papers II–IV)

General vitrectomy procedures

All surgical procedures on the rabbits were performed by experienced surgeons (HB and SC). Treatment was performed on the right eyes, with unoperated left eyes serving as controls. General anesthesia was induced with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg), and additional topical tetracaine (0,5%) was administered immediately preoperatively. The pupils were dilated with cyclopentolate (1%) and phenylephrine (10%) 30 minutes prior to the procedure, and the eyelids and the nictitating membrane were retracted with a blepharostat.

All sclerotomies were made 1 mm posterior to the limbus, with the infusion at 12 o'clock and two sclerotomies for the illumination and vitrectomy probes at 2 and 10 o'clock, respectively. The sclerotomies were made with 25G trocars (Alcon, Fort Worth, TX, USA or D.O.R.C., VC Zuidland, Netherlands) except in paper I, where the 2 o'clock incision was made with a conjunctival incision and subsequent 20G sclerotomy due to use of a larger vitrectomy probe. A continuous infusion of balanced salt solution (BSS) (Endosol®, Abbott Medical Optics or BSS plus, Alcon, Fort Worth, TX, USA) was used throughout the procedures. For visualization, a BIOM 90-D lens (Oculus Optikgeräte GmbH, Wetzlar, Germany) and standard endo-illuminating probes were used.

Accurus Surgical System® Alcon, Fort Worth, TX, USA) and a vitreous cutter (Innovit® or Accurus 2500, Alcon, Fort Worth, TX, USA) was used for the vitrectomy. Core vitrectomy with posterior vitreous detachment (PVD) was performed, and as much peripheral vitreous removed as possible, considering the comparatively large lens of the rabbit eye. At the end of surgery, sclerotomies and conjunctiva were sutured if larger incisions were used. In paper II, 25 mg gentamicin and 2 mg betamethasone were administered subconjunctivally. Chloramphenicol ointment (Chloromycetin, Pfizer Inc., New York, USA) was applied at the end of all operations. No other treatment was administered postoperatively.

Healaflow® in vivo (paper II)

For paper II, a fluid-air exchange was made after the core vitrectomy. Thereafter, Healaflow® was injected through a 25G needle.

Various tamponades in vivo (paper III)

For paper III, a fluid-air exchange was followed by injection of BSS, Healaflow®, or 1000 cSt silicone oil. Since Bio-Alcamid® was not injectable through the 25G system due to its high viscosity, the 2 o'clock trocar was for this purpose removed, and the sclerotomy widened to fit a 19G cannula.

Retinal detachment in vivo (paper IV)

For paper IV, the basic vitrectomy procedure described above was followed by the creation of a retinal break and subsequent detachment (RD). For this purpose, six different kinds of cannulas (including 2 subretinal micro-cannulas) were used to make a retinal break in the inferior mid-periphery of all operated eyes. The retinal break was created by forceful injection of dilute viscoelastic solution (0,85 ml sodium hyaluronate [Healon®, Johnson & Johnson Vision, Santa Ana, California, USA] and BSS to 3 ml) against the retina, or by mechanical manipulation of the retina with the tip of the cannula, keeping the size at a minimum. A subretinal injection of the viscoelastic fluid was then slowly made, aiming to create an RD of at least 2 disc diameters. The size of the existing retinal break was then slightly increased to mimic pathophysiological conditions.

Repeat surgery was performed the day after the initial surgery, using the same vitrectomy setup, with aspiration above the retinal break to remove subretinal fluid. After a fluid-air exchange, a tamponade (Healaflow® or 20% SF6) was injected into the posterior segment of the eyes. No laser or cryo retinopexy was performed. Eyes with significant perioperative complications, such as RD secondary to subretinally displaced infusion, significant iatrogenic retinal break, and significant lens touches were filled with BSS and analyzed separately. To validate the model and to obtain surgically treated controls, additional eyes were kept without reoperation.

Postoperative handling

All surgically treated eyes were examined on the first postoperative day, weekly or monthly, and at the end of the treatment period. The clinical evaluation included binocular ophthalmoscopy and intraocular pressure (IOP) measurement (Tono-Pen®, Reichert, Buffalo, NY or TonoVet®, Icare Finland Oy, Vantaa, Finland). In paper II and IV, the eyes were photographed during the follow up (Retcam®, Clarity Medical System, Pleasanton, CA, USA or Smartscope Pro®, Optomed Oy, Oulu, Finland).

Eyes with repeat surgery (paper IV) were examined the first postoperative day after each procedure. The rabbits were sacrificed at the end of the planned treatment period (42–105 days in paper II; 1 day, 1 week, and 1 month in paper III; and 1 month in paper IV). The treated eyes were enucleated, dissected and photographed along with a number of unoperated left eyes.

Electrophysiology

In paper II, the treated eyes were examined with full-field electroretinography (ERG) preoperatively, at 1 and 3 months postoperatively. During the recordings, the rabbits were under sedation with an intramuscular injection of Hypnorm® (fentanylcitrate 0,315 mg/ml and fluanisone 10 mg/ml). The pupils were dilated with cyclopentolate (1%). After 30 minutes of dark adaptation, topical anesthesia was administered, and a Burian-Allen bipolar ERG contact lens electrode applied to the corneas with 2% methyl-cellulose. A subcutaneous ground electrode was attached to the neck. The ERGs were obtained with a Nicolet Viking® analysis (Nicolet Biomedical Instruments, Madison Wisconsin) using a wide-band filter (-3 dB at 1 Hz and 500 Hz). The stimulations used were: single full-field flashes (30 µs) and dim blue light (Wratten filters # 47, 47A and 47B) [rod function], white light (0,8 cd·s/m2) without background [combined rod-cone function], and 30 Hz flickering white light (0,8 cd·s/m2) averaged from 20 sweeps without a background light [cone function].

Microscopical analyzes

Tissue handling

The explants (paper I) and the enucleated eyes (paper II-IV) were fixed for 4 h with 4% paraformaldehyde pH 7.3 in a 0.1 M Sørensen's phosphate buffer (PB). They were then repeatedly washed with 0.1 M Sørensen's PB using the same solution containing sucrose of rising concentrations (5–25%). The eyes of paper II-IV were dissected between the ora serrata regions, including the optic nerve head and visual streak. After sectioning at 12 µm on a cryostat, every 10th slide was stained with hematoxylin and eosin (H&E) according to standard procedures.

Immunohistochemistry

After a rinse with phosphate buffered saline (PBS) for 5 minutes, the retinal sections were incubated with PBS containing 0.1% Triton X and 1% bovine serum albumin for 20 minutes at room temperature. Thereafter, they were incubated overnight at 4°C together with antibodies. The slides were then rinsed and incubated with Texas Red- or FITC-conjugated antibodies for 45 minutes. After rinsing, they were mounted in anti-fading mounting media (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA). For double-labeling, the same procedures were followed but with both of the primary and secondary antibodies added at the same time in their respective steps. Negative controls were obtained by following the same procedure but without primary antibodies. The antibodies are summarized in Table I.

TUNEL

Apoptotic cells were studied using a commercial terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay system with fluorescein-conjugated dUTP (Boehringer Mannheim, Mannheim, Germany), used according to the manufacturer's instructions [Table 2].

Table 1: Antigen/antibody specifications

Antigen	Antibody name	Target structure	Species	Dilution	Source
GFAP	Anti-glial fibrillary acidic protein (G-A-5)	Astrocytes, activated Müller cells	Mouse monoclonal	1:200	Chemicon International, Temecula, CA, USA
Neurofilament 160 kDa (NF160)	Anti-neuro-filament 160 clone NN18	Ganglion and horizontal cells	Mouse monoclonal	1:500	Sigma, St. Louis, MO, USA
PKC	Phospho-PKC (pan)	Rod bipolar cells	Rabbit polyclonal	1:200	Cell Signaling, Beverly, MA, USA
Rhodopsin	Rho4D2	Rod photoreceptor	Mouse monoclonal	1:100	Courtesy of Prof. RS Molday, Vancouver, Canada
Vimentin	Mouse anti-vimentin	Müller cells	Mouse monoclonal	1:500	Chemicon International, Temecula, CA, USA
CD45	L12/201	Pan leukocyte	Mouse monoclonal	1:100	Bio-Rad, Oxford, UK
Galactein-3	Galactein-3 antibody	Mainly microglia	Chicken	1:100	Courtesy of Prof. H Leffler, Lund, Sweden
CD68	EBM11	Macrophages	Mouse monoclonal	1:200	Agilent DAKO, Santa Clara, CA, USA
CD20cy	L26	B-lymphocytes	Mouse monoclonal	1:200	Agilent DAKO, Santa Clara, CA, USA
Secondary antibody	**Antibody name**	**Target**	**Species**	**Dilution**	**Source**
FITC	Anti-mouse IgG FITC conjugate	Anti-mouse	Goat	1:200	Sigma, St Louis, MO, USA
FITC	Goat Anti-Rabbit IgM+IgG (H+L chain specific)	Anti-rabbit	Goat	1:200	Southern Biotechnology Associates, AL, USA
Texas red	Anti-IgY Texas Red conjugate	Anti-chicken	Rabbit	1:200	Abcam, Cambridge, UK

RESULTS

Vitreous substitutes *in vitro* (paper I)

For paper I, retinal explants were kept in culture with different potential vitreous substitutes for up to 10 Days *in vitro* (DIV). These gels (Healaflow®, PEG and Bio-Alcamid®) were compared to standard culturing conditions, i.e. medium only. Upon application to retinal explants, Healaflow® and PEG formed even films, whereas Bio-Alcamid® retained a lumpy, uneven texture and did not allow for complete coverage of the explant. The gels were saturated with medium, and their integrity and structure did not change during the study.

The standard retinal culture conditions imposed pathological morphological changes readily visible in hematoxylin and eosin (H&E) stained sections as early as after 2 DIV, progressing at later time-points. These changes in the cytoarchitecture included abnormal lamination, variability in thickness, and pyknosis in the outer and inner nuclear layers (ONL and INL). These changes increased at later time points with all groups. Retinas treated with Healaflow® was similarly or less affected by these degenerative changes compared to those kept under standard conditions at all time-points, whereas those kept with PEG, and especially, Bio-Alcamid® exhibited more severe degeneration. TUNEL labeling of retinas with medium only showed a progressive increase in apoptotic cells with time. Healaflow® showed similar findings at 2 DIV, but decidedly less TUNEL labeling at 5 and 10 DIV. PEG also displayed similar findings as control retinas at 2 DIV, as well as at 5

Figure 11. Retinal explant culture with Healaflow®.

Adult rat retinal explant in cell culture plate, kept in culture with Healaflow®.

DIV but very low labeling intensity at 10 DIV. Bio-Alcamid® showed higher levels of TUNEL- labeling at 2 and 5 DIV, and almost no labeling at 10 DIV.

Rhodopsin labeling in explants treated with medium only revealed a high number of labeled rod photoreceptors in the outer segments (OS) and the outer plexiform layer (OPL), with a low number of labeled photoreceptors in the ONL at 2 and 5 DIV. After 10 DIV, the labeling in the ONL was increased. Similar labeling patterns were seen with Healaflow® and PEG treatment, while Bio-Alcamid® exposure resulted in an earlier increase in pathological labeling in the ONL.

PKC labeling for rod bipolar cells revealed variable labeling in explants cultured with medium only, decreasing over time with no remaining labeled cells after 10 DIV. This decrease in labeling was faster in Healaflow® treated specimens, with only a few remaining labeled cells after 2 DIV, whereas PEG and Bio-Alcamid® treated explants did not display PKC labeling at any time-point. Neurofilament 160 labeled ganglion cells were seen both with and without gels, with no apparent differences between the different treatments or exposure time.

GFAP labeling of activated Müller cells in the inner retina was found to increase from 2 DIV and onwards in control cultures, with a peak in the labeling intensity at 5 DIV. Healaflow® treatment elicited a similar response at all time-points. In contrast to this pattern, PEG and Bio-Alcamid® exposed retinas exhibited high Müller reactivity already at 2 DIV, tapering off at later time-points especially with Bio-Alcamid® treatment. Vimentin labeling of Müller cell cytoskeletons was found in the inner parts of the retina to a similar extent in all groups, with increasing hypertrophy and disorganization over time.

Healaflow® *in vivo* (paper II)

The previously tested experimental vitreous substitute Healaflow® was tested in an *in vitro* rabbit model with follow up times of 1 to 3 months. The gel was found to be well suited for use with a standard vitrectomy setup, and it retained its structure after injection through a small gauge needle. There were no signs of uveitis or increased macroscopic inflammation during the follow-up period. The postoperative IOP was slightly elevated compared to earlier studies by our group, with transient peaks in the range of 21–27 mmHg in a minority of the cases. In four eyes, iatrogenic retinal breaks occurred during the surgery, in two cases along with limited RDs. These lesions did not progress during the follow-up. No distinct remnants of the gel could be identified upon dissection. Cataract development was seen exclusively in eyes with perioperative lens-touches.

Postoperative full-field ERGs at 1 month and 3 months did not reveal any changes in the amplitudes either of the isolated rod (Dim blue light stimulation), mixed rod and cone (White light), or isolated cone (Flickering light) responses compared to pre-operative ERGs.

The morphology of the postoperative retinas was normal as demonstrated by hematoxylin and eosin staining of cryosections, compared to unoperated left eyes. The iatrogenic retinal lesions were, however, visible on microscopy. Limited retinal detachments were also seen in two cases where the retina had been considered to be attached during the macroscopical examinations. Compared to control eyes, GFAP labeling revealed a minimal postoperative increase in activated Müller cells, although a more distinct increase was seen in eyes with peri-operative complications such as iatrogenic cataracts and retinal detachments. The TUNEL-assay revealed no significant labeling of apoptotic cells. No postoperative changes in PKC (rod bipolar cells) and Rhodopsin (photoreceptor cells) labeling occurred compared with unoperated control retinas.

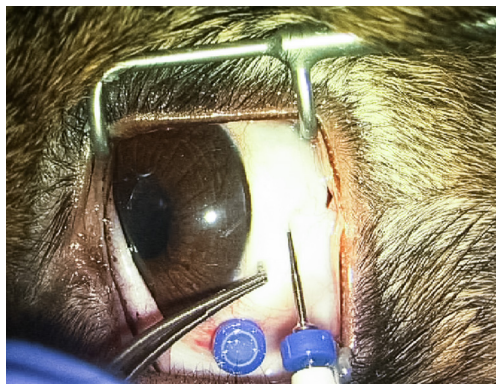

Figure 12. External view of the 25 G vitrectomy setup.
Blepharostat and 25 G trocar at the 12 o'clock position (for infusion) are already in place, and the trocar for the vitrectomy probe is in the process of installation.

Inflammatory responses *in vivo* (paper III)

In this study, rabbit eyes were vitrectomized and filled with various current and potential vitreous substitutes (BSS, silicone oil, Healaflow®, and Bio-Alcamid®). One eye filled with BSS sustained suspect endophthalmitis and was excluded from further analysis. Eyes treated with BSS, as well as those treated with Healaflow® and silicone oil exhibited mild to moderate signs of inflammation, such as conjunctival swelling and injection, at the first postoperative day but not at later time-points. The eyes treated with Bio-Alcamid® displayed increased macroscopic signs of inflammation in the anterior segment, as well as cloudiness and suspect infiltrates in the vitreous. Eyes which sustained significant perioperative retinal complications such as retinal breaks (n=13) or subretinal infusion with subsequent retinal detachment (n=2) were analyzed separately. These eyes were macroscopically comparable to the BSS and

Healaflow® groups. Cataract was common, mostly caused by known intraoperative lens-touches, and in some cases obscuring the vitreous and retina during the follow-up. Most of the eyes with significant perioperative retinal complications developed cataract during the study, and all eyes filled with Bio-Alcamid® developed cataract within 1 week.

The unoperated *in vivo* controls displayed normal morphology in H&E stained sections, with preserved structure, lamination, and no pyknosis. Treatment with BSS, Healaflow®, and silicone oil did not induce any notable changes in the overall morphology. There were, however, frequent cell clusters on the inner surface of the retina, particularly in silicone oil-filled eyes. Bio-Alcamid® exposure resulted in progressive, severe degenerative changes including regions of profound thinning, disturbed lamination, and degeneration already at 1 week after surgery, progressing at the 1-month time point. These eyes also revealed a higher amount of cells on the inner retinal surface. In the 13 eyes with perioperative retinal complications, corresponding lesions such as retinal ruptures and retinal detachments were seen with concurrent disturbances in the retinal lamination and severe degeneration in the affected areas.

Unoperated control eyes displayed very low Müller cell reactivity as demonstrated with GFAP labeling. Eyes filled with BSS or Healaflow® displayed low levels of labeling, peaking at 1 week, and then subsiding again to minimal levels. A similar pattern, but peaking at higher levels of labeling, was seen with silicone oil treatment. Conversely, exposure to Bio-Alcamid® resulted in very high and sustained GFAP labeling from 1 week and onwards. BSS treated eyes with significant perioperative retinal complications showed a comparably higher rate of labeling at all time points compared with BSS and Healaflow® treatments, increasing from the first post-operative control with persistent high labeling present from 1 week. No labeling of Galectin-3, a marker for neuroinflammation, was present in unoperated eyes. In contrast to this, all surgically treated eyes exhibited general diffuse labeling, especially in the inner retinal layers. There were no distinct differences between groups or different length of exposure.

Labeling for the pan-leukocyte marker CD45 revealed expression in all eyes, with and without surgery. In unoperated eyes, weak labeling of dendritic cells was present in the inner retina (GCL and IPL). All unoperated left eyes from surgically treated animals exhibited CD45 labeling in the inner retina and a low number of cells on the ILM. Post-operative retinas displayed increased labeling, frequently with additional labeled cells in the vitreous space. Treatment with BSS and Healaflow® elicited similar low to moderate responses at all time-points, whereas silicone oil provoked a comparably stronger response peaking at one week, with a higher number of cells on the ILM especially adjacent to the optic nerve head (ONH). Exposure to Bio-Alcamid® caused a very robust response of dendritic and round CD45+ cells often

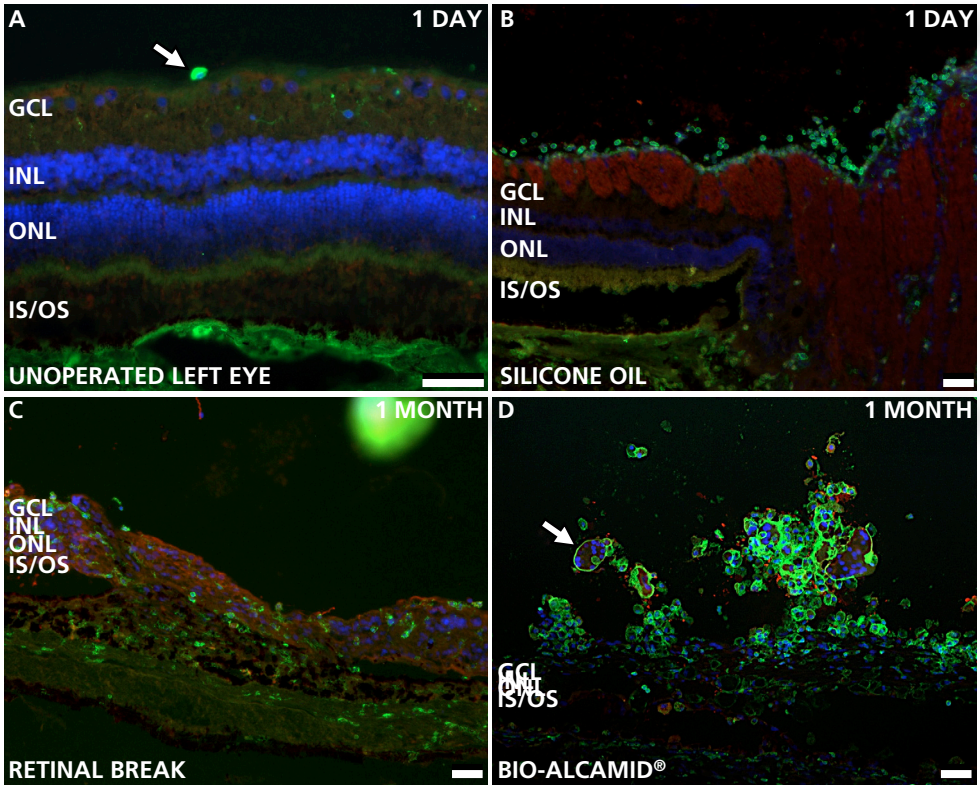

Figure 13. Immunohistochemistry of rabbit retina.

Examples of cryosections of rabbit retina co-labeled with CD45 [pan leukocyte] (green) and Galactein-3 [mainly microglia] (red);

A) Unoperated left eye 1 day after routine vitrectomy with BSS, arrow indicates infiltrating CD45+ cell. B) Silicone oil treatment after 1 day, clustering of CD45+ cells at the optic nerve head. C) Routine vitrectomy with BSS after 1 month, localized infiltration of CD45+ cells at a retinal break right. D) Bio-Alcamid® treatment after 1 month, arrow indicates multi-nucleated giant cell.

Abbreviations: ganglion cell layer (GCL) inner nuclear layer (INL), outer nuclear layer (ONL), inner and outer segments (IS/OS). Scale bar is 50 µm

throughout the entire retina, together with enlarged multi-nucleated giant cells in the vitreous. Eyes subjected to significant perioperative retinal complications displayed a heterogeneous, moderate response with higher rates of labeling in the vicinity of retinal lesions. Some of these eyes also displayed occasional clusters of cells on the ILM with a tendency to form giant cells.

Contrary to unoperated controls, most operated eyes displayed scattered CD68 positive cells in the inner retina, and in the vitreous. The responses elicited by BSS, Healaflow®, and silicone oil were all similar, no clear trends could be discerned over time. Bio-Alcamid exposure provoked extensive CD68 labeling at all time-points, especially in the most structurally degenerated areas. In eyes with significant

perioperative retinal complications, such as retinal breaks and RD, there were often regions with elevated CD68 expression, especially in the vitreous space and on the ILM.

Upon labeling for CD20, present in B-lymphocytes, no labeling was found in unoperated eyes. Occasional labeling was found in the vitreous space or GCL of some eyes treated with either BSS, Healaflow®, or silicone oil. The detected cells were almost exclusively found on the first postoperative day. Bio-Alcamid® exposed eyes displayed extensive CD20 labeling after a week, but only scarce labeling remained after a month. A few eyes with significant perioperative retinal complications exhibited CD20 expression early in the time-course, mainly in cases with a manifest RD.

Retinal detachment *in vivo* (paper IV)

In this study, a new repeat surgery model for studying the treatment of RDs with vitreous substitutes in rabbit eyes was developed. A number of different cannulas were tested for the purpose of creating retinal breaks and RDs, whereby subretinal microcannulas were found to offer the best control and precision. The repeat surgery was at times technically demanding due to conjunctival edema and reduced visualization caused by opacities in the crystalline lens and vitreous secondary to the prior operation. Vitreous hemorrhage was often present during repeat surgery, and a mild to moderate fibrinous reaction was seen in 7 of the 16 repeat surgery cases. As much as possible of the fibrinous membranes and hemorrhages in the vitreous were removed during the surgery. Two cases with repeat surgery developed endophthalmitis during the follow-up and were excluded from further analysis. Cataract development was common, especially with repeat surgery, where 9 of 16 eyes developed posterior subcapsular cataract related to known lens touches. One week after reoperation, 2 eyes treated with Healaflow® and 1 eye treated with SF6 developed slightly elevated intraocular pressure (IOP) in the range of 25–35 mmHg, returning to normal levels after a month.

Macroscopic examination of eyes without reoperation at 1 month revealed 2 out 8 eyes with bullous RDs and 2 more eyes with limited shallow detachments. Of the eyes treated with repeat surgery, 11 of 14 eyes had attached retinas at the end of the study. Upon treatment with SF6, there was progress to giant rupture with attached peripheral retina in 4 cases. The SF6 group also contained 1 case with a partial low RD, and 1 case with a retained a substantial intraoperative RD secondary to subretinal infusion. With Healaflow®, no cases displayed persisting RDs, but 1 eye developed a giant tear, similar to those presented after SF6 treatment, but significantly less extensive.

Dissection of the enucleated eyes confirmed an attached retina in 4 of 8 eyes without, and 11 of 14 eyes with repeat surgery. The non-repeat surgery eyes displayed 4 RDs (2 limited and 2 extensive), as well as 4 cases of retinas with limited retinal breaks. The eyes treated with SF6 included 3 cases of RD (1 limited and 2 extensive), 4 cases of giant ruptures, and 1 case of limited retinal break. All eyes treated with Healaflow® had attached retina, with 1 case of giant rupture, and limited retinal breaks in 5 cases.

The morphology of all eyes was largely normal in H&E sections, except in areas with RDs and retinal breaks. Detached retinas exhibited disturbed lamination and severe degeneration, frequently with immune cells in the retina, subretinal space, and in the vitreous in the vicinity of the RDs. Areas next to retinal breaks displayed similar, but less pronounced degenerative changes, whereas reattached areas were unaffected. Occasional inflammatory cells were present on the ILM in all surgically treated groups. Treatment with Healaflow® and SF6 elicited similar results, and repeat surgery did not induce any further degenerative changes or inflammatory cell infiltration.

The pan-leukocyte CD45-labeling was sporadically present in all unoperated eyes; either dendritic cells in the inner retina (GCL and IPL) or round cells on the ILM. All surgically treated eyes showed increased labeling in these locations, with accumulations of labeled cells in the areas of retinal lesions such as retinal breaks or RDs. No additional labeling was seen with repeat surgery, nor with any of the vitreous substitutes. Müller cell activation as demonstrated with GFAP labeling was present at low levels in unoperated controls, whereas surgery elicited a generally increased labeling in the inner retinal layers, sometimes reaching the OPL and ONL. RDs and retinal breaks provoked further increases in labeling, although repeat surgery did not. No differences were seen between SF6- or Healaflow®-treated eyes. TUNEL assay did not reveal significant DNA fragmentation in any of the studied eyes.

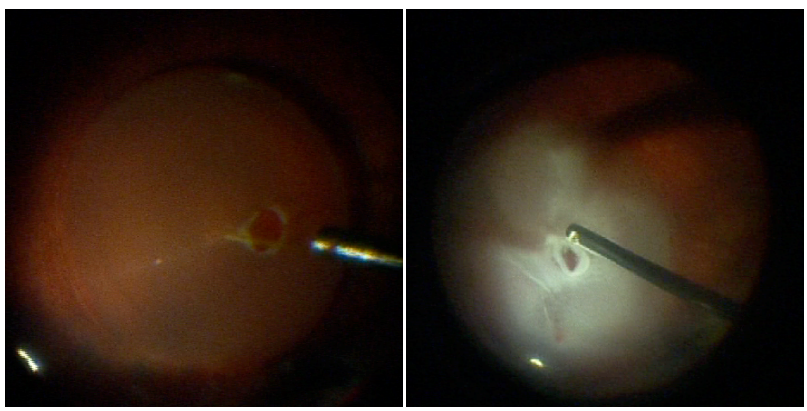

Figure 14. Surgical view of an experimental vitreous detachment.
Left: Creation of the retinal detachment (RD) with a subretinal micro-cannula.
Right: Repeat surgery of the same eye.

DISCUSSION

A search of the ideal vitreous substitute

To help us save and preserve the vision of patients suffering from vitreoretinal ailments such as retinal detachments (RDs), trauma, and proliferative diabetic retinopathy we need the right tools. The vitreous substitutes are key surgical aids in vitreoretinal surgery, ubiquitously used for tamponading retinal breaks and preventing the recurrence of RDs. The tamponades currently in clinical use are relatively effective in terms of anatomical results; however, they all have side effects and drawbacks, which affects the final visual outcome. In this project, we have embarked upon a quest, aiming to ultimately develop a more optimal vitreous substitute.

Although vitreoretinal surgery generally has a relatively high success rate, there is still significant room for improvement. A perfect vitreous substitute with more effective tamponading properties could potentially further increase the success rate of surgery while minimizing complications and patient discomfort during the healing period. In addition to improving the prognosis, and reducing suffering, socio-economical benefits may also be gained. By decreasing the need for prone positioning and reducing postoperative refractive errors, the rehabilitation process could be shortened, leading to speedier recovery and swifter return to work.

Studies into vitreous substitution have been ongoing since the early 20th century, and despite longstanding efforts, this task has proven to be challenging as evident by the impressive number of explored substances [Swindle 2007]. At first, the vitreous might look deceptively simple, but its composition and physiology are more intricate than what first meets the eye. Its interplay with other tissue is intimate, exemplified by the upkeep of physiological gradients of oxygen, nutrients and other substances vital for normal function [Stéfansson 1982, Stéfansson 2009]. Vitrectomy inevitably changes these interactions, and the addition of a foreign compound further affects the microenvironment. The often desired tamponading effect requires close contact with the retina and other delicate structures, placing high demands on the biocompatibility of the compound replacing the natural vitreous. These requirements, along with desired

physical and practical properties such as optical clarity, physiological refraction, and injectability are evidently not easily fulfilled.

In addition to the challenging physiological and practical demands on a candidate substance, the methodology for the translational process in the development of vitreous substitutes has been somewhat lacking. To alleviate this issue, we have devised and applied a number of new models for evaluating novel vitreous substitutes. We have also validated these models by comparing several previously studied experimental vitreous substitutes along with those in clinical use. Finally, we have during this process introduced and evaluated a very promising candidate for use as a novel vitreous substitute, Healaflow®.

The young and healthy vitreous is composed of a hyaluronic acid hydrogel reticulated by a meshwork of collagen fibers, thus reinforcing the gel. Theoretically, the physiological and physical properties of the natural vitreous would be optimally emulated by constructing a similarly composed gel. Such a compound is, however, not yet available. Cross-linked hyaluronic acid hydrogels are, however, a group of substances with the potential to fill this niche. Healaflow®, which has been extensively studied within this thesis, is one representative of these compounds with a previous track-record of favorable biocompatibility within its current use in glaucoma surgery [Schariot 2010, Roy 2012].

A new model for *in vitro* testing of vitreous substitutes

Several important steps in translation from bench-to-bedside need to be taken in order to introduce novel vitreous substitutes clinically, from physical property-testing and basic cytotoxicity assays to *in vivo*, and finally clinical trials.

Initial bench tests are essential to finding promising candidate compounds with focus on optical properties such as transparency and refractive index, and physical properties such as specific gravity and viscoelasticity. Current cytotoxicity assays often use RPE or endothelial cells which may provide useful information on the *in vitro* toxicity of the substances [Lloyd 1999, Malchiodi-Albedi 2005, Yang C-S 2008]. In this preliminary screen, unsuitable compounds can thereby be eliminated. However, clinical translation requires more certain proof of true biocompatibility which traditionally mandates the use of *in vivo* animal models [Coco 2018]. *In vivo* animal testing is, by its nature, often time-consuming and expensive, demanding considerable financial and personnel resources. For ethical reasons, it is also important to reduce, replace, and refine the severity of animaltesting. Thus, a more efficient initial

process to faster identify and eliminate unsuitable substances before the more costly and cumbersome *in vivo* animal trials is undoubtedly beneficial.

To bridge this gap in translation, and to further enhance the biocompatibility testing of candidate substances, we devised a new *in vitro* model based on adult rat retinal explants, described in paper I. This assay offers the possibility of relatively straightforward testing of the impact of vitreous substitutes on full-thickness neuroretinal sheets. These explants have a preserved cytoarchitecture which resembles the *in vivo* conditions more closely compared with existing cell-based assays, allowing for more precise predictions of biocompatibility [Bae 2012, Su 2011, Matteucci 2007, Lloyd 1999].

However, the adult retinal explant culture system paradigm involves several well-characterized degenerative changes, readily observable as early as 3 or 4 days *in vitro* (DIV), including gliosis and neuroretinal degeneration typified by GFAP upregulation, disruption of cell layers, and apoptosis [Kaempf 2008, Kobuch 2008, Taylor 2013]. By comparing these characteristics in retinal explants kept under standard conditions with those found in explants kept with vitreous substitute candidates, the *in vivo* biocompatibility can be inferred, and even beneficial effects evaluated by any attenuation of pathology.

In our newly developed *in vitro* model, explants kept with the two earlier studied potential vitreous substitutes displaying different biocompatibility profiles (PEG and Bio-Alcamid®), and a promising new compound (Healaflow®) were evaluated and compared to explants kept under standard culturing conditions [Pritchard 2010, Crafoord 2011]. Considerable differences in biocompatibility between the various groups were revealed, correlating well with the previous *in vivo* research. Among the evaluated substances, the most striking results were seen in cultures exposed to Bio-Alcamid®, displaying severe changes including gliosis, neuronal degeneration, and loss of structure—highlighting previous biocompatibility concerns [Crafoord 2011, Karim 2006, Nelson 2011]. On the other end of the spectrum, retinas cultured with Healaflow® matched, and sometimes even surpassed the preservation of structure of those kept with medium only. The third substance, PEG exhibited mild degenerative findings. Thus, a good correlation exists between the results of the presented *in vitro* explant culture system and *in vivo* findings, validating the model as a suitable pre-*in vivo* testing environment.

A new vitreous substitute tested *in vivo*

After successful *in vitro* trials, the logical next step in translation is *in vivo* trials, often with initial tests in rodents such as the iconic lab rats and guinea pigs. In surgical ophthalmic research, the animal of choice has often been the rabbit, for practical reasons as well as the anatomical and physiological similarities.

To further test the biocompatibility of Healaflow® we utilized the rabbit *in vivo* model previously described [Pritchard 2010, Crafoord 2011]. The results from this study are presented in paper II, wherein the gel was found to be easily incorporated into standard vitrectomy protocols. No clinical signs of toxic reactions, uveitis or secondary glaucoma were found during the follow-up time of up to 3 months. After this time, the retinal morphology and function were unchanged, highlighting the excellent biocompatibility of Healaflow®. The gel, furthermore, maintained its integrity for at least a couple of weeks after injection, indicating a potential use for at least short-term tamponade. A few iatrogenic retinal detachments were produced at surgery, but were self-limiting, giving credence to the notion of the tamponading properties of the gel.

Healaflow®, therefore, remained highly relevant for further work in the translational process to develop a clinically useful novel vitreous substitute.

An exploration into inflammatory responses elicited by vitreous substitutes

Several studies have highlighted inflammation as a commonly occurring phenomenon in the development of vitreous substitutes, often limiting their practical use [Oosterhuis 1966, Vote 2003, Liu 2017]. It is also well-established clinically that vitrectomy by itself elicits inflammation, necessitating the routine postoperative topical steroid regime to avoid uveitis [Yasuda 2016, Ben Yahida 2016]. Levels of cytokines such as IL-6, IL-8, MCP-1, and TGF-β1 are known to increase in the aqueous humor after vitrectomy, but the pathophysiology, however, is incompletely known [Hoerster 2013]. To further elucidate the underlying factors to these complications, we employed the previously described rabbit vitrectomy model to explore inflammatory responses elicited by current and experimental vitreous substitutes, and those of vitrectomy itself (paper III). Concurrent with this, we further validated the biocompatibility of Healaflow®.

Five immunohistochemical markers were chosen to reflect different aspects of the immunological response; GFAP and galactein-3 are representative of intrinsic retinal factors, while leucocytes positive for CD45, CD68, and CD20 indicate responses from the external immune system. The vitreous substitutes were selected according to their previously known, distinctly different biocompatibility profiles. The polyalkylimide hydrogel Bio-Alcamid® caused severe degenerative morphological changes and gliosis in our earlier studies [Crafoord 2011, paper I], and has been proven to cause chronic inflammation in clinical use as a tissue filler within plastic surgery [Karim 2006, Nelson 2011]. Silicone oil is currently in routine clinical use but has known biocompatibility issues in the long-term such as inflammation, retinal toxicity, and optic nerve atrophy [Asaria 2004, Papp 2007, Wickham 2007]. The commercially available cross-linked hyaluronic acid hydrogel Healaflow® exhibits, as we have previously shown, good biocompatibility [papers I and II]. These compounds were compared to balanced salt solution (BSS), the standard "minimal" treatment in a clinical setting.

Immunohistochemistry with these immune markers revealed immune activation detectable for at least a month after surgery in all groups. The intensity of the inflammatory reaction correlated well with the previously known biocompatibility profiles of the different vitreous substitutes—Healaflow®, similarly to BSS, elicited minimal inflammation, whereas a potent immune response was induced by exposure to silicone oil, and especially Bio-Alcamid®. These inflammatory markers also correlated well with the degree of injury found in the group with iatrogenic retinal lesions such as breaks and RDs.

The intrinsic retinal response included a diffuse upregulation of Galectin-3, a marker for neuroinflammation, but was predominantly characterized by an early gliotic response evident by the upregulation of GFAP. This response was transient and modest in eyes with Healaflow® and BSS, with the most prominent activation 7 days after surgery, while Silicone oil elicited a stronger, likewise transient reaction. Bio-Alcamid®, on the other hand, provoked even stronger, sustained inflammation. Müller cell activation and gliosis are common responses to retinal tissue injury, and has previously been described after vitrectomy as well as in experimental RDs [Anderson 1986, Durlu 1990, Guizzo 2008], while Galectin-3 has not, to our knowledge been described after vitrectomy, suggesting a neuroinflammatory response separate from gliosis [Ghosh 2018].

There was also an increased presence of cellular inflammation in all vitrectomized eyes, accompanying the retina-intrinsic response. This presence was readily detected with the pan-leukocyte antigen CD45, but also with the macrophage-linage antigen CD68, while significant CD20 labeling was present exclusively after exposure to Bio-Alcamid®. The expression of CD-antigen was congruent with GFAP-labeling, showing potent cellular immune responses in silicone oil, and Bio-Alcamid® filled eyes. A vigorous phagocytic response, together with the presence of multi-nucleated giant

cells indicates that the inflammation is, at least partly, due to a foreign body reaction. This pattern is in accordance with previously observed foreign body reactions and giant cell granulomas associated with silicone oil [Budde 2001, Srinivasan 2003, Wickham 2007]. Contrasting to this pattern, but similar to the GFAP-results, was the minimal presence of labeled immune cells in Healaflow® and BSS filled eyes. The structural and physiological similarities between Healaflow® and the natural vitreous may very well explain the absence of elevated immune activation when used as a vitreous substitute. To conclude, GFAP, CD45, and CD68 are relevant biomarkers for inflammation after vitrectomy and may be valuable in the search for novel vitreous substitutes.

A new *in vivo* model for treatment of retinal detachments

Ideally, the next step towards clinical translation involves *in vivo* testing in a relevant disease model which, in the case of vitreous substitutes has not been extensively explored so far. Research animals are generally young, healthy with eyes featuring an intact adhesion between the retina and the posterior vitreous membrane. In contrast, the pathophysiology of rhegmatogenous retinal detachment (RRD) includes posterior vitreous detachment (PVD) and retinal break. The pathophysiological process in the detached retina also involves progressive morphological changes such as neuronal degeneration, gliosis and proliferative vitreoretinopathy (PVR) affecting interactions with a tamponading agent [Lewis 2002, Khoroshilova-Maslova 2015].

A surprisingly small number of studies have evaluated the effects of potential vitreous substitutes on detached retinas. Among them, most have utilized retinal detachments created immediately prior to the application of the treatment [Yamana 2000, Teruya 2009, Hirata 2013, Yamamoto 2013, Schnichels 2017]. These protocols allow for practical testing of the tamponading effect but do not truly affect the interactions between the substances and a detached retina. To accomplish this, the retina must be detached over a period, allowing for the pathophysiological responses to form before the reattachment surgery.

The few studies that have been published using vitreous substitutes in retinal detachment models have primarily been designed to explore the pathophysiology of retinal detachment and reattachment, rather than to study vitreous substitutes. They have all been conducted in the same feline model, including vitrectomy and lensectomy before the creation of a retinal detachment with a microinjection of either BSS, or viscoelastic solution (Healon®). Reattachment surgery including fluid-air exchange

and injection with SF6 has been performed after time periods ranging from 1 hour up to 42 days [Anderson 1986, Lewis 2002, Lewis 2005]. Compared to the clinical situation, there are several major dissimilarities. For example, most clinical cases of RD present with a PVD and retinal breaks, and the lens is usually either intact or replaced with an intraocular lens after cataract surgery.

Our goal was to create a more relevant preclinical setting for the development of vitreous substitutes than those currently available i.e., a RRD treatment model with the retina detached using PVD and a break, and with a retained lens. The repeat surgery model presented in paper IV includes vitrectomy with PVD and creation of retinal detachments with a microinjection of dilute viscoelastic solution and retinal breaks, similar to the RRD model for porcine eyes described by Jackson [Jackson 2003]. After a period of detachment, in this study 24 hours, redetachment surgery with vitreous substitutes is performed. For reasons expanded upon previously, we used rabbits, but the principles can readily be applied in other species.

The method proved feasible, although not entirely without technical challenges due to the presence of conjunctival edema, vitreous hemorrhage, and cataract. These factors may be alleviated by further experience and refinement of the model. Eyes with repeat surgery displayed a higher number of attached retinas than eyes without, confirming the effect of the redetachment surgery in this setting. Due to a relatively low number of comparable cases, no definite conclusion can be made concerning the comparison between the evaluated vitreous substitutes, SF6 and Healaflow®. There was, however at least as high reattachment rate in the Healaflow® group as with the currently used SF6, implying a beneficial potential clinical effect. The SF6 group included a high rate of progression of retinal breaks to giant ruptures, possibly due to mechanical stress and the lack of retinopexy in the thin and avascular rabbit retina. The repeat surgery model in the rabbit eye may therefore be more suitable for gels than tamponading gases such as SF6.

Morphology and immunohistochemistry with GFAP and CD45 demonstrated similar retinal findings in eyes with, and without repeat surgery, as well as treatment with either of the two tamponades (SF6 and Healaflow®). The surgically treated retinas displayed a generally normal overall morphology with expected degenerative changes in, and in the vicinity of, the retinal detachments and lesions. Immunohistochemistry revealed general, and localized upregulation of GFAP as well as infiltration with CD45 labeled cells.

The repeat surgery model thus provides a more clinically relevant method for evaluating potential vitreous substitutes prior to the translation to clinical trials compared to models involving normal eyes. Healaflow® still remains a promising candidate for development into clinical use, but further studies are needed—potentially by using the repeat surgery model in porcine eyes, which are even more similar to the human counterpart.

A project with future implications

Recent medical research scandals have reiterated the need for prudent animal testing before new invasive therapies are introduced into human trials [Lancet 2018]. Despite rigorous testing, ethical guidelines and government regulations, some side effects and adverse reactions are still discovered after a long period of clinical use. One of the substances used in this thesis, Bio-Alcamid®, has a particularly illustrative history. It was enthusiastically received on its introduction as a synthetic tissue filler in plastic and reconstructive surgery, generally considered a safe and valuable device [Protopapa 2003, Claoue 2004, Ramires 2005, Lahiri 2007]. After several years of clinical use, reports of long-term complications such as chronic inflammation and excessive capsular formation surfaced, and the product was subsequently withdrawn [Karim 2006, Nelson 2011]. Before these concerns were evident, our group had initiated primary preclinical studies in order to test Bio-Alcamid® as tamponade for vitreoretinal surgery. The results in our *in vivo* rabbit vitrectomy model were disappointing, showing a surprising but significant lack of biocompatibility with inflammation and severe degeneration, and further development was therefore obviously discontinued [Crafoord 2011]. From these beginnings, our desire to characterize and predict these adverse events were born, along with a resolve to find better and more suitable substances.

During the course of this project, several new methods have been pioneered to increase the rigor of biocompatibility testing and to smoothen the translational process; from the *in vitro* retinal explant culture assay to the potential clinical impact of the *in vivo* repeat surgery model. Each step along this path contributes to a better understanding of the physiological and morphology impact of vitreous substitutes and furthermore offers potential economic and ethical benefits. Together these new methods represent our new paradigm for the translational development of future vitreous substitutes: sequential testing with a) *in vitro* retinal explant culture biocompatibility screening; b) *in vivo* biocompatibility trials in the rabbit vitrectomy model, evaluated with morphology, immunohistochemistry, and ERG); c) efficacy testing with the repeat surgery model in rabbit and/or porcine eyes; and d) human trials.

Through these stages of translation, a promising novel vitreous substitute, Healaflow® has proven its potential and excellent biocompatibility. With further research into its effect, it could potentially be a valuable addition as a short-term tamponade in clinical use in the near future.

CONCLUSIONS

- The herein presented *in vitro* retinal explant culture assay offers an innovative alternative to traditional cell-culture based toxicological testing wherein the bio-compatibility, and retinal impact of different vitreous substitutes can be tested in a more physiologically relevant environment. The bio-compatibility and retinal impact of previously evaluated substances (Bio-Alcamid® and PEG) correlate well with *in vivo* findings, whereas explants subjected to Healaflow® display similar or less degeneration than those kept under standard tissue culture conditions.

- In the previously established *in vivo* rabbit vitrectomy model, the cross-linked hyaluronic acid hydrogel Healaflow® exhibits excellent biocompatibility regarding retinal function and morphology.

- The use of vitreous substitutes and vitrectomy itself elicits intrinsic as well as extrinsic inflammatory tissue responses in the form of galectin-3 and GFAP upregulation in concert with infiltration of CD45 and CD68 positive cells. Retinas exposed to Healaflow® display inflammation comparable to BSS exposed counterparts, while Bio-Alcamid® and silicone oil elicit a response characterized by higher levels of gliosis and inflammatory cells.

- The *in vivo* repeat surgery model offers a new, clinically relevant way to study the practical use and effects of potential tamponades in the treatment of retinal detachment precluding clinical trials.

- The *in vitro* and *in vivo* biocompatibility profile of Healaflow® correlate well, suggesting that this compound is a promising candidate for translational development into clinical use as a vitreous substitute.

- The combination of the presented *in vivo* and *in vitro* methods comprise a new paradigm in the development of novel vitreous substitutes, potentially offering efficient and thorough evaluation before translation to clinical trials.

SVENSK SAMMANFATTNING

Blixtar. Svarta prickar, spindelben och dimmoln. Rörliga grumlingar framför ögat och liknade symptom drabbar varje dag många svenskar. Oftast är symptomen helt ofarliga men de kan också tyda på begynnande näthinneavlossning, vilket i värsta fall kan leda till blindhet.

Näthinneavlossning börjar i de flesta fall med en s.k glaskroppsavlossning. Detta tillstånd innebär att glaskroppen, den geléartade massa som fyller det inre av ögat, börjar skrumpna och släppa från näthinnan. Det är en åldersrelaterad process som kan vara oroväckande men oftast är helt ofarlig. Glaskroppen sitter normalt sett relativt löst fäst vid näthinnan, men om dragningen mot näthinnan blir för kraftig eller om det finns svagheter i denna, kan det uppstå hål eller större revor. Genom dessa kan vätska tränga in som i sin tur kan lösgöra näthinnan från underlaget, en näthinneavlossning. Om detta uppstår, får man i regel ännu mera framträdande och oroande symptom än vid enbart glaskroppsavlossning; synbortfall i form av mörka skuggor som gradvis blir större och till slut suddig syn. Normalt sett är det enda sättet att bota näthinneavlossning operation, vilken måste utföras relativt snabbt för att förhindra allvarliga synhandikapp eller t.o.m. blindhet.

Vitrektomi är en av de vanligaste operationsmetoderna vid näthinneavlossning och andra tillstånd som t.ex efter allvarligt trauma och diabeteskomplikationer. Denna operationsmetod innebär att man klipper och suger ut glaskroppen med små instrument, varvid draget mot näthinnan kan avlägsnas. För att täppa till, tamponera, eventuella hål i näthinnan och underlätta läkningsprocessen används oftast specialutvecklade gaser som så småningom försvinner av sig själv och ersätts av kroppsegen vätska. I svåra fall kan även silikonolja användas, vilken måste avlägsnas efter en tid. Både gaserna och oljan har avsevärda nackdelar; under behandlingstiden är synen alltid kraftigt nedsatt och behandlingen är förenad med biverkningar i form av inflammation, grå starr, tryckstegringar, toxisk påvekan på näthinnan och förtvining av synnerven. Dessutom måste man med dessa tamponader ofta för att få optimal effekt hålla huvudet i obekväma lägen under en viss del av behandlingstiden.

För att förbättra både läkning och minska biverkningarna har man under lång tid försökt utveckla nya, bättre sätt att ersätta glaskroppen på, men hittills har inget alternativ lyckats. Man skulle kunna tro att glaskroppen, som även ögonläkare ofta brukar se som en okomplicerad gelé, borde vara relativt enkel att efterlikna, men så tycks inte vara fallet. I själva verket är glaskroppens struktur mycket specifik och dess fysiologiska påverkan på ögats övriga vävnader komplex. Studier har visat att glaskroppen är viktig för ett flertal normala funktioner, bland annat genom att upprätthålla den viktiga mikromiljön i ögat. Exempelvis ökar risken för gråstarr om denna miljö rubbas, såsom efter en vitrektomi. Glaskroppens struktur är en så kallad hydrogel som huvudsakligen består av vatten, hyaluronsyra och ett nätverk av kollagenfibrer som likt armeringsjärn skapar stadga. Hyaluronsyra är känt för att vara skonsamt mot kroppen; t.ex. har preparat i gel-form, ursprungligen utvunna ur tuppkammar, länge använts inom gråstarr-kirurgi och för att lindra artros i leder.

Svårigheten med att hitta en välfungerande ersättning för ögats naturliga glaskropp, ett *glaskroppssubstitut*, härrör från glaskroppens komplexa samspel med ögats inre i kombination med den långa lista av andra önskvärda egenskaper som man kan upprätta; till exempel måste preparatet vara skonsamt mot näthinnan, vara möjligt att föra in i ögat under operationen och inte störa synen med grumligheter eller besvärande brytningsindex. För att efterlikna den naturliga glaskroppen har vi därför under hela det projekt som ligger till grund för denna avhandling utvärderat ett preparat, Healaflow®, som består av hyaluronsyra där molekylerna på artificiell väg sammanlänkats, *korsbundits*.

Förutom ovan nämnda svårigheter, har det tidigare funnits luckor i processen för att utveckla och föra över nya lovande glaskroppssubstitut från laboratoriet till kliniken – den så kallat *translationella* processen. Traditionellt sett har man först analyserat nya substanser kemiskt och fysikaliskt samt utfört toxikologiska försök på enskilda celler. Därefter har man övergått till försök på friska, unga försöksdjur och så småningom till kliniska studier på patienter. De stora hopp i den translationella processen som dessa steg inneburit, har gjort att viktig information om ögats reaktioner på den undersökta substansen missats. Detta kan vara en av förklaringarna till att vi ännu inte har någon bättre tamponad att tillgå än gas och olja.

Den viktigaste vävnad som glaskroppen interagerar med är näthinnan. Denna struktur liknar på många sätt hjärnan i sin uppbyggnad och är därmed komplex i sin struktur. Dess samspel med främmande substanser återspeglas inte fullt ut i metoder som baseras på enstaka celler. Av detta skäl utvecklade vi i **artikel I** en metod för att säkrare bedöma hur skonsamt, *biokompatibelt*, ett glaskroppssubstitut är för näthinnan. Metoden baserar sig på att isolerad vuxen näthinna från råtta exponeras för gelartade substanser i en odlingsmiljö. Näthinnorna kan sedan analyseras mikroskopiskt och med sk. immunohistokemi. Resultatet från detta försök visade sig stämma väl överens med tidigare fynd som vår grupp gjort i en vitrektomimodell på kanin där påverkan av polyetylenglykol (PEG) och Bio-Alcamid® studerades. I det

aktuella experimentet undersöktes även den korsbundna gelen Healaflow® som befanns påverka näthinnan minst och t.o.m skyddade den från patologisk påverkan.

Som ett led i den translationella utvecklingen provade vi också Healaflow® i den tidigare nämnda kanin-vitrektomi modellen, presenterad i **artikel II**. Gelen kunde lätt användas med sedvanliga kirurgiska metoder och den verkade vare sig påverka näthinnans elektrofysiologi (funktion) eller struktur negativt.

För att gå ännu mer på djupet i hur olika glaskroppssubstitut och vitrektomi i sig själv påverkar näthinnan gick vi sedan vidare med ytterligare försök i kanin-modellen, vilket redogörs för i **artikel III**. Här ville vi närmare utreda det inflammatoriska svaret i näthinnan efter påverkan av glaskroppssubstitut som tidigare visat olika biokompatibilitetsprofiler. I detta experiment jämförde vi Healaflow® och Bio-Alcamid® med två av de substanser som idag används i klinisk praxis – saltlösning (BSS) och silikonolja. Vid immunohistokemiska analyser av ett antal olika inflammatoriska markörer, för såväl näthinnans interna som det cell-baserade externa svaret, fann vi att Healaflow® utlöser ungefär lika lindrig inflammation som vitrektomi där BSS används, medan både silikonolja och Bio-Alcamid® frammanar ett betydligt kraftigare inflammatoriskt svar i näthinnan.

Som tidigare nämnts brukar man oftast i utvecklingsprocessen av potentiella glaskroppssubstitut gå direkt från försök i friska djurögon till kliniska prövningar. Detta tillvägagångssätt tar t.ex. inte hänsyn till att en avlossad näthinna sannolikt är känsligare och samspelar annorlunda med främmande substanser jämfört med en frisk motsvarighet. Dessutom kan man givetvis inte heller utvärdera någon behandlingseffekt. För att bättre värdera detta samspel och kunna bedöma substansens behandlingspotential utvecklade vi en ny näthinneavlossningsmodel i kanin, beskriven i **artikel IV**. I denna skapar vi i samband med vitrektomi först en glaskroppsavlossning, sedan ett hål i näthinnan och till sist en avlossning genom att utföra en mikroinjektion under densamma. Efter minst ett dygn görs en ny vitrektomi då näthinnan läggs på plats och glaskroppssubstitut injiceras. Vi jämförde i denna studie Healaflow® med en av de vanligaste behandlingsmetoderna i kliniken, SF6-gas. Metoden visade sig vara tekniskt något krävande, men klart genomförbart. Studien är av relativt preliminär karaktär, men Healaflow® visade sig i dessa försök ha minst lika god effekt som SF6.

Sammanfattningsvis presenteras här metoder som tillsammans utgör ett nytt translationellt tillvägagångssätt för att utveckla nya glaskroppssubstitut. Vi har med hjälp av detta utvärderat bl.a Healaflow® som visat sig ha stor potential för framtida klinisk användning vid kirurgisk behandling av avancerad näthinnesjukdom.

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- A love supreme, John Coltrane

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REFERENCES

Algvere PV, Jahnberg P, Textorius O (1999) The Swedish Retinal Detachment Register. I. A database for epidemiological and clinical studies. Graefes Arch Clin Exp Ophthalmol 237(2):137–44

Anderson DH Stern WH, Fisher SK, Erickson PA, Borgula GA (1981) The onset of pigment epithelial proliferation after retinal detachment. Invest Ophthalmol Vis Sci 21(1 pt 1):10–6

Anderson DH, Stern WH, Fisher SK Erickson PA Borgula GA (1983) Retinal detachment in the cat: the pigment epithelial-photoreceptor interface. Invest Ophthalmol Vis Sci 24(7): 906–26

Anderson DH, Guérin CJ, Erickson PA, Stern WH, Fisher SK (1986) Morphological recovery in the reattached retina. Invest Ophthalmol Vis Sci 27(2):168–83

Annaka M, Mortensen K, Vigild ME, Matsuura T, Tsuji S, Ueda T, Tsujinaka H (2011) Design of an injectable in situ gelation biomaterials for vitreous substitute. Biomacromolecules 12(11): 4011–21

Asaria RH, Kon CH, Bunce C, Sethi CS, Limb GA, Khaw PT, Aylward GW, Charteris DG (2004) Silicone oil concentrates fibrogenic growth factors in the retro-oil fluid. Br J Ophthalmol 88(11):1439–42

Bae SH, Che J-H, Seo J-M, Jeong J, Kim ET, Lee SW, Koo K-I, Suaning GJ, Lovell NH, Cho D-I, Kim SJ, Chung H (2012) In vitro biocompatibility of various polymer-based microelectrode arrays for retinal prosthesis. Invest Ophthalmol Vis Sci 53(6):2653–7

Baino F (2010) Towards an ideal biomaterial for vitreous replacement: Historical overview and future trends. Acta Biomater 7(3):921–25

Balazs EA, Freeman MI, Klöti R, Meyer-Schwickerath G, Regnault F, Sweeney DB (1972) Hyaluronic acid and replacement of vitreous and aqueous humor. Mod Probl Ophthalmol 10:3–21

Barbazetto IA, Liang J, Chang S, Zheng L, Spector A, Dillon JP (2004) Oxygen tension in the rabbit lens and vitreous before and after vitrectomy. Exp Eye Res 78(5):917–924

Ben Yahia S, Kahloun R, Abroug N, Kaibi I, Laadhari G, Jelliti B, Khairallah M (2016) Comparative effect of topical diclofenac and topical dexamethasone on anterior chamber flar e and postoperative pain following rhegmatogenous retinal detachment surgery. Int Ophthalmol 36(5):623–8

Bettin P, Di Matteo F, Rabiolo A, Fiori M, Ciampi C, Bandello F (2016) Deep Sclerectomy With Mitomycin C and Injectable Cross-linked Hyaluronic Acid Implant: Long-term Results. J Glaucoma 25(6):e625–9

Budde M, Cursiefen C, Holbach LM, Naumann GO (2001) Silicone oil-associated optic nerve degeneration. Am J Ophthalmol 131(3)392–4

Caffé AR, Ahuja P, Holmqvist B, Azadi S, Forsell J, Holmqvist, I Söderpalm AK, van Veen T (2001) Mouse retina explants after long-term culture in serum free medium. J Chem Neuroanat 22(4):263–73

Chang S (2006) LXII Edward Jackson lecture: open angle glaucoma after vitrectomy. Am J Ophthalmol 141(6):1033–1043

Chirila TV, Tahija S, Hong Y, Vijayasekaran S, Constable IJ (1994) Synthetic polymers as materials for artificial vitreous body: review and recent advances. J Biomater Appl 9(2):121–37

Claoue BL, Rabineau P (2004) The polyalkamide gel: experience with Bio-Alcamid®. Semin Cutan Med Surg 23(4):236–240

Coco RM, Srivastava GK, Andrés-Iglesias C, Medina J, Rull F, Fernandez-Vega-Gonzalez A, Fernandez-Bueno I, Dueñas A, Pastor J (2018) Acute retinal toxicity associated with a mixture of perfluorooctane and perfluorohexyloctane: failure of another indirect cytotoxicity analysis. Br J Ophthalmol 2018;0:1–6

Crafoord S, Andreasson S, Ghosh F (2011) Experimental vitreous tamponade using polyalkyl-imide hydrogel. Graefes Arch Clin Exp Ophthalmol 249: 1167–74

Cutler NL (1947) Vitreous transplantation. Trans Am Acad Ophthalmol Otolaryngol 52:253–259

Del Amo EM, Urtti A (2015) Rabbit as an animal model for intravitreal pharmacokinetics: Clinical predictability and quality of the published data. Exp Eye Res 137:111–24

Denlinger J, El-Mofty A, Balazs E (1980) Replacement of the liquid vitreus with sodium hyaluronate in monkeys II. Long-term evaluation. Exp Eye Res 30:101–117

Deutschmann R (1906) Zur operativen behandlung der netzhautablosung. Klin Monastbl Augenheilkd 44:364–70

Diederen RMH, La Heij EC, Lemmens MAM, Kijlstra A, Vente J, Hendrikse F (2008) Cyclic GMP in the pig vitreous and retina after experimental retinal detachment. Mol Vis 14:255–61

Durlu YK, Ishiguro S, Yoshida A, Mito T, Tsuchiya M, Tamai M (1990) Response of Müller cells following experimental lensectomy-vitrectomy. Graefes Arch Clin Exp Ophthalmol 228(1): 44–8

Duvvuri S, Janoria KG, Pal D, Mitra AK (2007) Controlled delivery of ganciclovir to the retina with drug-loaded Poly(d,L-lactide-co-glycolide) (PLGA) microspheres dispersed in PLGA-PEG-PLGA Gel: a novel intravitreal delivery system for the treatment of cytomegalovirus retinitis. J Ocul Pharmacol Ther 23(3):264–74

Elsing SH, Fekrat S, Green WR, Chang S, Wajer SD, Haller JA (2001) Clinicopathologic findings in eyes with retained perfluoro-n-octane liquid. 108(1):45–8

Engelsberg K, Ehinger B, Wasselius J Johansson K (2004) Apoptotic cell death and microglial cell responses in cultured rat retina. Graefe's Arch Clin Exp Ophthalmol 242(3):229–39

Foster WJ, Aliyar HA, Hamilton P, Ravi N (2006) Internal Osmotic Pressure as a Mechanism of Retinal Attachment in a Vitreous Substitute. J Bioact Compat Polym 21(3):221–35

Gao Q, Mou S, Ge J, To C-H, Hui Y, Liu A, Wang Z, Long C, Tan J (2008) A new strategy to replace the natural vitreous by a novel capsular artificial vitreous body with pressure-control valve. Eye (Lond) 22(3):461–8

García-Layana A, García-Arumí J, Ruiz-Moreno JM, Arias-Barquet L, Cabrera-López F, Figueroa MS (2015) A review of current management of vitreomacular traction and macular hole. J Ophthalmol 2015:809640

Gerke E, Meyer-Schwickerath G, Wessing A (1984) Healon in retinal detachment with proliferative vitreoretinopathy. Graefes Arch Clin Exp Ophthalmol 221(5):241–3

Ghoraba HH, Zaky AG, Heikal MA, Elgemai EEM, Abd Al Fatah HM (2017) Silicone Oil-Related Visual Loss. Ophthalmologica 238(1–2):59–67

Ghosh F, Abdshill H, Arnér K, Vos, U, Taylor L (2018) Retinal neuroinflammatory induced neuronal degeneration - Role of toll-like receptor-4 and relationship with gliosis. Exp Eye Res 169:99–110

Gjörloff KW, Andréasson S, Ehinger B (2004) Standardized full-field and multifocal electroretinography in rabbits. Doc Ophthalmol 109:163–68

Guizzo R, Paques MW, Anhezini L, Simon CR, Scott IU, Jorge R, Santos WF (2008) Neuroprotective effects of oral lamotrigine administration on rabbit retinas after pars plana vitrectomy and silicone oil injection. Retina 28(4): 638–44

Haimann MH, Burton TC, Brown CK (1982) Epidemiology of retinal detachment. Arch Ophthalmol 100(2):289–92

Heimann H, Stappler T, Wong D (2008) Heavy tamponade 1: a review of indications, use, and complications. Eye (Lond) 22(10):1342–59

Hirata A, Yamamoto S, Okinami S (2013) Use of an ophthalmic viscosurgical device for experimental retinal detachment in rabbit eyes. J Funct Biomater 4(1):6–13

Hoerster R, Hermann MM, Rosentreter A, Muether PS, Kirchhof B, Fauser S (2013) Profibrotic cytokines in aqueous humour correlate with aqueous flare in patients with rhegmatogenous retinal detachment. Br J Ophthalmol 97(4):450–3

Holekamp NM, Shui Y-B, Beebe DC (2005) Vitrectomy surgery increases oxygen exposure to lens: a possible mechanism for nuclear cataract formation. Am J Ophthalmol 139(2):302–10

Iribarne M, Canto-Soler MV, Torbidoni V, Suburo AM (2007) Controlling retinal pigment epithelium injury after experimental detachment of the retina. Invest Ophthalmol Vis Sci 48(3):1348–54

Jackson TL, Hillenkamp J, Williamson TH, Clarke KW, Almubarak AI, Marshall J (2003) An experimental model of rhegmatogenous retinal detachment: surgical results and glial cell response. Invest Ophthalmol Vis Sci 44(9):4026–34

Jiang X, Peng Y, Yang C, Liu W, Han B (2018) The feasibility study of an in situ marine polysaccharide-based hydrogel as the vitreous substitute. J Biomed Mater Res A 106(7):1997–2006

Kaempf S, Walter P, Salz AK, Thumann G. (2008) Novel organotypic culture model of adult mammalian neurosensory retina in co-culture with retinal pigment epithelium. J. Neurosci. Methods. 173(1):47–58

Kanski JJ, Daniel R (1973) Intravitreal silicone injection in retinal detachment. Br J Ophthalmol. 57(8): 542–5

Kanski, J J (1975) Intravitreal hyaluronic acid injection. A long-term clinical evaluation. Br J Ophthalmol 59(5):255–6

Karim RB, Hage JJ, van Rozelaar L, Lange CAH, Raaijmakers J (2006) Complications of polyalkylimide 4% injections (Bio-Alcamid): a report of 18 cases. J Plast Reconstr Aesthet Surg 59(12): 1409–14

Khoroshilova-Maslova IP, Leparskaya NL, Nabieva MM, Andreeva LD (2015) Experimental Modeling of Proliferative Vitreoretinopathy. An Experimental Morphological Study. Bull Exp Biol Med 159(1):100–2

Kirchhof B, Wong D, Van Meurs J, Hilgers RD, Macek M, Lois N, Schrage NF (2002) Use of perfluorohexyloctane as a long-term internal tamponade agent in complicated retinal detachment surgery. Am J Ophthalmol 133(1):95–101

Killey FP, Edelhauser HF, Aaberg TM (1978) Intraocular sulfur hexafluoride and octofluorocyclobutane. Effects on intraocular pressure and vitreous volume. Arch Ophthalmol 96(3):511–15

Kirchhof B, Wong D, Van Meurs J, Hilgers RD, Macek M, Lois N, Schrage NF (2002) Use of perfluorohexyloctane as a long-term internal tamponade agent in complicated retinal detachment surgery. Am J Ophthalmol 133(1):95–101

Kobuch K, Herrmann WA, Framme C, Sachs HG, Gabel VP, Hillenkamp J (2008) Maintenance of adult porcine retina and retinal pigment epithelium in perfusion culture: characterisation of an organotypic in vitro model. Exp Eye Res. 86(4):661–668

Koster R, Stilma JS (1986) Healon as intravitreal substitute in retinal detachment surgery in 40 patients. Doc Ophthalmol 64:13–1

Kuhn, Ferenc (2014) The timing of reconstruction in severe mechanical trauma. Ophthalmic Res 51(2): 67–72

Lahiri A, Waters R (2007) Experience with Bio-Alcamid®, a new soft tissue endoprosthesis. J Plast Reconstr Aesthet Surg 60(6):663–667

Lancet, the (2018) The final verdict on Paolo Macchiarini: guilty of misconduct. Lancet 392(10141):2

Lewis GP, Charteris DG, Sethi CS, Leitner WP, Linberg KA, Fisher SK (2002) The ability of rapid retinal reattachment to stop or reverse the cellular and molecular events initiated by detachment. Invest Ophthalmol Vis Sci 43(7):2412–2420

Lewis GP, Sethi CS, Carter KM, Charteris DG, Fisher SK (2005) Microglial cell activation following retinal detachment: a comparison between species. Mol Vis (11):491–500

Lin X, Sun X, Wang Z, Jiang Z, Liu Y, Wang P, Gao Q (2016) Three-Year Efficacy and Safety of a Silicone Oil-Filled Foldable-Capsular-Vitreous-Body in Three Cases of Severe Retinal Detachment. Transl Vis Sci Technol 5(1):2[1–8]

Liu Y, Ke Q, Chen J, Wang Z, Xie Z, Jiang Z, Ge J, Gao Q (2010) Sustained mechanical release of dexamethasone sodium phosphate from a foldable capsular vitreous body. Invest Ophthalmol Vis Sci 51(3):1636–42

Liu Z, Fu G, Liu A (2017) The relationship between inflammatory mediator expression in the aqueous humor and secondary glaucoma incidence after silicone oil tamponade. Exp Ther Med 14(6):5833–5836

Lloyd AW, Dropcova S, Faragher RG, Gard PR, Hanlon GW, Mikhalovsky SV, Olliff CJ, Denyer SP, Letko E, Filipec M (1999) The development of in vitro biocompatibility tests for the evaluation of intraocular biomaterials. J Mater Sci Mater Med 10(10-11):621–627

Loporchio D, Mukkamala L, Gorukanti K, Zarbin M, Langer P, Bhagat N (2016) Intraocular foreign bodies: A review. Surv Ophthalmol 61(5):582–96

Lucas DR, Trowell OA (1958) In vitro culture of the eye and the retina of the mouse and rat. J Embryol Exp Morphol 1958 6(1):178–82

Machemer R (1968) Experimental retinal detachment in the owl monkey. IV. The reattached retina. Am J Ophthalmol 66(6):1075–91

Machemer R (1995) The development of pars plana vitrectomy: a personal account. Graefes Arch Clin Exp Ophthalmol 233(8):453–68

Mackiewicz J, Mühling B, Hiebl W, Meinert H, Maaijwee K, Kociok N, Lüke C, Zagorski Z, Kirchhof B, Joussen AM (2007) In vivo retinal tolerance of various heavy silicone oils. Invest Ophthalmol Vis Sci 48(4):1873–83

Malchiodi-Albedi F, Matteucci A, Formisano G, Paradisi S, Carnovale-Scalzo G, Scorcia G, Hoerauf H (2005) Induction of apoptosis in rat retinal cell cultures by partially fluorinated alkanes. Am J Ophthalmol 139(4):737–9

Mandal N, Lewis GP, Fisher SK, Heegaard S, Prause JU, La Cour M, Vorum H, Honoré B (2015) Proteomic Analysis of the Vitreous following Experimental Retinal Detachment in Rabbits. J Ophthalmol 2015:e583040

Marmor MF, Abdul-Rahim AS, Cohen DS (1980) The effect of metabolic inhibitors on retinal adhesion and subretinal fluid resorption. Invest Ophthalmol Vis Sci 19(8):893–903

Marmor MF, Fulton AB, Holder GE, Miyake Y, Brigell M, Bach M (2009) ISCEV Standard for full-field clinical electroretinography (2008 update). Doc Ophthalmol 118(1):69–77

Matteucci A, Formisano G, Paradisi S, Carnovale-Scalzo G, Scorcia, G, Caiazza S, Hoerauf H, Malchiodi-Albedi F (2007) Biocompatibility assessment of liquid artificial vitreous replacements: relevance of in vitro studies. Surv Ophthalmol 52(3):289–99

McMenamin PG (1997) The distribution of immune cells in the uveal tract of the normal eye. Eye (Lond) 11(Pt 2):183–93

Morescalchi F, Costagliola C, Duse S, Gambicorti E, Parolini B, Arcidiacono B, Romano MR, Semeraro F (2014) Heavy silicone oil and intraocular inflammation. Biomed Res Int 2014:574825

Nakamura K, Refojo MF, Crabtree DV, Pastor J, Leong FL (1991) Ocular toxicity of low-molecular-weight components of silicone and fluorosilicone oils. Invest Ophthalmol Vis Sci 32(12): 3007–20

Neffendorf JE, Gupta B, Williamson TH (2017) The role of intraocular gas tamponade in rhegmatogenous retinal detachment: A Synthesis of the Literature. Retina 38(Suppl 3):1–13

Nelson L, Stewart KJ (2011) Early and late complications of polyalkylimide gel (Bio-Alcamid)®. J Plast Reconstr Aesthet Surg 64(3):401–4

Oosterhuis JA, van Haeringen NJ, Jeltes IG, Glasius E (1966) Polygeline as a vitreous substitute. I. Observations in rabbits. Arch Ophthalmol. 76(2):258–65

Papp A, Kiss EB, Tímár O, Szabó E, Berecki A, Tóth J, Páli J (2007) Long-term exposure of the rabbit eye to silicone oil causes optic nerve atrophy. Brain Res Bull 74(1-3):130–33

Parmley VC, Barishak YR, Howes EL Jr, Crawford JB (1986) Foreign-body giant cell reaction to liquid silicone. Am J Ophthalmol 101(6):680–3

Pritchard CD, Crafoord S, Andréasson S, Arnér KM, O'Shea T M, Langer R, Ghosh FK (2010) Evaluation of viscoelastic poly(ethylene glycol) sols as vitreous substitutes in an experimental vitrectomy model in rabbits. Acta Biomater 7: 936–943

Protopapa C, Sito G, Caparole D, Cammarota N (2003) Bio-Alcamid® in drug- induced lipodystrophy. J Cosmet & Laser Ther 5(3–4):1–5

Ramires P, Miccoli M, Panzarini E, Dini L, Protopapa C (2005) In vitro and in vivo biocompatibility evaluation of a polyalkylimide hydrogel for soft tissue augmentation. J Biomed Mater Res B Appl Biomater 72(2):230–238

Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP (2004) Global data on visual impairment in the year 2002. Bull World Health Organ 82(11):844–51

Roy S, Thi HD, Feusier M, Mermoud A (2012) Crosslinked sodium hyaluronate implant in deep sclerectomy for the surgical treatment of glaucoma. Eur J Ophthalmol 22(1):70–6

Sakai T, Lewis GP, Linberg KA, Fisher SK (2001) The ability of hyperoxia to limit the effects of experimental detachment in cone-dominated retina. Invest Ophthalmol Vis Sci 42(13): 3264–73

Santhanam S, Liang J, Struckhoff J, Hamilton PD, Ravi N (2016) Biomimetic hydrogel with tunable mechanical properties for vitreous substitutes. Acta Biomater 43:327–337

Schariot GB (2010) Canaloplasty Re-establish the Natural Outflow in Patients with Chronic Open-Angle Glaucoma. J Current Glau Prac 4(2):97–102

Schatz B, El-Shabrawi Y, Haas A, Langmann G (2004) Adverse side effects with perfluorohexyloctane as a long-term tamponade agent in complicated vitreoretinal surgery. Retina 24(4):567–73.

Schnichels S, Schneider N, Hohenadl C, Hurst J, Schatz A, Januschowski K, Spitzer MS (2017) Efficacy of two different thiol-modified crosslinked hyaluronate formulations as vitreous replacement compared to silicone oil in a model of retinal detachment. PLoS One 12(3):e0172895

Schramm C, Spitzer MS, Henke-Fahle S, Steinmetz G, Januschowski K, Heiduschka P, Geis- Gerstorfer J, Biedermann T, Bartz-Schmidt KU, Szurman P (2012) The cross-linked biopolymer hyaluronic acid as an artificial vitreous substitute. Invest Ophthalmol Vis Sci 53(2):613–21

Sebag, J [ed.] (2014)Vitreous: in health and disease. Springer ScienceþBusiness Media B.V

Shafer DM (1976) Human vitreous transplantation. Ann R Coll Surg Engl 58(1):25–23

Siegfried CJ, Shui Y-B, Holekamp NM, Bai F, Beebe DC (2010) Oxygen distribution in the human eye: relevance to the etiology of open-angle glaucoma after vitrectomy. Invest Ophthalmol Vis Sci 51(11):5731–8

Sigler EJ, Randolph JC, Charles S, (2014) Foreign body response within postoperative perfluoro-N-octane for retinal detachment repair: clinical features, grading system, and histopathology. Retina 34(2):237–46

Srinivasan S, Singh AK, Desai SP, Talbot JF, Parsons MA (2003) Foreign body episcleral granulomas complicating intravitreal silicone oil tamponade: a clinicopathological study. Ophthalmology 110(9)1837–40

Stappler T, Morphis G, Irigoyen C, Heimann H (2011) Is there a role for long-term silicone oil tamponade for more than twelve months in vitreoretinal surgery? Ophthalmologica 226(Suppl I):36–41

Stéfansson E, Landers MB 3d, Wolbarsht ML (1982) Vitrectomy, lensectomy, and ocular oxygenation. Retina 2(3):159–66

Stéfansson E (2009) Physiology of vitreous surgery. Graefe's Arch Clin Exp Ophthalmol 247(2):147–163

Stenzel KH, Dunn MW, Rubin AL, Miyata T (1969) Collagen gels: design for a vitreous replacement. Science 164(3885):1282–3

Streilein JW (2003) Ocular immune privilege: therapeutic opportunities from an experiment of nature Nat Rev Immunol 3(11):879–89

Strettoi E, Masland RH (1995) The organization of the inner nuclear layer of the rabbit retina. J Neuroscience 15:875–886

Su, W-Y, Chen K-H, Chen Y-C, Lee Y-H, Tseng C-L, Lin F-H (2011) An injectable oxidated hyaluronic acid/adipic acid dihydrazide hydrogel as a vitreous substitute. J Biomater Sci Polym Ed 22(13):1777–97

Sun X, Xu X, Wang F, Zhang X, Yu Z, Lu H, Ho PC (2007) Effects of nerve growth factor for retinal cell survival in experimental retinal detachment. Curr Eye Res 32(9):765–72

Suri S, Banerjee R (2006) In vitro evaluation of in situ gels as short term vitreous substitutes. J Biomed Mater Res A 79(3):650–64

Swindle KE, Ravi N (2007) Recent advances in polymeric vitreous substitutes. Expert Rev Ophthalmol 2(2):255–265

Swindle-Reilly KE, Shah M, Hamilton PD, Eskin TA, Kaushal S, Ravi N (2009) Rabbit study of an in situ forming hydrogel vitreous substitute. Invest Ophthalmol Vis Sci 50(10):4840–6

Tao Y, Tong X, Zhang Y, Lai J, Huang Y, Jiang Y-R, Guo B-H (2013) Evaluation of an in situ chemically crosslinked hydrogel as a long-term vitreous substitute material. Acta Biomater 9(2):5022–30

Taylor L, Arnér K, Engelsberg K, Ghosh F (2013) Effects of glial cell line-derived neurotrophic factor on the cultured adult full-thickness porcine retina. Curr Eye Res. 38(4);503–515

Teruya K, Sueda J, Arai M, Tsurumaru N, Yamakawa R, Hirata A, Hirose T (2009) Patching retinal breaks with Seprafilm in experimental rhegmatogenous retinal detachment of rabbit eyes. Eye (Lond) 23(12):2256–9

Thackaberry EA, Farman C, Zhong F, Lorget F, Staflin K, Cercillieux A, Miller PE, Schuetz C, Chang D, Famili A, Daugherty AL, Rajagopal K, Bantseev V (2017) Evaluation of the Toxicity of Intravitreally Injected PLGA Microspheres and Rods in Monkeys and Rabbits: Effects of Depot Size on Inflammatory Response. Invest Ophthalmol Vis Sci 58(10):4274–4285

Thompson JT (2003) The role of patient age and intraocular gases in cataract progression following vitrectomy for macular holes and epiretinal membranes. Trans Am Ophthalmol Soc 101:485–98

Vatne HO, Syrdalen P (1986) The use of sodium hyaluronate (Healon) in the treatment of complicated cases of retinal detachment. Acta Ophthalmol 64(2):169–72

Vaziri K, Schwartz SG, Kishor KS, Flynn HW Jr (2016) Tamponade in the surgical management of retinal detachment. Clin Ophthalmol 10:471–6

Verardo MR, Lewis GP, Takeda M, Linberg KA, Byun J, Luna G, Wilhelmsson U, Pekny M, Chen D-F, Fisher SK (2008) Abnormal reactivity of muller cells after retinal detachment in mice deficient in GFAP and vimentin. Invest Ophthalmol Vis Sci 49(8)3659–65

Versura P, Cellini M, Torreggiani A. Bernabini B, Rossi A, Moretti M, Caramazza R (2001) The biocompatibility of silicone, fluorosilicone and perfluorocarbon liquids as vitreous tamponades. An ultrastructural and immunohistochemical study. Ophthalmologica 215(4): 276–83

Vote B, Wheen L, Cluroe A, Teoh H, McGeorge A (2003) Further evidence for proinflammatory nature of perfluorohexyloctane in the eye. Clin Experiment Ophthalmol 31(5):408–14

Wathier M, Johnson MS, Carnahan MA, Baer C, McCuen BW, Kim T, Grinstaff MW (2006) In situ polymerized hydrogels for repairing scleral incisions used in pars plana vitrectomy procedures. ChemMedChem 1(8):821–5

Werner L, Chew J, Mamalis N (2006) Experimental evaluation of ophthalmic devices and solutions using rabbit models. Vet Ophthalmol 9 (5):281–91

Wickham LJ, Asaria RH, Alexander R, Luthert P, Charteris DG (2007) Immunopathology of intraocular silicone oil: retina and epiretinal membranes. Br J Ophthalmol 91(2):258–262

Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27(5):1047–53

Yamamoto S, Hirata A, Ishikawa S, Ohta K, Nakamura K-I, Okinami S (2013) Feasibility of using gelatin-microbial transglutaminase complex to repair experimental retinal detachment in rabbit eyes. Graefes Arch Clin Exp Ophthalmol 251(4):1109–14

Yamana T, Kita M, Ozaki S, Negi A, Honda Y (2000) The process of closure of experimental retinal holes in rabbit eyes. Graefes Arch Clin Exp Ophthalmol 238(1)81–7

Lloyd AW, Dropcova S, Faragher RG, Gard PR, Hanlon GW, Mikhalovsky SV, Olliff CJ, Denyer SP, Letko E, Filipec M (1999) The development of in vitro biocompatibility tests for the evaluation of intraocular biomaterials. J Mater Sci Mater Med 10(10/11):621–7

Yang H, Wang R, Gu Q, Zhang X (2008) Feasibility study of chitosan as intravitreous tamponade material. Graefe's Arch Clin Exp Ophthalmol 246(8):1097–1105

Yasuda K, Motohashi R, Kotake O, Nakagawa H, Noma H, Shimura M (2016) Comparative effects of topical diclofenac and betamethasone on inflammation after vitrecto my and cataract surgery in various vitreoretinal diseases. J Ocul Pharmacol Ther 32(10): 677–684

Zhou R, Caspi RR (2010) Ocular immune privilege. F1000 Biol Rep 2:3[1–3]

APPENDIX

Paper I

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BASIC SCIENCE

A new model for in vitro testing of vitreous substitute candidates

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Abstract
Purpose To describe a new model for in vitro assessment of novel vitreous substitute candidates.
Methods The biological impact of three vitreous substitute candidates was explored in a retinal explant culture model; a polyalkylimide hydrogel (Bio-Alcamid®), a two component hydrogel of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PEG) and a cross-linked sodium hyaluronic acid hydrogel (Healaflow®). The gels where applied to explanted adult rat retinas and then kept in culture for 2, 5 and 10 days. Gel-exposed explants were compared with explants incubated under standard tissue culture conditions. Cryosections of the specimens were stained with hematoxylin and eosin, immunohistochemical markers (GFAP, Vimentin, Neurofilament 160, PKC, Rhodopsin) and TUNEL.
Results Explants kept under standard conditions as well as PEG-exposed explants displayed disruption of retinal layers with moderate pyknosis of all neurons. They also displayed moderate labeling of apoptotic cells. Bio-Alcamid®-exposed explants displayed severe thinning and disruption of retinal layers with massive cell death. Healaflow®-treated explants displayed normal retinal lamination with significantly better preservation of retinal neurons compared with control specimens, and almost no signs of apoptosis. Retinas exposed to Healaflow® and retinas kept under standard conditions showed variable labeling of GFAP with generally low expression and some areas of upregulation. PEG-exposed retinas showed increased GFAP labeling and Bio-Alcamid®-exposed retinas showed sparse labeling of GFAP.
Conclusions Research into novel vitreous substitutes has important implications for both medical and surgical vitreoretinal disease. The in vitro model presented here provides a method of biocompatibility testing prior to more costly and cumbersome in vivo experiments. The explant culture system imposes reactions within the retina including disruption of layers, cell death and gliosis, and the progression of these reactions can be used for comparison of vitreous substitute candidates. Bio-Alcamid® had strong adverse effects on the retina which is consistent with results of prior in vivo trials. PEG gel elicits reactions similar to the control retinas whereas Healaflow® shows protection from culture-induced trauma indicating favorable biocompatibility.

Keywords Vitreous substitute · Immunohistochemistry · Retinal culture · Vitreoretinal surgery · Hyaluronic acid · Polyethylene oxide · Polyal kylimide

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Introduction

Vitrectomy is a common procedure for several eye disorders capable of severely impacting the vision of affected patients and has an important role in the treatment of conditions such as rhegmatogenous retinal detachment, severe diabetic retinopathy, penetrating ocular trauma, macular holes and epiretinal membranes. The removal of vitreous tissue during vitreoretinal surgery mandates its replacement, either in the form of water or various tamponading agents. The compounds currently in widespread clinical use such as balanced salt

solutions, gases, silicon oils and perflourocarbon liquids all have considerable disadvantages, with complications such as cataract formation, uveitis, increased intraocular pressure [1] and cytotoxicity [2, 3]. Further, current tamponading agents are either resorbed spontaneously after a few weeks or are not suitable for long-term use [4–9], and may require strict body positioning postoperatively.

The search for improved vitreous substitutes has been ongoing since the early days of the 20th century [10]. Early attempts were made to transplant animal and human vitreous [11] and investigations have been made into numerous semi-synthetic [12–14] and synthetic [15] molecules, although few of them have reached a clinical setting and none have fulfilled the requirements for long-term biocompatibility.

Traditionally the interactions of vitreous substitutes with eye tissues have been studied in various animal models in vivo. Such trials are, however, costly, time consuming and might be considered ethically problematic. In some cases in vivo experiments have been precluded by preclinical toxicological assays, mainly targeting apoptosis in cultures of cells from tissues outside the eye [16, 17], isolated retinal pigment epithelium (RPE) cells [18, 19] or dissociated cells from embryonal retinas [20]. The validity of these findings in relation to a clinical setting is, however, unclear since they represent a large transitional step regarding the impact on the adult neuroretinal sheet [21]. Therefore, a means to investigate the biological impact of vitreous substitutes more similar to the in vivo situation is desirable.

For this paper we wanted to explore a novel in vitro model for investigating the biological impact of vitreous substitutes on the neuroretina. To this end we have used the well-established retinal explant model to study three polymer hydrogels of different chemical composition that theoretically may be considered as potential vitreous substitutes; 1) Cross-linked hyaluronic acid (Healaflow®), clinically used in glaucoma surgery [22, 23]; 2) Poly (ethylene glycol) (PEG), widely used in different biochemical applications [24, 25]; and, 3) Polyalkylimide (Bio-Alcamid®), clinically used in reconstructive surgery [26–29].

Materials and methods

Three vitreous substitute candidates were investigated in the retinal explant culture model: a cross-linked sodium hyaluronic acid (22,5 mg/ml) hydrogel (Healaflow®); a two component hydrogel of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PEG) and a polyalkylimide hydrogel (Bio-Alcamid®). The gels where applied to explanted adult rat retinas and then kept in culture for 2, 5, and 10 days in vitro (DIV). Gel-exposed explants were compared with explants incubated under standard conditions (medium only). Cryosections of the specimens were stained with hematoxylin and eosin, immunohistochemical markers (GFAP, Vimentin, PKC, NF160, Rhodopsin) and TUNEL.

Animals

Retinas from adult Sprague–Dawley rats were used. All proceedings and animal treatment were in accordance with the guidelines and requirements of the government committee on animal experimentation at Lund University and with the Association for Research in Vision and Ophthalmology (ARVO) statement on the use of animals in ophthalmic and vision research.

Gels

Healaflow® (Anteis S.A., Plan Les Ouates, Switzerland) is a commercially available translucent hydrogel, clinically used in glaucoma filtering surgery as a space-filler and to limit postoperative fibrosis [22, 23]. The hydrogel consists of over 97% water, sodium hyaluronic acid (22.5 mg/ml) of non-animal origin cross-linked with BDDE (1.4-Butanediol diglycidyl ether), and phosphate- and NaCl-salts to maintain a physiological pH (7.0) and osmolarity (305 mOsm/kg). The estimated specific gravity is circa 1.03, and the refractive index i=1.341.

A custom made two component cross-linked hydrogel (PEG) consisting of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PBS) was prepared by mixing PEGDA in PBS into ETTMP-1300 in PBS [30]. PEG is a synthetic water-soluble polymer approved by the FDA for biomedical use in different applications including injectable hydrogels. It has been investigated for use in intravitreal drug delivery, repair of scleral incisions and the sealing of retinal breaks in retinal detachment surgery [24, 25].

Bio-Alcamid® (Polymekon, Brindisi, Italy) is a commercially available clear hydrogel in clinical use as tissue filler for plastic and reconstructive surgery, mainly for lipoatrophic and posttraumatic conditions. The hydrogel consists of approximately 4% reticulated polyalkylimide and approximately 96% non-pyrogenic water (pH 6.9), it contains no free monomers and is considered physically and chemically stable [29]. In vivo a collagen capsule surrounding the implanted Bio-Alcamid® is formed.

Tissue handling and culture procedure (Fig. 1)

The rats were euthanized with CO_2 with subsequent decapitation, enucleation and immediate immersion of the eyes in an ice-cold CO2-independent medium (Gibco, Paisley, UK). The neuroretinas were dissected from the retinal pigment epithelium (RPE) and the vitreous with fine forceps, and either half or the entire neuroretinas were subsequently explanted on

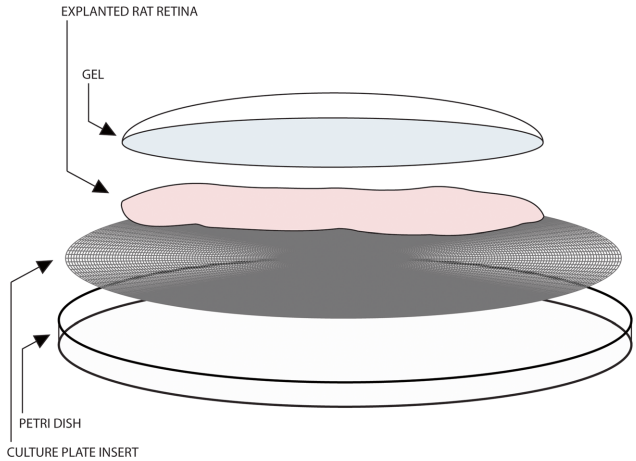


Fig. 1 Schematic overview of the retinal explant culture system with the vitreous substitute candidate

to culture plate inserts (Millicell Isopore-PCF 0.4 μm, 30 mm; Millipore, Billerica, ME) with the photoreceptor layer against the membrane, and covered by 50–100 μl gel (Healaflow®, PEG, or Bio-Alcamid®) depending on the size of the explant. The explants were cultured in 2 ml of Dulbecco's modified Eagle's medium (DMEM)/F12 medium–l-glutamine (Gibco) supplemented with 10% fetal calf serum, with a drop of enriched medium applied directly onto the gels to ensure saturation. The cultures were also supplemented with 2 mM l-glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma-Aldrich, St Louis, MO), and the retinas were kept at 37 °C at 95% humidity and 5% CO2. Four explants in each group (standard conditions, Healaflow®, PEG, and Bio-Alcamid®) were kept in culture for 2 days and six explants in each group were kept for 5 or 10 days, with exchange of half the culture medium after 3, 5, 7 and 9 days. No exchange of gel was made during the change of medium.

Immunohisto chemistry

In preparation for further histological studies the explants were fixed for 1 h in 4% formalin (pH 7.3) in 0.1 M Sørensen phosphate buffer (PB). The specimens were then washed with 0.1 M Sørensen PB; this was repeated with the same solution containing sucrose of increasing concentrations (5–25 %). Specimens were sectioned to 12 μm on a cryostat and every tenth slide was stained with hematoxylin and eosin according to standard procedures.

For immunohistochemical staining sections were washed at room temperature with 0.1 M of sodium PBS (pH 7.2) with 0.1% Triton X–100 (PBS/Triton), and thereafter incubated overnight at 4 °C with antibodies against the following antigens; Rhodopsin [rod photoreceptors] (Rho4D2, a kind gift from Prof. R.S. Molday, Vancouver, Canada; monoclonal, diluted 1:100), phospho-protein kinase C [PKC, rod bipolar cells] (K01107M; Cell Signaling, USA; diluted 1:200), Neurofilament 160 KDa [NF1 60, ganglion and horizontal cells] (clone NN18; Sigma, St. Louis, MO, USA; diluted 1:500), glial fibrillary acidic protein [GFAP, activated Müller cells] (clone G-A-5; Millipore, Sundbyberg, Sweden; diluted 1:200 with PBS/Triton with 1% bovine serum albumin) and vimentin [Müller cells] (Chemicon, USA; 1: 500). After incubation with the antibodies and rinse with PBS/Triton the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 45 min, rinsed and mounted in an anti-fading mounting media (Vectashield, Vector laboratories, Inc., Burlingame, CA, USA). Negative controls were obtained by performing the same procedure as above but without any primary antibodies. Antibodies are summarized in Table I. For identification of apoptotic cells a commercial terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay system with fluorescein-conjugated dUTP (Boehringer Mannheim, Mannheim, Germany) was used on the retinal sections according to the manufacturers instruction.

Results

Retinal explant cultures

All gels (Healaflow®, PEG and Bio-Alcamid®) could successfully be applied to the explanted retinal tissue. Healaflow® and PEG formed even films over the retinal explants whereas Bio-Alcamid® retained a thick, uneven

Table I Specification of immounohistochemical markers

Antigen	Antibody name	Target structure	Species	Dilution	Source
GFAP	Anti-glial fibrillary acidic protein	Astrocytes, activated Müller cells	Mouse monoclonal	1200	Chemicon International, CA, USA
Neurofilament 160 KDa (NF160)	Anti-Neurofilament 160 clone NN18	Ganglion and horizontal cells	Mouse monoclonal	1500	Sigma, St. Louis, MO, USA
PKC	Phospho-PKC (pan)	Rod bipolar cells	Rabbit polyclonal	1200	Cell Signaling, Beverly, MA, USA
Rho4D2	Rod photoreceptor	Rod photoreceptor	Mouse monoclonal	1:100	Kind gift of Prof. RS Molday, Vancouver, Canada
Vimentin	Mouse anti-vimentin	Müller cells	Mouse monoclonal	1:500	Chemicon International, CA, USA
Secondary antibody	**Antibody name**	**Target**	**Species**	**Dilution**	**Source**
FITC	Anti-mouse IgG FITC conjugate	Anti-mouse	Goat	1:200	Sigma, St Louis, MO, USA
FITC	Goat Anti-Rabbit IgM + IgG (H + L chain specific	Anti-rabbit	Goat	1:200	Southern Biotechnology Associates, AL, USA

texture that did not cover the explants completely even after a prolonged time. The PEG gel was found to benefit from a 20 min incubation time prior to administration of the medium, allowing for some gelation and preventing dissolution. The gels remained translucent and could be visualized at every exchange of the medium and were confirmed to be macroscopically saturated with the colored medium by means of visual inspection. Two of the explant-cultures suffered infection and were excluded from further analysis.

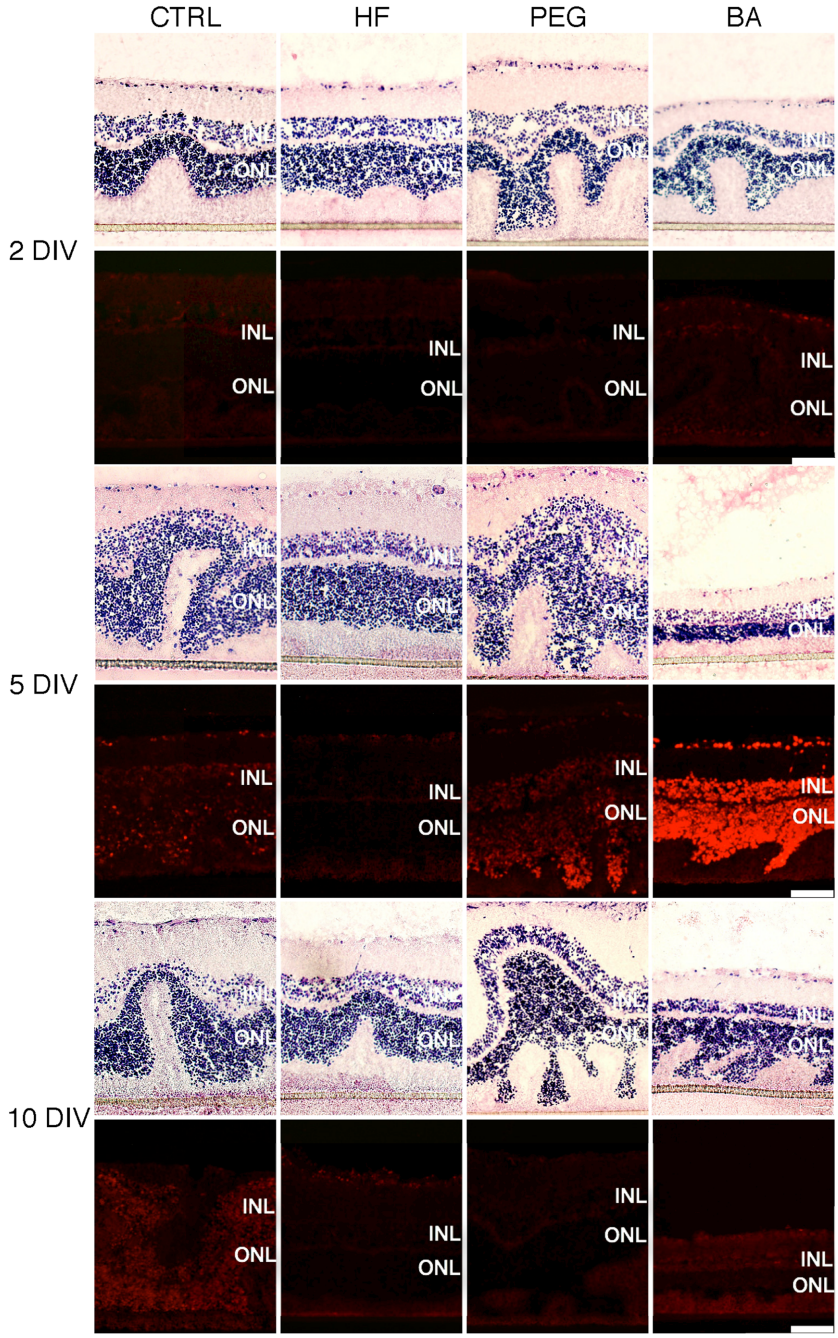
Cytoarchitecture and cell death (Fig. 2)

After two DIV hematoxylin- and eosin-stained sections of explants kept under control conditions as well as PEG-exposed explants displayed an abnormal retinal lamination with a wavy appearance of the outer nuclear layer (ONL). The ONL also displayed variable thickness, displacement towards the inner retina and moderate pyknosis. Inner retinal layers displayed some variability in total thickness and moderate pyknosis. Healaflow®-treated explants showed almost normal retinal lamination with significantly better preservation of retinal neurons compared with control specimens, whereas Bio-Alcamid®-exposed explants displayed a highly variable cytoarchitecture with severe thinning and disruption of all retinal layers in most parts, and a less disrupted structure in minor areas. TUNEL labeling at 2 DIV demonstrated no or almost no apoptotic cells with explants kept under control conditions, with Healaflow® and with PEG, and some apoptosis with explants cultured with Bio-Alcamid®. After 5 and 10 DIV a progressive increase in pyknosis and laminar disruption was seen in all groups. Retinas kept under standard conditions, and especially with Healaflow®, exhibited less pyknosis and laminar disruption than those treated with PEG and Bio-Alcamid®. TUNEL labeling of 5 DIV explants kept under control conditions and those subjected to PEG displayed moderate signs of apoptosis. Healaflow®-treated retinas showed almost no TUNEL labeling whereas explants treated with Bio-Alcamid® displayed massive cell death. At 10 DIV intense TUNEL labeling was observed in explants cultured under standard conditions, low labeling with Healaflow®-treatment and very low labeling in the PEG- and Bio-Alcamid®-treated cultures.

Rod photoreceptors (Fig. 3)

Rhodopsin-labeled photoreceptor cells in standard cultures displayed high intensity labeling of the outer segments (OS) and in the outer plexiform layer (OPL), with mild intensity labeling present in the ONL. Similar patterns of labeling were seen at 2 and 5 DIV. At 10 DIV stronger labeling was seen in the ONL. The Rhodopsin labeling pattern of Healaflow®- and PEG-exposed explants was comparable to the standard

Fig. 2 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with standard conditions (CTRL), Healaflow® (HF), PEG-gel (PEG) and Bio-Alcamid® (BA). Hematoxylin and eosin staining (top rows), and TUNEL staining (bottom rows). Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=25 μm ▶


CTRL
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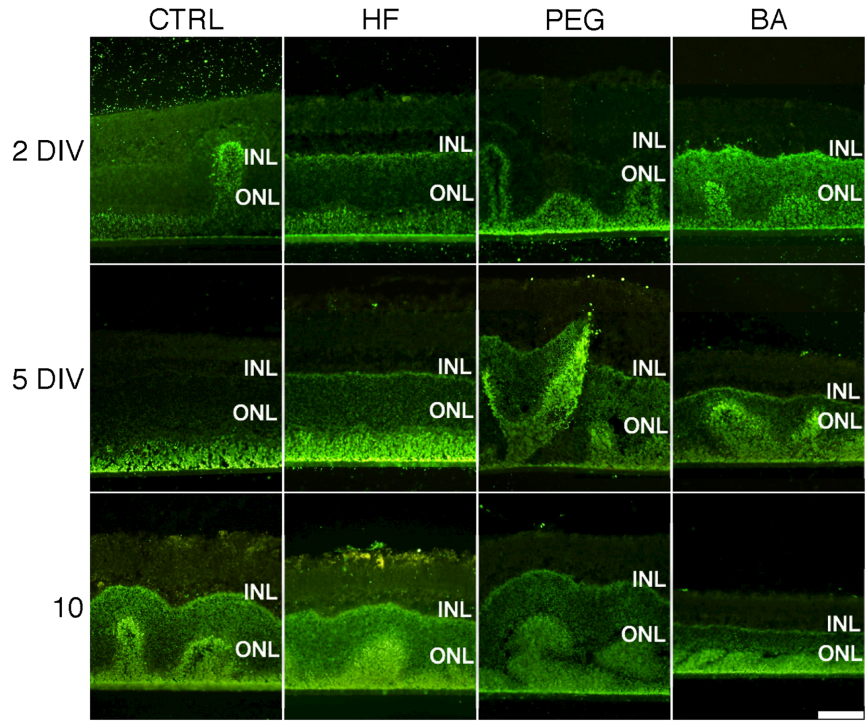


Fig. 3 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with: standard conditions (CTRL), Healaflow® (HF), PEG-gel (PEG) and Bio-Alcamid® (BA). Immunohistochemical staining for Rhodopsin. Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=25 μm

control. Bio-Alcamid® explants displayed intense labeling of the entire ONL already at 2 DIV.

Inner retinal cells (Fig. 4)

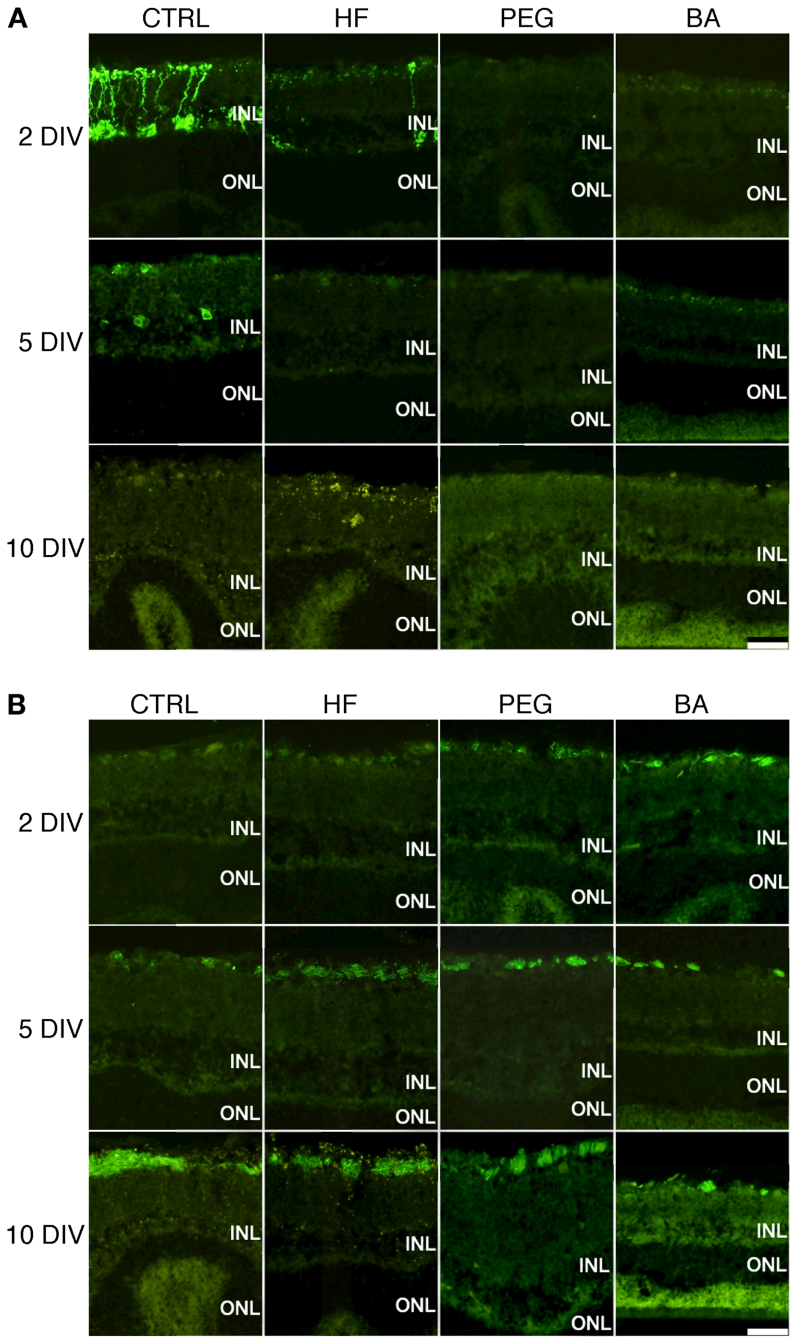
PKC labeling for rod bipolar cells at 2 DIV displayed a high variability with the most intense labeling towards the peripheral edge of the control explants. In 5 DIV specimens only a few PKC-labeled cell bodies were found whereas 10 DIV specimens did not show any remaining rod bipolar cells. In Healaflow®-treated specimens at 2 DIV a few PKC-labeled rod bipolar cells were found, but in older explants no such cells were found. PEG and Bio-Alcamid® explants did not display any PKC-labeled rod bipolar cells.

Neurofilament 160-labeled ganglion cells were seen in all retinal cultures with no clear differences between the different tested gels. No difference was observed between different incubation times.

Müller cells (Fig. 5)

GFAP labeling, indicative of Müller cell activation, showed very low intensity in most parts of the control retinas at 2 DIV but intense labeling was present in astrocytes located in the innermost retina. A generally low labeling intensity was seen at 5 DIV with some areas of moderate to high labeling of Müller cells (shown in Fig. 4). At 10 DIV some areas of moderate labeling was seen with mostly fragmentary labeling having a tortuous appearance of the Müller cell fibers. Healaflow®-subjected retinas displayed patterns similar to those of the control retinas at all timepoints, although there

Fig. 4 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with: standard conditions (CTRL), Healaflow® (HF), PEG-gel (PEG) and Bio-Alcamid® (BA). Immunohistochemical staining: **a** PKCpan; **b** Neurofilament 160. Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=12.5 μm ▶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was a tendency towards a slightly lower labeling intensity at 5 DIV.

The retinas exposed to PEG and Bio-Alcamid® displayed a high labeling intensity in the inner retina with labeled Müller cell fibers occasionally reaching the ONL at 2 DIV. After 5 DIV moderate, variable expression both in the inner retina and in fibrils was exhibited on the PEG exposure. Bio-Alcamid®-exposed retinas exhibited low labeling intensity, almost exclusively in the inner retina. At 10 DIV cultures with PEG showed moderate, variable expression and those cultured with Bio-Alcamid® displayed only weak labeling present in the inner retina.

Vimentin labeling of Müller cell cytoskeletons was present in fibers through the inner parts of the retina, in some areas through to the ONL, with some labeling in the innermost retina. No significant differences were seen between the different groups but increased hypertrophy and disorganization of Müller cell fibers was seen over time with the labeling pattern appearing almost granular at later time points.

Fig. 5 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with: standard conditions (**CTRL**), Healaflow® (**HF**), PEG-gel (**PEG**) and Bio-Alcamid® (**BA**). Immunohistochemical staining: **a** *GFAP*; **b** Vimentin. Abbreviations: inner nuclear layer (*INL*), outer nuclear layer (**ONL**). Scale bar=12,5

Discussion

Summary

In this study a new in vitro model for evaluating the effect of potential vitreous substitutes on adult neuroretinal sheets was explored. Three potential candidates were evaluated and compared to retinal explants cultured under standard conditions. Clear differences were seen between the groups with similar effects observed in explants cultured under standard conditions and with Healaflow®, and more degenerative findings in cultures with PEG and, particularly, Bio-Alcamid®. The relative degenerative morphological and immunohistochemical changes for the different gels compared to standard conditions are summarized as qualitative compound scores in Table 2.

The in vitro model

Research into novel vitreous substitutes has important implications for both medical and surgical vitreoretinal disease. An in vitro assay analysed using immunohistochemistry and morphological stainings can determine the biocompatibility and safety of potential vitreous substitutes. This may provide better predictions of the effects of novel substances on the retina than the traditional, more simplistic in vitro assays currently in use [16–19, 21, 31].

The in vitro model presented here provides a method of biocompatibility testing prior to more costly and cumbersome in vivo experiments [20]. In retinal explant cultures under standard conditions there are several well-characterized reactions easily observable as early as 3 or 4 DIV [32–34]. These reactions include gliosis and neuroretinal degeneration and can be visualized by GFAP upregulation, disruption of the cell layers and the labeling of apoptotic cells. Using these reactions elicited by the explant culture system under standard conditions and comparing them to different vitreous substitute candidates indicates the biocompatibility of the substances in vivo.

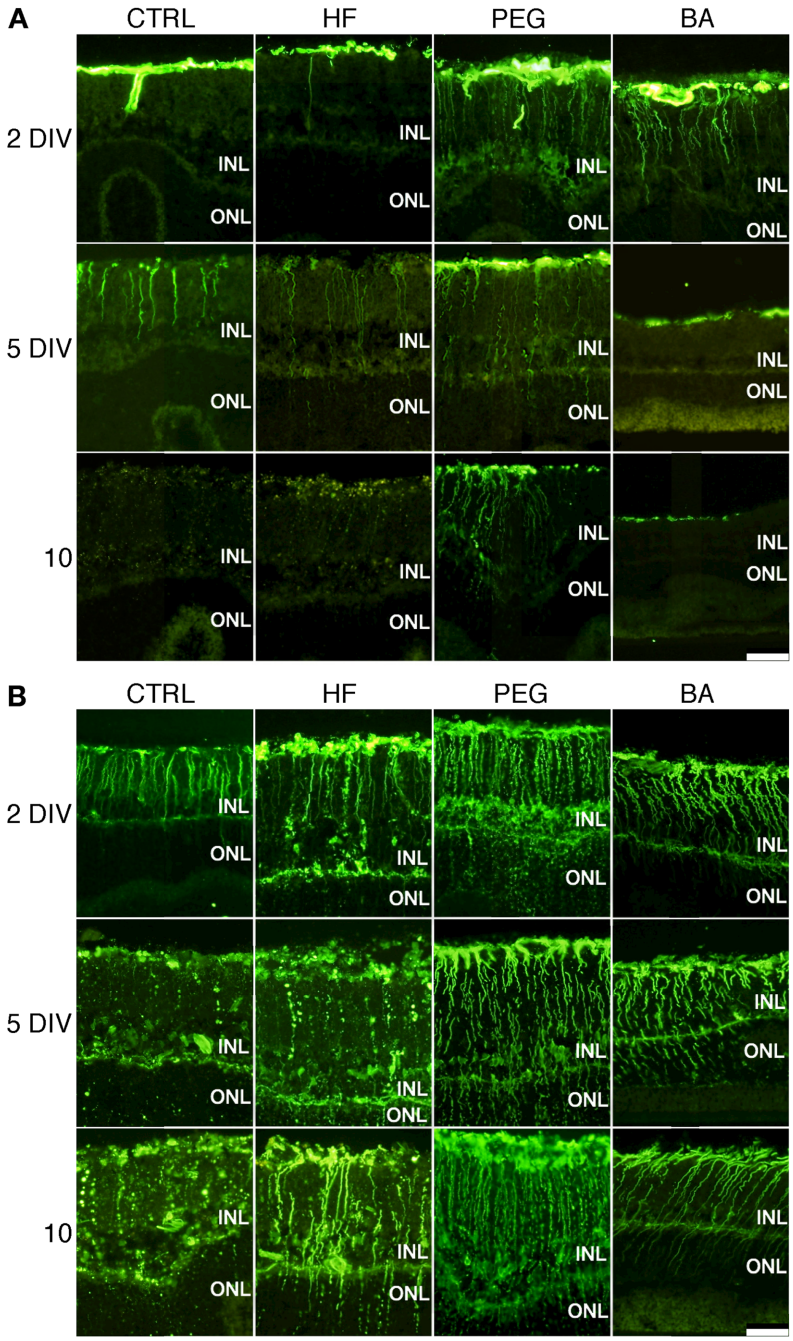
Our previous results and our hypothesis

The vitreous is often simplistically seen as a mere space filler inside the eye bulb. There is, however, evidence of a more intricate and purposeful composition [10] with important physiological implications on the micro-milieu of the retina including the upkeep of salt and nutrient gradients, physical support and more [35, 36]. An ideal vitreous substitute would replicate these influences on the neuroretina and surrounding tissues as well as provide a tamponading effect after vitrectomy [10].

In two recent papers our group investigated two promising potential intravitreal substitutes in an in vivo rabbit model: Polyalkylimide (Bio-Alcamid®) [37] and a poly (ethylene glycol) (PEG) hydrogel [38].

Bio-Alcamid® is a translucent hydrogel with high biocompatibility [26, 27] used in plastic surgery and in clinical use forms a surrounding collagen capsule giving it a degree of isolation from the surrounding tissue [28]. The synthetic polymer hydrogel PEG is used in different biomedical application, has been tested for intravitreal administration of drugs [24, 39] and is FDA approved for use intravitreally. The in vivo trials showed favorable biocompatibility but inadequate stability in vivo using PEG where the substance was largely tolerated with minor changes in retinal cytoarchitecture and GFAP-upregulation, and minor electrophysiological changes [38]. On the other hand, Bio-Alcamid® displayed suitable physical properties but caused severe functional and morphological retinal damage with increased GFAP expression and cell death (TUNEL) [37].

The use of derivates of sodium hyaluronic acid in vitreoretinal surgery predates their ubiquitous use in cataract and anterior segment surgery [12, 14, 40–42], but their use in a clinical setting has been limited mainly due to concern about short term side effects and retention time [41, 43]. Healaflow® is a commercially available compound consisting of a cross-linked sodium hyaluronic acid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ONL

Table 2 Relative compound score for the degenerative retinal changes for the different gels compared to standard conditions ranging from – to ++

Gels	Cytoarchitecture and cell death	Rod photoreceptors	Inner retinal cells	Müller cell activation
Healaflow®	_	0	+	_
PEG	+	0	++	+
Bio-Alcamid®	++	++	++	++

hydrogel and is FDA approved for use in glaucoma surgery [22, 23]. The composition of Healaflow® is akin to natural vitreous: a reinforced hydrogel of hyaluronic acid with similar physical properties and thus considered a plausible candidate for vitreous substitution.

In vivo vs. in vitro: our earlier results and others

It seems to us that a good correlation exists between the results of this in vitro explant culture system and earlier results for all the tested substances.

In this setting retinal explants cultured with Healaflow® compare very well to specimens cultured under standard conditions and even seem to lessen the trauma caused by the culture process. This is consistent with the excellent biocompatibility of hyaluronic acid seen in other studies [18, 19]. Hyaluronic acid is one of the main constituents of natural vitreous and is consistently well-tolerated in different biomedical applications. Healaflow® may exert a protective effect from culture-induced trauma on the retinal explants by providing a more physiologically similar microenvironment in vitro. Additionally, the positive effect on the retina could be due to biomechanical factors via physical interaction from the gel that might prevent retinal folds and keep the explants under tension. This is a factor that previously has been showed to favorably affect retinas in vitro [44].

PEG gel elicits reactions similar to the control retinas with comparable changes in the cytoarchitecture but with earlier, more intense TUNEL labeling, consistent with previous in vivo findings [38]. In the retinal explant cultures with the longest duration (10 DIV) there was a decrease in the amount of apoptotic cells observed at earlier time points. This may be due to a loss of viable cells as cell death occurred earlier than for Healaflow® and standard conditions, indicating a stronger adverse reaction to these gels than what is caused by the culture procedure.

Bio-Alcamid® caused severe retinal damage in vivo [37] and negatively affected the morphology of cells and cell layers, induced cell death and induced GFAP upregulation very early in vitro. Some of the variability in cytoarchitecture for retinas treated with Bio-Alcamid® might have been due to uneven coverage of the gel. The adverse effects may in part be influenced by uneven exposure to the medium, but cytotoxic effects from the gel itself are likely to play a part in this process. The explanted retinas were less affected in minor areas that may not have been in direct contact with the gel, although this is difficult to discern due to the loss of gel in the preparation and sectioning procedures. This is in accordance with previous studies that demonstrated pathological changes in the retina in vivo, primarily in parts more likely to have been in direct contact with the gel [37], suggesting at least in part a toxic or immunological response. Recently, clinical use of Bio-Alcamid® in reconstructive surgery has become increasingly controversial due to late complications such as inflammation, infection and excessive capsule formation [45–48].

Conclusion

The retinal explant assay described in this paper has the potential to be a useful tool for preliminary study of vitreous substitute candidates prior to more costly andtime-consumingin vivo testing. In addition, it may reduce the need for laboratory animals and limit the severity of the experiments from an ethical standpoint by excluding unfit substances from further testing, thereby providing refinement of the tests. In vivo tests will still be essential before testing on human subjects but this assay may minimize the translational step which would prove valuable and beneficent in vetting out unsuitable biomaterials.

A need for better vitreous substitutes still remains and more suitable substances would be highly valuable. Healaflow® and, to a lesser extent, PEG seem to be promising candidates for further development and further in vivo testing of these and similar substances is clearly indicated.

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1986) and the U.S. Animal Welfare Act, as amended, were followed, as well as the current version of the Swedish Law on the Protection of Animals, where applicable.

References

1. Killey FP, Edelhauser HF, Aaberg TM (1978) Intraocular sulfur hexafluoride and octofluorocyclobutane. Effects on intraocular pressure and vitreous volume. Arch Ophthalmol 96(3):511–15
2. Nakamura K, Refojo MF, Crabtree DV, Pastor J, Leong FL (1991) Ocular toxicity of low-molecular-weight components of silicone and fluorosilicone oils. Invest Ophthalmol Vis Sci 32(12):3007–20
3. Colthurst MJ, Williams RL, Hiscott PS, Grierson I (2000) Biomaterials used in the posterior segment of the eye. Biomaterials 21(7):649–65
4. Versura P, Cellini M, Bernabini TA, Rossi A, Moretti M, Caramazza R, (2001) The biocompatibility of silicone, fluorosilicone and perfluorocarbon liquids as vitreous tamponades. An ultrastructural and immunohistochemical study. Ophthalmologica 215(4):276–83
5. Vote B, Wheen L, Cluroe A, Teoh H, McGeorge A (2003) Further evidence for proinflammatory nature of perfluorohexyloctane in the eye. Clin Experiment Ophthalmol 31(5):408–14
6. Schatz B, El-Shabrawi Y, Haas A, Langmann G (2004) Adverse side effects with perfluorohexyloctane as a long-term tamponade agent in complicated vitreoretinal surgery. Retina 24(4):567–73
7. Joussen AM, Wong D (2008) The concept of heavy tamponades-chances and limitations. Graefes Arch Clin Exp Ophthalmol 246(9): 1217–24
8. Heimann H, Stappler T, Wong D (2008) Heavy tamponade 1: a review of indications, use, and complications. Eye (Lond) 22(10): 1342–59
9. Mackiewicz J, Mühling B, Hiebl W, Meinert H, Maaijwee K, Kociok N, Lüke C, Zagorski Z, Kirchhof B, Joussen AM (2007) In vivo retinal tolerance of various heavy silicone oils. Invest Ophthalmol Vis Sci 48(4):1873–83
10. Swindle KE, Ravi N (2007) Recent advances in polymeric vitreous substitutes. Expert Rev Ophthalmol 2(2):255–265
11. Shafer DM (1976) Human vitreous transplantation. Ann R Coll Surg Engl 58(1):25–23
12. Kanski JJ (1975) Intravitreal hyaluronic acid injection : A long-term clinical evaluation. Br J Ophthalmol 59(5):255–6
13. Balazs EA, Freeman MI, Klöti R, Meyer-Schwickerath G, Regnault F, Sweeney DB (1972) Hyaluronic acid and replacement of vitreous and aqueous humor. Mod Probl Ophthalmol 10:3–21
14. Denlinger J, El-Mofty A, Balazs E (1980) Replacement of the liquid vitreus with sodium hyaluronate in monkeys II. Long-term evaluation. Exp Eye Res 30:101–117
15. Kanski JJ, Daniel R (1973) Intravitreal silicone injection in retinal detachment. Br J Ophthalmol 57(8):542–5
16. Foster WJ, Aliyar HA, Hamilton P, Ravi N (2006) Internal osmotic pressure as a mechanism of retinal attachment in a vitreous substitute. J Bioact Compat Polym 21(3):221–35
17. Bae SH, Che J-H, Seo J-M, Jeong J, Kim ET, Lee SW, Koo K-I, Suaning GJ, Lovell NH, Cho D-I, Kim SJ, Chung H (2012) In vitro biocompatibility of various polymer-based microelectrode arrays for retinal prosthesis. Invest Ophthalmol Vis Sci 53(6):2653–7
18. Su W-Y, Chen K-H, Chen Y-C, Lee Y-H, Tseng C-L, Lin F-H (2011) An injectable oxidated hyaluronic acid/adipic acid dihydrazide hydrogel as a vitreous substitute. J Biomater Sci Polym Ed 22(13): 1777–97
19. Schramm C, Spitzer MS, Henke-Fahle S, Steinmetz G, Januschowski K, Heiduschka P, Geis- Gerstorfer J, Biedermann T, Bartz-Schmidt KU, Szurman P (2012) The cross-linked biopolymer hyaluronic acid as an artificial vitreous substitute. Invest Ophthalmol Vis Sci 53(2): 613–21
20. Malchiodi-Albedi F, Matteucci A, Formisano G, Paradisi S, Carnovale-Scalzo G, Scorcia G, Hoerauf H (2005) Induction of apoptosis in rat retinal cell cultures by partially fluorinated alkanes. Am J Ophthalmol 139(4):737–9
21. Matteucci A, Formisano G, Paradisi S, Carnovale-Scalzo G, Scorcia G, Caiazza S, Hoerauf H, Malchiodi-Albedi F (2007) Biocompatibility assessment of liquid artificial vitreous replacements: relevance of in vitro studies. Surv Ophthalmol 52(3):289–99
22. Schariot GB (2010) Canaloplasty re-establish the natural outflow in patients with chronic open-angle glaucoma. J Current Glau Prac 4(2): 97–102
23. Roy S, Thi HD, Feusier M, Mermoud A (2012) Crosslinked sodium hyaluronate implant in deep sclerectomy for the surgical treatment of glaucoma. Eur J Ophthalmol 22(1):70–6
24. Duvvuri S, Janoria KG, Pal D, Mitra AK (2007) Controlled delivery of ganciclovir to the retina with drug-loaded Poly (d, L-lactide-co-glycolide) (PLGA) microspheres dispersed in PLGA-PEG-PLGA Gel: a novel intravitreal delivery system for the treatment of cytomegalovirus retinitis. J Ocul Pharmacol Ther 23(3):264–74
25. Wathier M, Johnson MS, Carnahan MA, Baer C, McCuen BW, Kim T, Grinstaff MW (2006) In situ polymerized hydrogels for repairing scleral incisions used in pars plana vitrectomy procedures. ChemMedChem 1(8):821–5
26. Ramires P, Miccoli M, Panzarini E, Dini L, Protopapa C (2005) In vitro and in vivo biocompatibility evaluation of a polyalkylimide hydrogel for soft tissue augmentation. J Biomed Mater Res B Appl Biomater 72(2):230–238
27. Protopapa C, Sito G, Caparole D, Cammarota N (2003) Bio-Alcamid® in drug- induced lipodystrophy. J Cosmet & Laser Ther 5(3–4):1–5
28. Lahiri A, Waters R (2007) Experience with Bio-Alcamid®, a new soft tissue endoprosthesis. J Plast Reconstr Aesthet Surg 60(6):663–667
29. Claoue BL, Rabineau P (2004) The polyalkamide gel: experience with Bio-Alcamid®. Semin Cutan Med Surg 23(4):236–240
30. Pritchard CD, O'Shea TM, Siegwart DJ, Eliezer C, Anderson DG, Reynolds FM, Thomas JA, Slotkin JR, Woodard EJ, Langer R (2011) An injectable thiol-acrylate poly (ethylene glycol) hydrogel for sustained release of methylprednisolone sodium succinate. Biomaterials 32(2):587–97
31. Lloyd AW, Dropcova S, Faragher RG, Gard PR, Hanlon GW, Mikhalovsky SV, Olliff CJ, Denyer SP, Letko E, Filipec M (1999) The development of in vitro biocompatibility tests for the evaluation of intraocular biomaterials. J Mater Sci Mater Med 10(10–11):621–627
32. Kaempf S, Walter P, Salz AK, Thumann G (2008) Novel organotypic culture model of adult mammalian neurosensory retina in co-culture with retinal pigment epithelium. J Neurosci Methods 173(1):47–58
33. Kobuch K, Herrmann WA, Framme C, Sachs HG, Gabel VP, Hillenkamp J (2008) Maintenance of adult porcine retina and retinal pigment epithelium in perfusion culture: characterisation of an organotypic in vitro model. Exp Eye Res 86(4):661–668
34. Taylor L, Arnér K, Engelsberg K, Ghosh F (2013) Effects of glial cell line-derived neurotrophic factor on the cultured adult full-thickness porcine retina. Curr Eye Res 38(4):503–515
35. Stefansson E (2009) Physiology of vitreous surgery. Graefe's Arch Clin Exp Ophthalmol 247(2):147–163
36. Chang S (2006) LXII Edward Jackson lecture: open angle glaucoma after vitrectomy. Am J Ophthalmol 141(6):1033–1043
37. Crafoord S, Andreasson S, Ghosh F (2011) Experimental vitreous tamponade using polyalkyl-imide hydrogel. Graefes Arch Clin Exp Ophthalmol 249:1167–74
38. Pritchard CD, Crafoord S, Andréasson S, Arnér KM, O'Shea TM, Langer R, Ghosh FK (2010) Evaluation of viscoelastic poly (ethylene

glycol) sols as vitreous substitutes in an experimental vitrectomy model in rabbits. Acta Biomater 7:936–943
39. Rauck BM, Friberg TR, Medina Mendez CA, Park D, Shah V, Bilonick RA, Wang Y (2014) Biocompatible reverse thermal gel sustains the release of intravitreal bevacizumab in vivo. Invest Ophthalmol Vis Sci 55(1):469–76
40. Koster R, Stilma JS (1986) Comparison of vitreous replacement with Healon and with HPMC in rabbits´eyes. Doc Ophthalmol 61:247–253
41. Koster R, Stilma JS (1986) Healon as intravitreal substitute in retinal detachment surgery in 40 patients. Doc Ophthalmol 64:13–1
42. Gerke E, Meyer-Schwickerath G, Wessing A (1984) Healon in retinal detachment with proliferative vitreoretinopathy. Graefes Arch Clin Exp Ophthalmol 221:241–3
43. Vatne HO, Syrdalen P (1986) The use of sodium hyaluronate (Healon) in the treatment of complicated cases of retinal detachment. Acta Ophthalmol 64:169–72
44. Taylor L, Moran D, Arnér K, Warrant E, Ghosh F (2013) Stretch to see: lateral tension strongly determines cell survival in long-term cultures of adult porcine retina. Invest Ophthalmol Vis Sci 54(3): 1845–56
45. Rosengren A, Danielsen N, Bjursten LM (1999) Reactive capsule formation around soft-tissue implants is related to cell necrosis. J Biomed Mater Res 46(4):485–64
46. Christensen L, Breiting V, Janssen M, Vuust J, Hogdall E (2005) Adverse reactions to injectable soft tissue permanent fillers. Aesthetic Plast Surg 29(1):34–48
47. Karim RB, Hage JJ, van Rozelaar L, Lange CAH, Raaijmakers J (2006) Complications of polyalkylimide 4 % injections (Bio-Alcamid): a report of 18 cases. J Plast Reconstr Aesthet Surg 59(12):1409–14
48. Nelson L, Stewart KJ (2011) Early and late complications of polyalkylimide gel (Bio-Alcamid)®. J Plast Reconstr Aesthet Surg 64(3):401–4

Paper II

Graefes Arch Clin Exp Ophthalmol
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BASIC SCIENCE

A cross-linked hyaluronic acid hydrogel (Healaflow®) as a novel vitreous substitute

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Abstract
Purpose Vitrectomy requires the substitution of the natural vitreous, as well as tamponading of retinal breaks. Clinically available alternatives such as gas and silicone oil have side effects such as inflammation, secondary glaucoma, cataract, and a need for head posturing. In this study, a hydrogel of cross-linked sodium hyaluronic acid (Healaflow®) is evaluated for use as a novel vitreous substitute.
Methods A combined 25-20-gauge pars plana vitrectomy with posterior vitreous detachment was performed in the right eye of twelve pigmented rabbits, with subsequent injection of approximately 1 ml Healaflow®. Clinical evaluation, measurement of intraocular pressure (IOP), and full-field ERG were performed postoperatively. The rabbits were sacrificed at different time-points between 42 and 105 days. After enucleation, the eyes were examined macroscopically, photographed, and prepared for histological examination with routine microscopy and immunohistochemistry.
Results Healaflow® was successfully used with standard surgical procedures and remained translucent but did lose most of its viscosity during the postoperative period. One rabbit was lost due to unrelated causes. In two eyes iatrogenic partial retinal detachments were seen, and in two eyes significant cataract developed due to intra-operative complications. ERG-recordings revealed no toxic effect on rod or cone function. Routine microscopy and immunohistochemistry demonstrated normal morphology with some Müller cell activation (up-regulation of glial acidic fibrillary protein, GFAP) compared to unoperated eyes and no significant DNA-fragmentation (TUNEL-assay).
Conclusions Healaflow® did not affect retinal morphology or function negatively during long-term use as a vitreous substitute, making it highly interesting in this setting. An estimated retention time of a few weeks suggests potential for use as a short-term tamponade. Future work will include an increased ratio of cross-linking to prolong the structural integrity of the gel.

Keywords Vitreous substitute · Vitreoretinal surgery · Hyaluronic acid · Immunohistochemistry

Introduction

Vision threatening disorders such as rhegmatogenous retinal detachment, severe diabetic retinopathy, penetrating ocular trauma, macular holes, and epiretinal membranes often require surgical intervention such as vitrectomy. An infusion of Balanced Saline Solution (BSS) is used during the surgery, but it is often necessary to replace the BSS with a tamponading agent to prevent fluid from re-entering retinal breaks during the healing process. The clinically used substances, such as gases and silicone oils, have several unwanted side effects. Among them are complications such as low vision, cataract formation, uveitis [1], increased intraocular pressure [2], and cytotoxicity [3–5]. These substances are either resorbed by the body after a relatively short time, or are otherwise unsuited for long-term use and therefore removed [6–9], and may require strict posturing of the head for an extended period of time postoperatively. Additionally, there is evidence that physiological changes in the vitreous could

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play a role in different pathological processes such as post-vitrectomy cataract formation [10], and glaucoma [11]. These changes include altered diffusion gradients of nutrients and other biologically important substances [12], and could feasibly be alleviated by improved vitreous substitutes.

Clearly, there is a need for more suitable vitreous substitutes, both to enhance the biocompatibility and lessen the side effects, and possibly to allow for safe long-term substitution. This is especially true in complex cases that require silicone oils, which are considerably more prone to induce side effects than the more extensively used gases, although a vitreous substitute that would permit faster visual rehabilitation and less patient discomfort than the gases would also be highly beneficial. The task to devise an optimal vitreous substitute is demanding, requiring consideration of a variety of parameters such as clinical usability, physical properties, biomechanics, and the biochemical micromilieu. Studies in vitreous substitution have been ongoing since the early 20th century, with experimental use of substances ranging from transplanted animal and human vitreous to more recent semi-synthetic [13–15], and synthetic [16] substances. So far, none of these substances have proved more useful than the clinically available alternatives.

A potentially fruitful way to devise a substance able to fulfill these requirements is to mimic the healthy vitreous. The natural vitreous is a hydrogel consisting of hyaluronic acid reticulated with collagen fibers, thus reinforcing the gel. A similarly structured synthetic gel, such as a cross-linked hyaluronic acid hydrogel, could provide beneficial physiological properties [17]. Healaflow® is a commercially available substance of this type, developed for glaucoma surgery. In a previous *in vitro* study it was tolerated well by retinal explant cultures [18], and it seems to harbor potential for use as a novel vitreous substitute. In this *in vivo* study, we explore Healaflow® as a novel vitreous substitute in a previously established rabbit vitrectomy model [19, 20].

Materials and methods

Animals

Twelve pigmented rabbits, aged 4 months, were used in the experiments. All proceedings and animal treatment were in accordance with the guidelines and requirements of the government committee on animal experimentation at Lund University and with the ARVO (The Association for Research in Vision and Ophthalmology) statement on the use of animals in ophthalmic and vision research.

Gel properties

Healaflow® (Anteis S.A., Plan Les Ouates, Switzerland) is a commercially available transparent hydrogel, clinically used in glaucoma filtering surgery as a space-filler, and to limit postoperative fibrosis [21, 22]. The hydrogel consists of over 97 % water, sodium hyaluronic acid (22.5 mg/ml) of non-animal origin cross-linked with BDDE (1.4-Butanediol diglycidyl ether), and phosphate- and NaCl-salts to maintain physiological pH (7.0) and osmolarity (305 mOsm/kg). Estimated specific gravity is circa 1.03, and refractive index i = 1.341.

Surgery

Experienced surgeons performed all surgical procedures under general anesthesia, where a combination of ketamine (35 mg/kg) and xylazine (5 mg/kg) was given intramuscularly. The right eyes of the rabbits were vitrectomized, while the left eyes served as controls. The pupils of the right eyes were dilated with cyclopentolate (1 %) and phenylephrine (10 %), and anesthetized with topical tetracaine (0,5 %) just before surgery. Sclerotomies were made 1 mm posterior to limbus with two transconjunctival 25 G trocars for infusion and illumination purposes, and either a transconjunctival 25 G trocar or a conjunctival incision and subsequent 20 G sclerotomy for the main instrument. Balanced salt solution (BSS, Endosol®, Abbott Medical Optics) was used as a continuous infusion. A BIOM 90-D lens (Oculus) and a standard endo-illuminating probe were used for visualization. With the Accurus Surgical System® (Alcon, Fort Worth, TX, USA) and a vitreous cutter (Innovit®, Alcon) posterior vitreous detachment (PVD) was initiated by positioning the vitrectomy probe at the margin of the disk, and applying suction and traction with the vitrectomy probe. PVD was, if possible, visually confirmed, and a central vitrectomy performed, leaving peripheral parts of the vitreous intact due to the increased risk of traumatic cataract with the comparatively large lens of the rabbit. After fluid-air exchange, approximately 1 ml Healaflow® was injected through a 25 G needle. The sclerotomies and conjunctiva were sutured if needed, and 25 mg gentamicin and 2 mg betamethasone were administered subconjunctivally. No other postoperative treatment was given.

Postoperative handling

Clinical evaluation with ophthalmoscopy, Retcam® photography, intraocular pressure (IOP) measurement (Tono-Pen®), and full-field ERG was performed postoperatively at intervals up to 105 days. The rabbits were sacrificed at different time-points between 42 and 105 days. After enucleation, the eyes were examined macroscopically, photographed, and prepared for histological examination and immunohistochemistry.

Electrophysiology

Full-field electroretinograms (ERG) from the right eyes were obtained preoperatively, at 1 and 3 months after surgery for six of the rabbits, and at 1 month postoperatively for the rest of the rabbits. A Nicolet Viking® analysis system (Nicolet Biomedical Instruments, Madison, Wisconsin) was used for the recordings as described previously [23, 24]. The rabbits were sedated with an intramuscular injection of Hypnorm® (fentanylcitrate 0.315 mg/ml and fluanisone 10 mg/ml). Pupil dilation was performed with cyclopentolate (1 %), and topical anesthesia applied. After 30 minutes of dark adaptation, a Burian-Allen bipolar ERG contact lens electrode was applied on the cornea with 2 % methylcellulose lubrication, and a subcutaneous ground electrode attached to the neck. Responses were obtained with a wide band filter (−3 dB at 1 Hz and 500 Hz). Stimulations were performed with single full-field flashes (30 μs), with dim blue light (Wratten filters # 47, 47A and 47B), and white light (0.8 cd.s/m2) without a background. Cone responses were obtained using 30 Hz flickering white light (0.8 cd s/m2) averaged from 20 sweeps without a background light. The luminances of the different light stimuli refer to the light reflected from the Ganzfeld sphere.

Tissue handling and immunohistochemistry (Table 1)

The rabbits were euthanized at different time-points between 42 and 105 days and the eyes were enucleated, dissected, and fixation performed for 1 h in 4 % formalin, pH 7.3 in a 0.1 M Sørensen's phosphate buffer (PB). The specimens were washed with 0.1 M Sørensen's PB, and then washed again using the same solution containing increasing concentrations of sucrose (5–25 %). The specimens were cryosectioned with a section thickness of 12 μm, and every 10th slide was stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry, sections were washed three times in room temperature with 0.1 M of sodium phosphate-buffered saline pH 7.2 (PBS) with 0.1 % Triton X-100 (PBS/Triton), and incubated with PBS/Triton with 1 % bovine serum albumin (BSA). The sections were thereafter incubated overnight at + 4 °C with antibodies (diluted in PBS/Triton with 1 % BSA) against the following antigens; Rhodopsin [rod photoreceptors] (Rho4D2, a kind gift from Prof. R.S. Molday, Vancouver, Canada; monoclonal, diluted 1:100), phospho-protein kinase C [PKC, rod bipolar cells] (K01107M; Cell Signaling, USA, diluted 1:200), and glial fibrillary acidic protein [GFAP, activated Müller cells] (clone G-A-5; Millipore, Sundbyberg, Sweden, diluted 1:200). The slides were rinsed with PBS/Triton, and incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies (Sigma-Aldrich, St.Louis, MO, USA, diluted 1:200) for 45 min, rinsed again, and mounted in antifading mounting media (Vectashield, Vector laboratories, Inc., Burlingame, CA, USA). Negative controls were obtained by performing the same procedure without primary antibodies. The antibodies are summarized in Table 1. For identification of apoptotic cells, a commercial terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay system with fluorescein-conjugated dUTP (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions.

Results

Macroscopic findings (Fig. 1 and Tables 2 and 3)

Healaflow® was easily incorporated in the standard surgical procedure and could successfully be injected through small gauge needles. During the surgeries, iatrogenic retinal breaks occurred in four cases and the lens was accidentally touched in two cases. One rabbit was lost due to unrelated causes.

No signs of excessive conjunctival swelling or uveitis were seen. Postoperative IOP (15–25 mmHg, see Table 3) was slightly elevated compared to earlier results [19]. In the two

Table 1 Specification of immunohistochemical markers

Antigen	Antibody name	Target structure	Species	Dilution	Source
GFAP	Anti-glial fibrillary acidic protein	Astrocytes, activated Müller cells	Mouse monoclonal	1:200	Chemicon International, CA, USA
PKC	Phospho-PKC (pan)	Rod bipolar cells	Rabbit polyclonal	1:200	Cell Signaling, Beverly, MA, USA
Rhodopsin	Rho4D2	Rod photoreceptor	Mouse monoclonal	1:100	Kind gift of Prof. RS Molday, Vancouver, Canada
Secondary antibody	Antibody name	Target	Species	Dilution	Source
FITC	Anti-mouse IgG FITC conjugate	Anti-mouse	Goat	1:200	Sigma, St Louis, MO, USA
FITC	Goat Anti-Rabbit IgM + IgG (H + L chain specific)	Anti-rabbit	Goat	1:200	Southern Biotechnology Associates, AL, USA

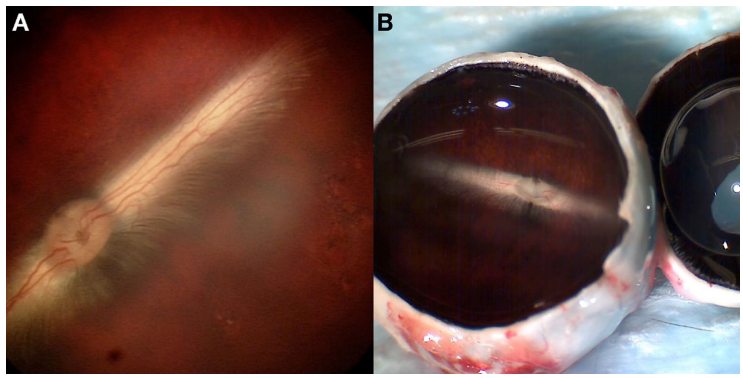

Fig. 1 Postoperative results after 3 months of Healaflow® treatment. (**a**) Retcam image of the central retina, the retina remained attached despite iatrogenic retinal ruptures. (**b**) The retina and vitreous cavity after dissection

eyes with traumatic cataracts, lens material was observed in the vitreous cavity, and iatrogenic partial retinal detachments were seen. These retinal detachments stayed partial for the duration of the follow-up. None of the other cases of operative complications developed retinal detachment. The untreated lesions were seen to heal with pigmented scar formation. No intact gels were visible after dissection, although some gel remnants more viscous than water were seen.

Table 2 Summary of operative and postoperative data

Case	Follow-up time (days)	Preoperative complications	Cataract	Postop status (macro- and microscopically)
1	105	Two retinal lesions	No	Pigmented lesions. Normal histology, no upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
2	3 (deceased)	None	No	N/a
3	105	One retinal lesion	Slight	Pigmented lesions. Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
4	105	None	No	Normal histology, no upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
5	105	None	No	Macroscopically normal. Small RD in histology, upregulation of GFAP in some areas. PKC and Rhodopsin normal. TUNEL negative.
6	105	One retinal lesion	Slight	Macroscopically normal. Small RD in histology, upregulation of GFAP in some areas. PKC and Rhodopsin normal. TUNEL negative.
7	56	None	No	Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
8	42	Traumatic cataract	Severe	Cataract, lens material in vitreous cavity. Limited retinal detachment (RD) with retinal degeneration. Moderate upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
9	56	One retinal lesion	No	Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
10	56	None	No	Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
11	56	None	No	Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.
12	43	Traumatic cataract	Severe	Cataract, lens material in vitreous cavity. Limited retinal detachment (RD) with retinal degeneration. Moderate upregulation of GFAP PKC and Rhodopsin normal. TUNEL negative.

Table 3 Postoperative intraocular pressure (mmHg)

Case	35 days in vivo (DIV)	68 days in vivo (DIV)	105 days in vivo (DIV)
1	27	18	16
2	N/a	N/a	N/a
3	19	16	15
4	21	17	16
5	18	16	20
6	18	26	12
7	25	N/a	N/a
8	15	N/a	N/a
9	16	N/a	N/a
10	16	N/a	N/a
11	18	N/a	N/a
12	N/a	N/a	N/a

Electrophysiology (Fig. 2)

Full-field ERG-recordings from the vitrectomized rabbits were reproducible and easily detected. There were no observed changes in the amplitudes either of the isolated rod (Dim blue light stimulation), mixed rod and cone (White light), or isolated cone (Flickering light) responses 1 and 3 months after surgery compared to preoperative ERGs.

Histology and immunohistochemistry (Fig. 3 and Table 2)

Routine microscopy with hematoxylin and eosin staining demonstrated normal morphology. The previously seen iatrogenic retinal detachment and retinal lesions were also observable during light microscopy. In two cases, signs of limited retinal detachments were seen although the retina had been deemed attached during the gross examination of the enucleated eyes.

GFAP (glial acidic fibrillary protein) labeling showed minimal signs of Müller cell up-regulation compared to unoperated eyes. In the eyes with traumatic cataracts and major retinal detachments, the GFAP expression was more prominent. No apoptotic cell labeling was observed in any of the eyes using the TUNEL-assay. Labeling with PKC (rod bipolar cells) and Rhodopsin (photoreceptor cells) revealed no differences compared to unoperated eyes.

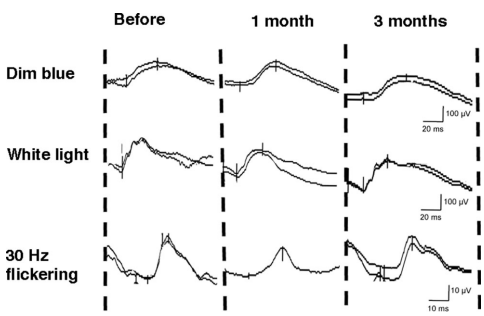

Fig. 2 Electrophysiology on rabbit eyes treated with Healaflow®. Compounded image of electroretinograms (ERG) before surgery, 1 month after surgery, and 3 months after surgery

Discussion

The search for an ideal vitreous substitute has been constantly ongoing since the early days of ophthalmology. Many different approaches have been tried with varying success, but due to the demanding task of combining a clinically usable substance with the correct physical and biochemical properties, there has been no definitive breakthrough so far. The clinically available substances all have considerable drawbacks, including side effects such as retinal toxicity, cataract, secondary glaucoma, and uveitis.

One of the reasons for the difficulties in devising a well-tolerated vitreous substitute is the complex and highly organized environment inside the eye globe. The vitreous cavity has traditionally been viewed in a highly simplified manner, as a space merely filled with a homogenous and inert gel, the vitreous. This simplistic view has gradually changed, and evidence now suggests that the vitreous is highly organized and more physiologically active than previously thought [25, 26]. One of the main functions of the vitreous is to uphold gradients of important nutrients such as oxygen [27], and other substances such as VEGF [10]. After vitrectomy, this environment is inevitably changed, and the addition of a vitreous substitute will further change the properties of the physiological milieu [10]. To prevent fluid from entering retinal breaks a tamponading effect is desirable, mandating close contact with the retina and other sensitive structures. Naturally, this places high demands on the biocompatibility of the substances used as vitreous substitutes.

In two previous papers, we have presented our experience with two potential vitreous substitutes in an *in vivo* vitrectomy rabbit model, Polyalkylimide (Bio-Alcamid®) and a poly(ethylene glycol) (PEG) hydrogel. In our study Bio-Alcamid® proved to have many suitable properties but caused severe retinal damage [19]. The PEG hydrogel, on the other hand, displayed an acceptable biocompatibility but had an impractically short retention time [20].

Hyaluronic acid is one of the main constituents of the natural vitreous, and related substances have a long history as candidates for vitreous substitutes, predating their routine usage in anterior segment surgery [13, 15, 28]. Due to limited retention time and short-term side effects [29, 30], none of

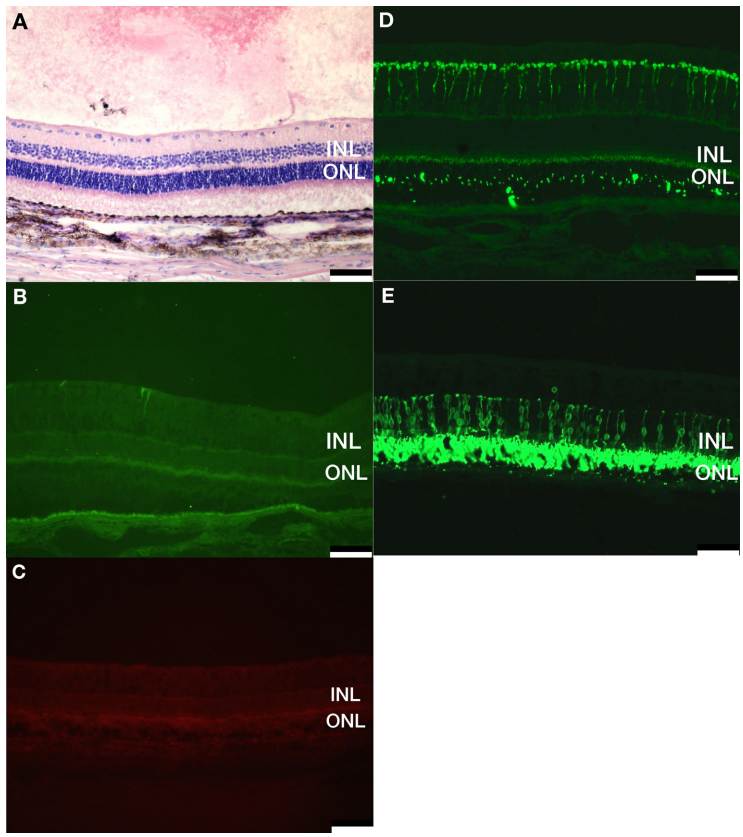

Fig. 3 Cryosections of rabbit retina after treatment with Healaflow® for 2–3 months. Labeled with; Hematoxylin and eosin (**a**), glial fibrillary acidic protein [GFAP, activated Müller cells] (**b**), terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay [DNA fragmentation] (**c**), phospho-protein kinase C [PKC, rod bipolar cells] (**d**), and Rhodopsin [rod photoreceptors] (**e**). Abbreviations: inner nuclear layer (**INL**), outer nuclear layer (**ONL**). Scale bar is 50 μm

these substances have found their way into routine surgery. Healaflow® is a cross-linked sodium hyaluronic acid hydrogel developed and FDA approved for use in glaucoma surgery [21, 22]. The cross-linking provides the compound with physical properties and structure similar to the collagen and hyaluronic acid meshwork of the natural vitreous [26].

In a recent paper, we described a novel model for *in vitro* testing of vitreous substitutes, wherein explanted rat retinas kept with Bio-Alcamid®, the PEG-hydrogel, and Healaflow®, respectively, were compared to retinas kept with culture medium only. The results were consistent with earlier *in vivo* trials for the former two substances, which displayed varying degrees of retinal damage, whereas retinas kept with Healaflow® exhibited less culture-induced trauma such as gliosis, loss of structure, and apoptosis compared to those kept with medium only [18]. This suggests excellent biocompatibility, a result confirmed in the current *in vivo* study. These findings are consistent with other recent animal studies using cross-linked hyaluronic acid hydrogels [17, 31], making this group of substances highly relevant for the development of vitreous substitutes.

In this study, we evaluated Healaflow® for use as a potential vitreous substitute in the rabbit model. The gel was easily incorporated with standard surgical procedures, and well tolerated with no signs of uveitis. Healaflow® was not seen to cause any long-term change in the intraocular pressure, although further studies are required to completely evaluate its effect.

Healaflow® did not affect retinal morphology or function negatively, making it highly interesting in this setting. The gel appeared to maintain its viscous structure in the vitreous cavity for at least a couple of weeks, potentially allowing for an effective short-term tamponade. The fact that the iatrogenic retinal detachments were self-limiting on long-term follow up may support the notion of a tamponading effect, although further studies are clearly warranted to confirm this phenomenon. Future work will include an increased ratio of cross-linking of

the gel to prolong the time of structural integrity even further, potentially allowing for a longer retention time *in vivo*.

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Compliance with ethical standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

This article does not contain any studies with human participants performed by any of the authors.

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References

1. Vote B, Wheen L, Cluroe A, Teoh H, McGeorge A (2003) Further evidence for proinflammatory nature of perfluorohexyloctane in the eye. Clin Experiment Ophthalmol 31(5):408–414
2. Killey FP, Edelhauser HF, Aaberg TM (1978) Intraocular sulfur hexafluoride and octofluorocyclobutane. Effects on intraocular pressure and vitreous volume. Arch Ophthalmol 96(3):511–515
3. Nakamura K, Refojo MF, Crabtree DV, Pastor J, Leong FL (1991) Ocular toxicity of low-molecular-weight components of silicone and fluorosilicone oils. Invest Ophthalmol Vis Sci 32(12):3007–3020
4. Colthurst MJ, Williams RL, Hiscott PS, Grierson I (2000) Biomaterials used in the posterior segment of the eye. Biomaterials 21(7):649–665
5. Papp A, Kiss EB, Tímár O, Szabó E, Berecki A, Tóth J, Páli J (2007) Long-term exposure of the rabbit eye to silicone oil causes optic nerve atrophy. Brain Res Bull 74(1–3):130–133
6. Versura P, Cellini M, Torreggiani A, Bernabini B, Rossi A, Moretti M, Caramazza R (2001) The biocompatibility of silicone, fluorosilicone and perfluorocarbon liquids as vitreous tamponades. An ultrastructural and immunohistochemical study. Ophthalmologica 215(4):276–283
7. Schatz B, El-Shabrawi Y, Haas A, Langmann G (2004) Adverse side effects with perfluorohexyloctane as a long-term tamponade agent in complicated vitreoretinal surgery. Retina 24(4):567–573
8. Joussen AM, Wong D (2008) The concept of heavy tamponades-chances and limitations. Graefes Arch Clin Exp Ophthalmol 246(9):1217–1224
9. Heimann H, Stappler T, Wong D (2008) Heavy tamponade 1: a review of indications, use, and complications. Eye (Lond) 22(10): 1342–1359
10. Stéfansson E (2009) Physiology of vitreous surgery. Graefes Arch Clin Exp Ophthalmol 247(2):147–163
11. Chang S (2006) LXII Edward Jackson lecture: open angle glaucoma after vitrectomy. Am J Ophthalmol 141(6):1033–1043
12. Holekamp NM, Shui Y-B, Beebe DC (2005) Vitrectomy surgery increases oxygen exposure to lens: a possible mechanism for nuclear cataract formation. Am J Ophthalmol 139(2):302–310
13. Kanski JJ (1975) Intravitreal hyaluronic acid injection. A long-term clinical evaluation. Br J Ophthalmol 59(5):255–256
14. Balazs EA, Freeman MI, Klöti R, Meyer-Schwickerath G, Regnault F, Sweeney DB (1972) Hyaluronic acid and replacement of vitreous and aqueous humor. Mod Probl Ophthalmol 10:3–21
15. Denlinger J, El-Mofty A, Balazs E (1980) Replacement of the liquid vitreus with sodium hyaluronate in monkeys II. Long-term evaluation. Exp Eye Res 30:101–117
16. Kanski JJ, Daniel R (1973) Intravitreal silicone injection in retinal detachment. Br J Ophthalmol 57(8):542–545
17. Schramm C, Spitzer MS, Henke-Fahle S, Steinmetz G, Januschowski K, Heiduschka P, Geis-Gerstorfer J, Biedermann T, Bartz-Schmidt KU, Szurman P (2012) The cross-linked biopolymer hyaluronic acid as an artificial vitreous substitute. Invest Ophthalmol Vis Sci 53(2):613–621
18. Barth H, Crafoord S, O'Shea TM, Pritchard CD, Langer R, Ghosh F (2014) A new model for in vitro testing of vitreous substitute candidates. Graefes Arch Clin Exp Ophthalmol 252(10):1581–1592
19. Crafoord S, Andreasson S, Ghosh F (2011) Experimental vitreous tamponade using polyalkyl-imide hydrogel. Graefes Arch Clin Exp Ophthalmol 249:1167–1174
20. Pritchard CD, Crafoord S, Andréasson S, Arnér KM, O'Shea TM, Langer R, Ghosh FK (2010) Evaluation of viscoelastic poly(ethylene glycol) sols as vitreous substitutes in an experimental vitrectomy model in rabbits. Acta Biomater 7:936–943
21. Schariot GB (2010) Canaloplasty re-establish the natural outflow in patients with chronic open-angle glaucoma. J Current Glau Prac 4(2):97–102
22. Roy S, Thi HD, Feusier M, Mermoud A (2012) Crosslinked sodium hyaluronate implant in deep sclerectomy for the surgical treatment of glaucoma. Eur J Ophthalmol 22(1):70–76
23. Gjörloff KW, Andréasson S, Ehinger B (2004) Standardized full-field and multifocal electroretinography in rabbits. Doc Ophthalmol 109:163–168
24. Marmor MF, Fulton AB, Holder GE, Miyake Y, Brigell M, Bach M (2009) ISCEV standard for full-field clinical electroretinography (2008 update). Doc Ophthalmol 118(1):69–77
25. Le Goff MM, Bishop PN (2008) Adult vitreous structure and postnatal changes. Eye 22(10):1214–1222
26. Swindle KE, Ravi N (2007) Recent advances in polymeric vitreous substitutes. Expert Rev Ophthalmol 2(2):255–265
27. Stéfansson E, Landers MB 3rd, Wolbarsht ML (1982) Vitrectomy, lensectomy, and ocular oxygenation. Retina 2(3):159–166
28. Gerke E, Meyer-Schwickerath G, Wessing A (1984) Healon in retinal detachment with proliferative vitreoretinopathy. Graefes Arch Clin Exp Ophthalmol 221:241–243
29. Koster R, Stilma JS (1986) Healon as Intravitreal substitute in retinal detachment surgery in 40 patients. Doc Ophthalmol 64:13–1
30. Vatne HO, Syrdalen P (1986) The use of sodium hyaluronate (Healon) in the treatment of complicated cases of retinal detachment. Acta Ophthalmol 64:169–172
31. Su W-Y, Chen K-H, Chen Y-C, Lee Y-H, Tseng C-L, Lin F-H (2011) An injectable oxidated hyaluronic acid/adipic acid dihydrazide hydrogel as a vitreous substitute. J Biomater Sci Polym Ed 22(13):1777–1797

Paper III

Paper IV

Substituting the vitreous

Blinding and debilitating vitreoretinal disease often necessitate surgical intervention with vitrectomy, wherein the vitreous is removed. In order to optimally treat our patients, we need to replace it with a highly biocompatible substance with the right properties.

The work presented in this thesis explores new compounds and innovative methods for comprehensive translational development of novel vitreous substitutes with the ultimate goal of clinical use.

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