Investigation of the emulsification of silicone oil used in vitrectomy in the presence of hydrophilic ophthalmic media

Ph.D. Thesis

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List of abbreviations

AH	aqueous humor
AIDS	acquired immune deficiency syndrome
BCVA	best corrected visual acuity
BSS	balanced salt solution
C3F8	perfluoropropane
CMV	cytomegalovirus
CT	computed tomography
FDA	Food and Drug Administration
FCVB	foldable capsular vitreous body
ILM	internal limiting membrane
IOFB	intraocular foreign body
IOP	intraocular pressure
LMWC	low-molecular-weight components
PDMS	polydimethylsiloxanes
PDR	proliferative diabetic retinopathy
PPV	pars plana vitrectomy
PVR	proliferative vitreoretinopathy
RD	retinal detachment
SD	standard deviation
VB	porcine vitreous
Zave	average droplet size

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1. Introduction

Silicone oil endotamponade has frequently been applied in the last decades since its introduction by Armaly [1] and Cibis [2] in 1962, providing internal tamponade by intravitreal injection. Both Machemer and Scott believed that silicone oil could dissect preretinal membranes and work against retinal traction as a stand-alone therapeutic agent [3,4]. Using Scott's technique to dissect membranes with an intravitreal injection of silicone oil without vitrectomy, others also reported good results [5,6]. Due to later development of pars plana vitrectomy by Machemer, silicone oil use became even more widespread, particularly for complicated retinal detachments.

In 1994, a form of silicone oil was approved by the FDA for the treatment of AIDS-related disorder, complicated retinal detachment related to cytomegalovirus retinitis. In 1997, the FDA approved a commercial formulation of silicone oil, Silikon 1000 (Alcon, Fort Worth, TX), for use as a prolonged retinal tamponade in selected cases of complicated retinal detachment [7].

After implantation, silicone oil forms a spherical bubble in the vitreous cavity, but some residual vitreous can remain at the vitreous base, especially in phakic patients. Intraocular silicone oil is in contact with the residual vitreous, and aqueous humor secreted by the ciliary body. Silicone oil, however, may undergo emulsification in 4–72 % of the cases and lead to vision-threatening complications affecting nearly all ocular structures. Complications may include corneal decompensation, band keratopathy, acute and chronic changes in intraocular pressure, lens opacities, epiretinal membrane, retinopathy, optic neuropathy, and extraocular extension (such as migration into the optic nerve, chiasm and even into the cerebral ventricular system) [8–13]. Silicone oil may infiltrate the optic nerve and even the subarachnoid space, too[14]. The emulsification of silicone oil depends on the interfacial tension between the oil and the hydrophilic phases (aqueous, vitreous or balanced salt solution (BSS)), which means decreased interfacial tension results in increasing emulsification tendency. The time course and level of emulsification are rather variable [15], but it was established to occur within the first year and mainly after the 5th postoperative month [16].

Besides the physicochemical characteristics of silicone oils, biological factors such as biological environment and mechanical effects can influence the development of

emulsification. On the other hand, based on literature information, until now there is no data on how the complex ocular biological media can influence emulsification.

2. Literature background

2.1 Anatomical background

2.1.1 Ciliary body

The ciliary body is a ring-shaped structure approximately 6–7 mm wide that extends from the base of the iris and becomes continuous with the choroid at the ora serrata. The ciliary body is subdivided into 2 parts: the anterior is pars plicata, which includes the ciliary processes and the more posterior is the pars plana. The pars plicata is richly vascularized and consists of the ciliary processes, which are the sites of aqueous humor production. The normal flow of aqueous humor is from the posterior chamber, through the pupil, and into the anterior chamber. The pars plana is a relatively avascular, smooth, pigmented zone. It is 4 mm wide and extends from the ora serrata to the ciliary processes. The safest posterior surgical approach to the vitreous cavity is through the pars plana, located 3–4 mm from the corneal limbus.

2.1.2 Aqueous humor

Aqueous humor (AH) is secreted by the ciliary epithelium lining the ciliary processes and fills the anterior and posterior chambers of the eye. This clear fluid provides nutrition, removes excretory products from metabolism, transports neurotransmitters, stabilizes the ocular structure and contributes to the regulation of the homeostasis of these ocular tissues. Aqueous humor also permits inflammatory cells and mediators to circulate in the eye in pathological conditions, as well as drugs to be distributed to different ocular structures [17].

The major components of aqueous humor are organic and inorganic ions, carbohydrates, glutathione, ascorbate, urea, amino acids and proteins, oxygen, carbon dioxide and water. The greatest differences in aqueous humor relative to plasma are the concentrations of protein and ascorbate. Protein is 200 times less in AH than in plasma, while ascorbate – which helps protect against light-induced oxidative damage – is 20 to 50 times higher in AH [18,19].

Important antioxidant substances derived by diffusion from the blood, such as glutathione, can also be found in aqueous humor. Several molecules involved in the maintenance of the extracellular matrix, such as collagenase, have been identified in human aqueous humor. In addition, growth factors have been detected in aqueous humor, as well as

receptors for many of these factors on target tissues, including transferrin, transforming growth factors, endothelin-1 [20].

2.1.3 Vitreous

Vitreous humor is a transparent crosslinked hydrogel filling the vitreous cavity, which is a space between the lens and the retina. It is in contact with the aqueous on its frontal surface. The vitreous is tightly connected to the surrounding tissues everywhere, most strongly in the area of the vitreous base, around the papilla, in the macula, and along the main retinal vessels. The vitreous base is an annular three-dimensional structure at the height of the ora serrata with an extent of about 2-3 mm in all directions. The ora serrata is a border line between the ciliary epithelium of the ciliary body called the pars plana and the ten differentiated layers of the retina. In terms of its function, it plays a primarily supportive role.

The functions of the vitreous are the following.

- The large concentration of hyaluronic acid in the vitreous humor protects the eye from shocks, which arise due to mechanical stress and vibrations.
- The highly transparent nature of vitreous humor allows 90 % transmission of visible and near-infrared light [21].
- The vitreous acts as a barrier to biochemical substances and cells, thereby inhibiting inflammation and neovascularization [22].
- The vitreous helps the metabolism of the surrounding tissue by transporting essential molecules and minimizes the risk of cataract formation by protecting the lens from oxidative damage [23].

Ageing leads to vitreous liquefaction and detachment of the vitreous from the retinal surface. The separation of the vitreous from the retina can induce retinal tears. The liquefied vitreous can pass through these holes/tears causing retinal detachment.

2.2 Pars plana vitrectomy

Pars plana vitrectomy (PPV) as a technique has revolutionized retinal surgery since its advent in 1970. This innovation allowed the treatment of previously blinding retinal diseases. PPV is a technique in vitreoretinal surgery that enables access to the posterior segment for treating pathologies such as rhegmatogenous retinal detachment, macular hole, epiretinal

membranes, diabetes-related vitreous hemorrhage or tractional retinal detachment, endophthalmitis and ocular trauma in a controlled, closed system. The procedure derives its name from the fact that the vitreous is removed (i.e. vitreous + ectomy = removal of the vitreous) and the instruments are introduced into the eye through the pars plana.

David Kasner first described vitrectomy, or removal of the vitreous body, using an open-sky technique in 1969 [24]. Two years later, Robert Machemer created the first closed system vitrectomy setup – which enabled intraocular pressure control – using 17-gauge instruments with a pars plana approach, the beginning of what became known as pars plana vitrectomy [25,26]. In 1974, Conor O'Malley and Ralph Heintz developed the three-port system [27]. They used 20-gauge instruments - one port had an infusion line sewn into place, while the remaining two ports were utilized for introduction of a light source and a vitreous instrument such as a cutter. At the completion of the procedure, these ports were traditionally closed with an absorbable suture. Subsequent innovations include the development of a trocar-cannula system for insertion of instruments by Robert Machemer and Dyson Hickingbotham, 23-gauge instrumentation by Gholam Peyman and Claus Eckardt, 25-gauge instrumentation by Gildo Fujii and Eugene de Juan, and 27-gauge instrumentation by Yusuke Oshima and colleagues in 2010 [28–37].

Over the decades, not only did sclerotomy become smaller, but progress was also made through other innovations, including endoscopic light amplification by stimulated emission of radiation (endoLASER), perfluorocarbon liquid, wide-field viewing systems and improved illuminating systems (e.g. Xenon). Each of these advances made it easier to treat diseases that were previously difficult or impossible to treat [38].

2.3 Endotamponades

During vitrectomy, the vitreous is removed. Since the vitreous is not able to regenerate or replenish, the vitreous has to be replaced by some kind of vitreous substitute. Artificial vitreous tamponades are applied in order to re-establish intraocular volume and provide a temporary or permanent tamponade to the retina after vitrectomy. They also play an important role in separating membranes adherent to the retina, manipulating retinal detachments, and mechanically flattening detached retinas. An ideal vitreous substitute would need appropriate viscosity, have good biocompatibility, optical clarity, a suitable refractive index, and suitable

rheological properties, while being non-toxic to the retina [39] and would be able to perform its physiological function in the eye for a long time or throughout the patient's life.

The first endotamponades that were used before the era of vitrectomy were injected into the eye without removing the vitreous. The use of gas or air, introduced by Rosengren in 1938, is still employed by many ophthalmologists as a single procedure [40]. All over the world, many researchers have developed different types of vitreous substitutes or tamponade agents. Each developed substitute can be associated with several advantages and disadvantages, and until now there is no ideal vitreous substitute available [41].

Nowadays vitreous tamponades are composed of gases (e.g., air and perfluorocarbon gases) and liquids (e.g., silicone oil and perfluorocarbon liquids). Gases provide a short- to mid-term and silicone oil provides a long-standing endotamponade.

2.3.1 Silicone oils

2.3.1.1 Historical perspective of silicone oils

Based on the experimental work of Stone, Armaly [1], and Cibis [2], silicone oils had already been applied in retinal detachment surgery before the advent of modern pars plana vitreous surgery in the early 1960s. Silicone oils have relatively high interfacial tension against water and own hydraulic capabilities, thus Cibis envisioned silicone oil both as an extended intraocular tamponade and a surgical instrument. Silicone oils can be used as a tamponade in order to push the retina back to its original place against the tractional force of preretinal membranes; and as an instrument to aid to separate membranes. With this new approach, previous inoperable cases could end with great anatomical and visual success.

The application of silicone oils in vitreoretinal surgery was widespread mostly in the USA. Their widespread use has also increased the number of complications associated with long-lasting silicone tamponade. This, along with the early death of Cibis, has resulted in a retreat of the retinal surgical use of silicone oils for a time. Only a few retinal surgeons, most notably John Scott [42,43] in England, continued to use silicone oil in vitreoretinal surgery, their work helped expand Cibis' work and provided additional experience with silicone oil as a long-lasting tamponade. With the advent of modern vitreous microsurgery in the 1970s, some researchers, like Jean Haut in France, Relja Zivojnovic [44] in the Netherlands and Peter Leaver[45]

in England, successfully combined pars plana vitrectomy techniques with the use of silicone oil as an endotamponade.

As the use of silicone oil spread worldwide, other innovations, such as inferior peripheral iridectomy and relaxing retinotomy in selected cases reduced the rates of complications; and further increased the likelihood of successful retinal reattachment. While silicone oil was used with increasing frequency in the treatment of complex retinal detachments in Europe, retinal surgeons in the USA focused their attention on improving fluid – gas exchange techniques and on the development of longer-acting gases for extended intraocular tamponade.

By the mid-1980s, the relative safety and efficacy of these two types of tamponades had not been determined. Uncontrolled clinical studies suggested that the guaranteed and extended nature of silicone oil tamponade might improve the anatomical results for complex cases, but that the ultimate visual results might be compromised by complications related to its use. Stephen Ryan initiated the implementation of The Silicone Study, which sought to compare the safety and efficacy of silicone oil to long-acting gas as a tamponade in the repair of complex rhegmatogenous retinal detachments with severe proliferative vitreoretinopathy (PVR).

2.3.1.2 Characterization of silicone oils

Silicone oils for ophthalmic use are composed of polydimethylsiloxanes (PDMS), which are synthetic polymers. They are highly hydrophobic materials, thus they result in high interfacial tension with water (44 mN/m) [46]. The viscosity of silicone oils depends on the molecular chain length (molecular weight), longer molecular length can mean higher viscosity. The higher viscosity of silicone oils as endotamponade is beneficial because their migration, their emulsification tendency can be reduced [47–49]. The viscosity range of the currently used silicone oils is in between 1000 and 5700 mPa·s [50]. In general, SO with higher viscosity has a lower tendency to emulsify [46]. The molecular weights of the most commonly used silicone oils as endotamponade are usually between 37 and 65 kDa [51].

The density of ophthalmic silicone oils can be very critical, it is usually around 0.97 g/cm^3, which is lower than that of water and the aqueous phase in the vitreous. Due to its lower density, it is positioned on top of the aqueous phase in the vitreous space, so it is more

advantageous as a retinal tamponade for the treatment of superior retinal breaks. In the case of inferior retinal tamponade, heavy silicone oils are applied in clinical practice [52,53], their density is lower than that of water, so they are located normally at the inferior part of the vitreous cavity. These silicone oils were developed by substituting some of the methylated groups of conventional silicone oils with fluorinated groups, this modification resulted in higher specific gravity.

Silicone oils can be used in the indications of several specific ophthalmic clinical situations, which can also be attributed to the fact that their use is much more advantageous than intraocular gases. The advantages of silicone oils are:

- their volume does not change over time,
- they are thought to require less strict positioning compared to intraocular gases,
- they can be used for children and for adults unable to be positioned optimally postoperatively.

Unlike gases, the volume of silicone oils is not affected much by variations in atmospheric pressure; thus, they are preferred in patients who have to fly during the postoperative period. Although not proven or contraindicated, most ophthalmic physicians use silicone oil primarily in retinal detachments with a high risk for repair failure, including cases involving viral retinitis, proliferative vitreoretinopathy, giant retinal tears, and tractional retinal detachments [54].

The most often used silicone oils are summarized in Table 1.

Table 1. Silicone oils in clinical use [55,56]

Silicone oil	**Composition**	**Characteristics** (density g/cm^3 viscosity mPa·s)	**Clinical use**
Siluron® 1000 (Fluoron)	100 % Poly-dimethylsiloxane (PDMS)	0.97 900 – 1200	Retinal detachments with giant tears, PVR, or proliferative diabetic retinopathy (PDR); or traumatic retinal detachment.
Siluron® 2000 (Fluoron)	95 % Siluron® 1000 + 5 % PDMS (2,5 Mio. mPa·s)	0.97 2000-2400	
Siluron® 5000 (Fluoron)	100 % Poly-dimethylsiloxane (PDMS)	0.97 4800-5500	
Siluron®Xtra (Fluoron)	90 % Siluron® 1000 + 10 % PDMS (2,5 Mio. mPa·s)	0.97 4100-4800	
Densiron 68® (Fluoron)	30.5 % F6H8 and 69.5 % Siluron 5000	1.06 1400	Inferior and posterior retinal holes; retinal detachments with giant tears, PVR, or PDR; or traumatic retinal detachment.
Densiron®Xtra (Fluoron)	69.5 % PDMS (containing 10 % Siluron Xtra), 30.5 % F6H8	1.06 1200	
Oxane® 1300 (Bausch and Lomb)	100 % Poly-dimethylsiloxane (PDMS)	0.98 1000	PVR C State; giant tears with PVR, PDR, retinal detachment (RD) by perforating trauma, intra-operative intra-vitreal haemorrhage; epiretinal membranes
Oxane® 5700 (Bausch and Lomb)	100 % Poly-dimethylsiloxane (PDMS)	0.98 5000	
Oxane® HD (Bausch and Lomb)	11.9 % RMN3 and 88.1 % Oxane® 5700	1.02 3300	Inferior and posterior retinal breaks; RD complicated by severe PVR, penetrating trauma, giant tears; inferior retinectomy for anterior PVR
ADATO® SIL-OL 5000 (Bausch and Lomb)	100 % long chain PDMS	0.96-0.98 5000-5900	PVR, PDR, cytomegalovirus (CMV) retinitis, giant tears, Acquired Immune Deficiency Syndrome (AIDS) related CMV retinitis and other viral infections.

2.3.2 Emulsification of silicone oils

Emulsification is a clinically significant complication of the usage of silicone oils as tamponade. The mechanism of their emulsification is still not completely known. It is hypothesized that small oil droplets come off the main oil bubble. The initiator of this process can be shear forces induced by saccadic eye movements [51]. The small dispersed droplets can be stabilized by various endogenous substitutes (different blood components) and the small droplets can migrate to different parts of the eye or the human body.

There are several factors that can promote or prevent silicone oil emulsification after retinal detachment repair, including proteins, surfactants, contaminants, and shear forces [57].However, the duration of tamponade remains the most significant one. After emulsification has occurred, keratopathy [6,54,58] and glaucoma [10,54,59–63] are the most common complications. The emulsification and migration of silicone oil can also affect the retina, the optic nerve, and it may even migrate into the brain [14,64]. The minimalization of the residence time of silicone oil in the eye is the most important factor in reducing its complications.

To avoid complication caused by silicone oil emulsification and migration, a novel vitreous substitute was developed by the State Key Laboratory of Ophthalmology (Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China) called foldable capsular vitreous body (FCVB). FCVB contains a vitreous-like capsule, drain tube, and drain valve. After implanting the FCVB in the vitreous cavity, silicone oil is injected into the capsule through the drainage valve, and the capsule is inflated [65]. With this method, silicone oil can closely fit the inner structure of the eyeball, but it can completely cut off direct contact between silicone oil and eye tissues, avoid silicone oil emulsification and migration, reduce damage to the function of the ciliary body, and provide 360° full-filled support retina. A retrospective analysis reported by Chen et al. involved 26 patients with severe ocular trauma and one with recurrent retinal detachment who underwent 23G pars plana vitrectomy and FCVB implantation combined with silicone oil tamponade [66]. In this study, the final retinal reattachment rate was 92.59 %. The initial VA of all the patients was <0.05 and the final VA scores were either maintained or slightly lower than the initial VA scores. This novel technique can help avoid eye enucleation and maintain the shape of the eyeball but, of course, further studies are required.

3. Aims

Vitreous substitution is deemed to be a highly challenging and interesting research topic in ophthalmology. The use of silicone oils in vitreoretinal surgery, especially in complicated cases, is essential nowadays too. I present its importance and difficulty through my case reports.

The experimental aims of my Ph.D. work were to develop an *in vitro* model for the complex investigation of the phenomenon of silicone oil emulsification in the presence of potential ophthalmic hydrophilic phases obtained from porcine eyes. The aims were as follows (Figure 1.).

1. Measurement of the emulsification ability of the biological media (aqueous humor, vitreous) and BSS.

   The purpose was to investigate the potential media (hydrophilic phase) and silicone oil and their mixture/emulsions by means of:

   - surface tension,
   - zeta potential,
   - microscopic measurements,
   - rheological measurements.

2. Evaluation of the emulsification effect of the vitreous.

   The plan was to analyze the formation and stability of the emulsion formed with the vitreous and silicone oils, therefore:

   - zeta potential,
   - macroscopic investigations were performed,
   - the stability of the emulsions was followed.

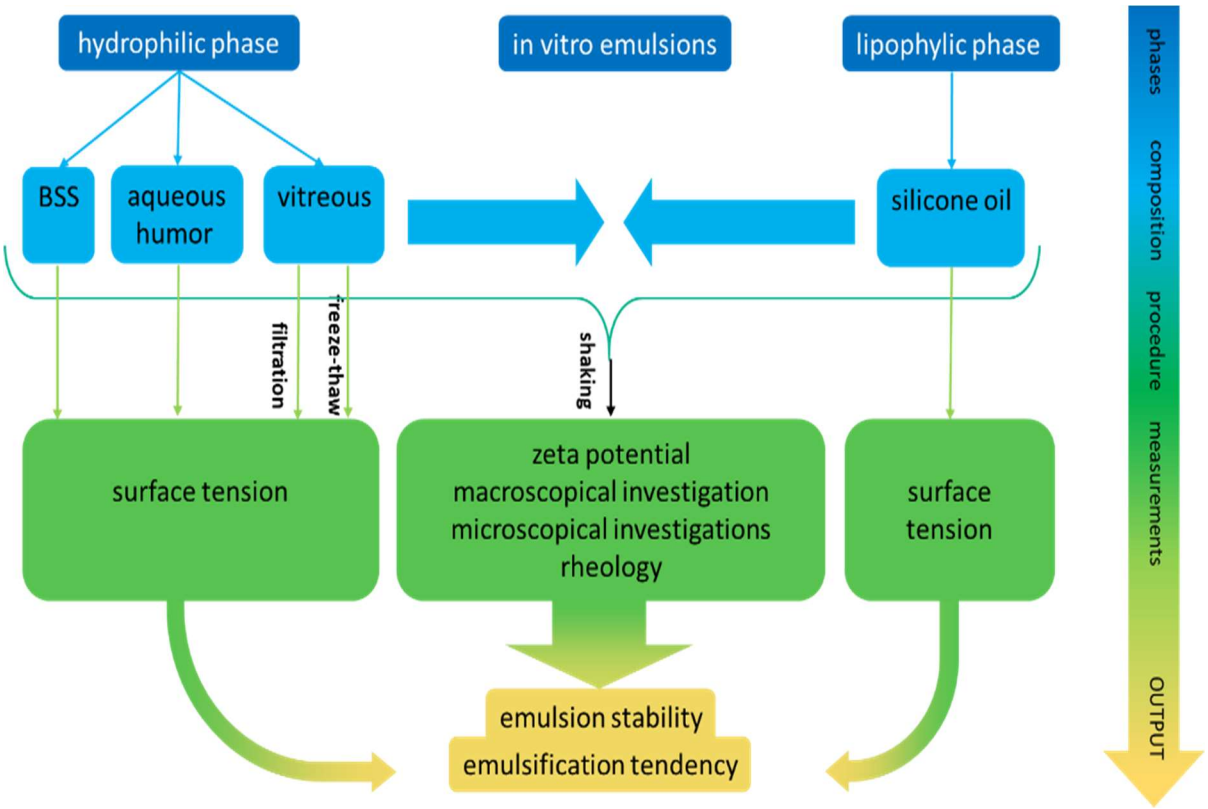

Figure 1. Structure of the measurement of the emulsification ability of the hydrophilic phases.

4. Application of silicone oils in vitreoretinal surgery: Case studies

4.1 Case study 1

17-year-old male patient with myopia in both eyes reported one-week-long visual disturbance in the left eye. Retinal detachment was noted (Figure 2.). Scleral buckling surgery was performed. Visual acuity showed immediate improvement following surgery with mild resolution of the subretinal fluid, but on postoperative day two PVR formation accelerated and new retinal tears were found.

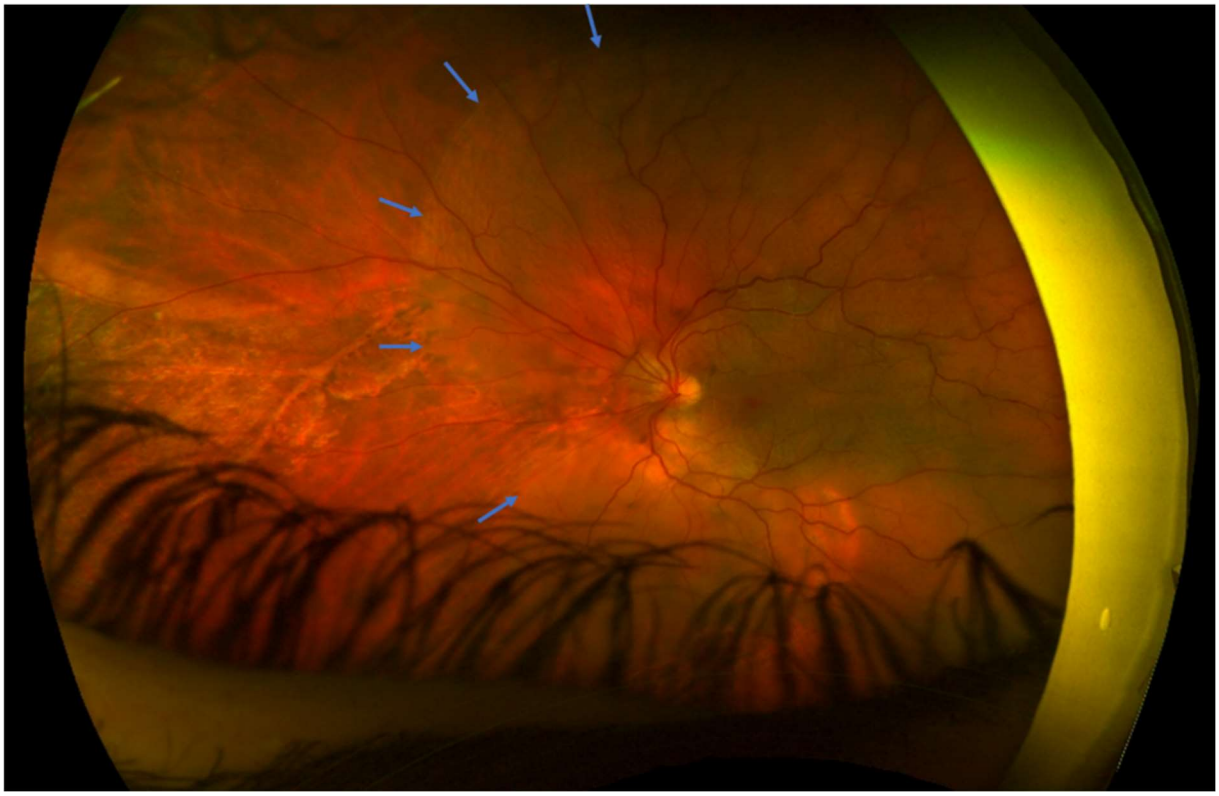

Figure 2. Retinal detachment (blue arrow: border of the retinal detachment).

Additional vitrectomy was performed with repositioning the buckle and peeling of the ILM, additional endolaser treatment and ultimately silicone oil implantation.

Best corrected visual acuity (BCVA) at postoperative 1 month is 0.3, retina is attached, retinal breaks are closed (Figure 3. blue arrow: indentation of the buckle). The removal of silicone oil is pending.

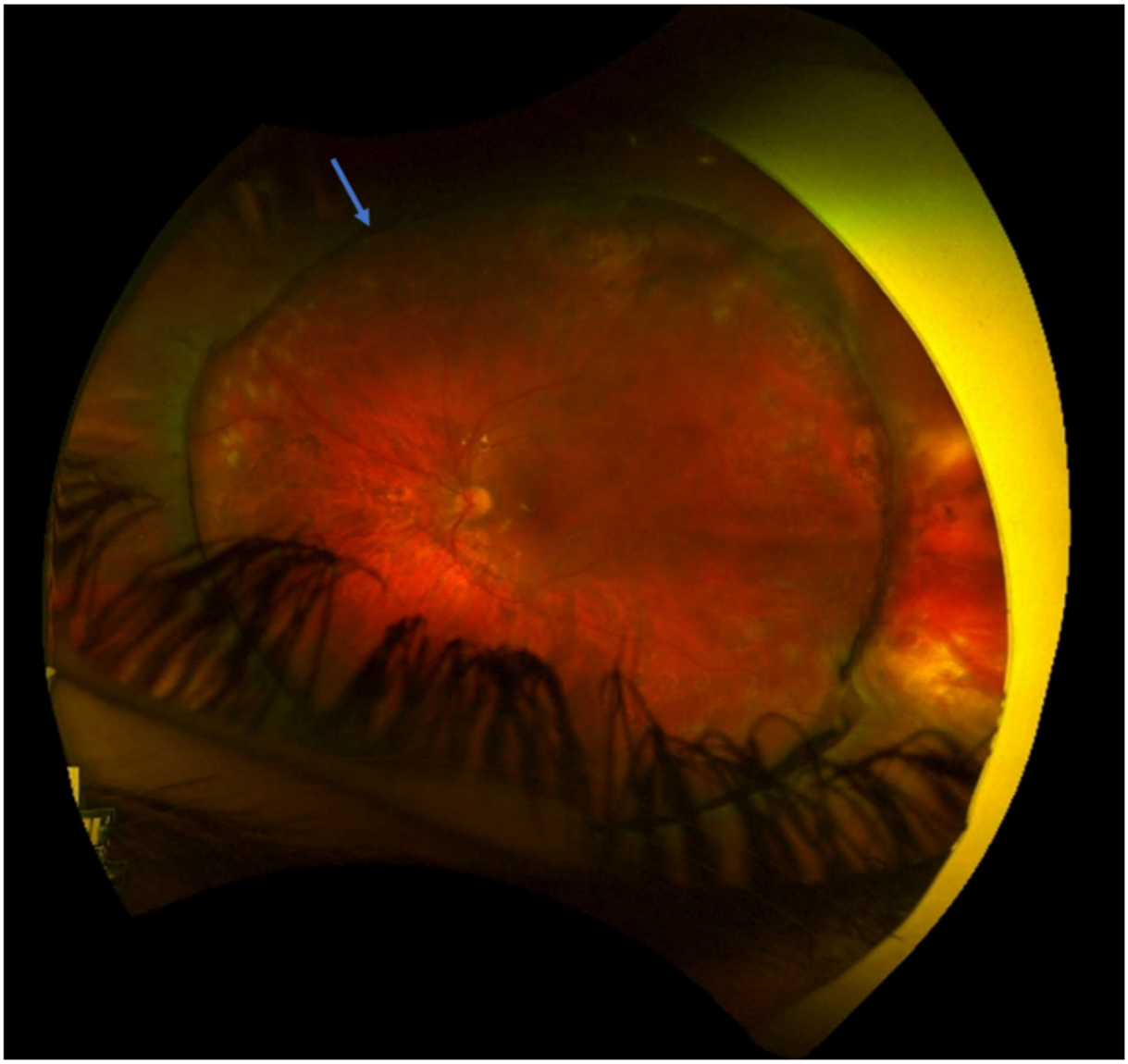

Figure 3. One month later, retina is attached, retinal breaks are closed (blue arrow: indentation of the buckle).

4.2 Case study 2

A 39-year-old female patient suffered perforating injury with intraocular foreign body (IOFB) as a bystander of a household accident. She presented with a 5 mm lacerated corneoscleral wound with iris prolapse, incipient traumatic cataract, vitreous haemorrhage and penetrating wound in the superotemporal part of the macula (Figure 4.).

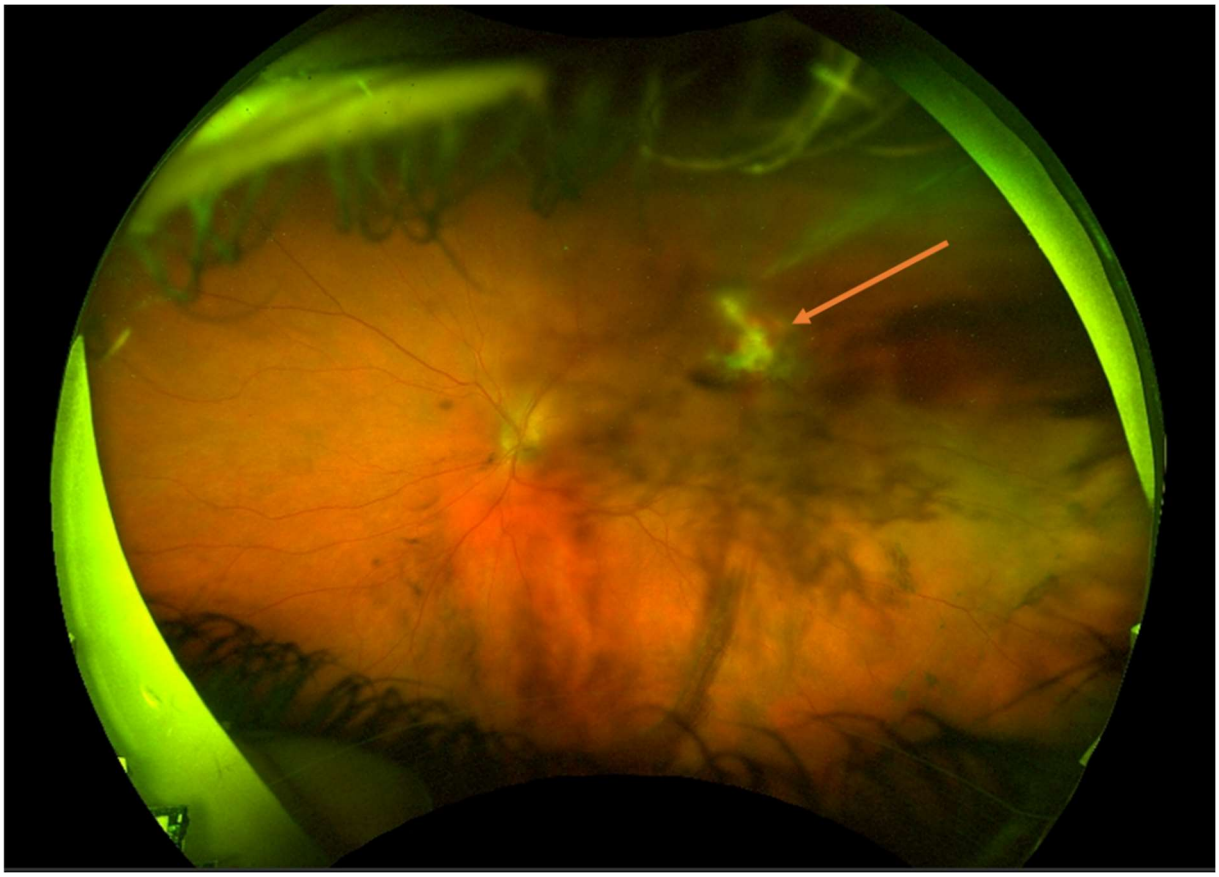

Figure 4. Exit wound in the superotemporal part of the macula (orange arrow).

Computed tomography (CT) imaging revealed a 4 mm metallic foreign body located in the superior temporal quadrant of the orbit located intraconally between the globe and the lateral rectus muscle.

The wound was addressed in general anaesthesia followed by 23-gauge vitrectomy. After evacuation of the vitreous bleeding, a star-shaped exit wound was found at the site of the temporal macula.

Fluid exiting the posterior wound resulted in severe proptosis and deformation of the globe (Figure 5.). Due to the inability of proper posterior reconstruction, surgery was terminated without detachment of the posterior vitreous with spontaneous closure to be expected. With frequent follow-up, retinal detachment occurred by postoperative day 20.

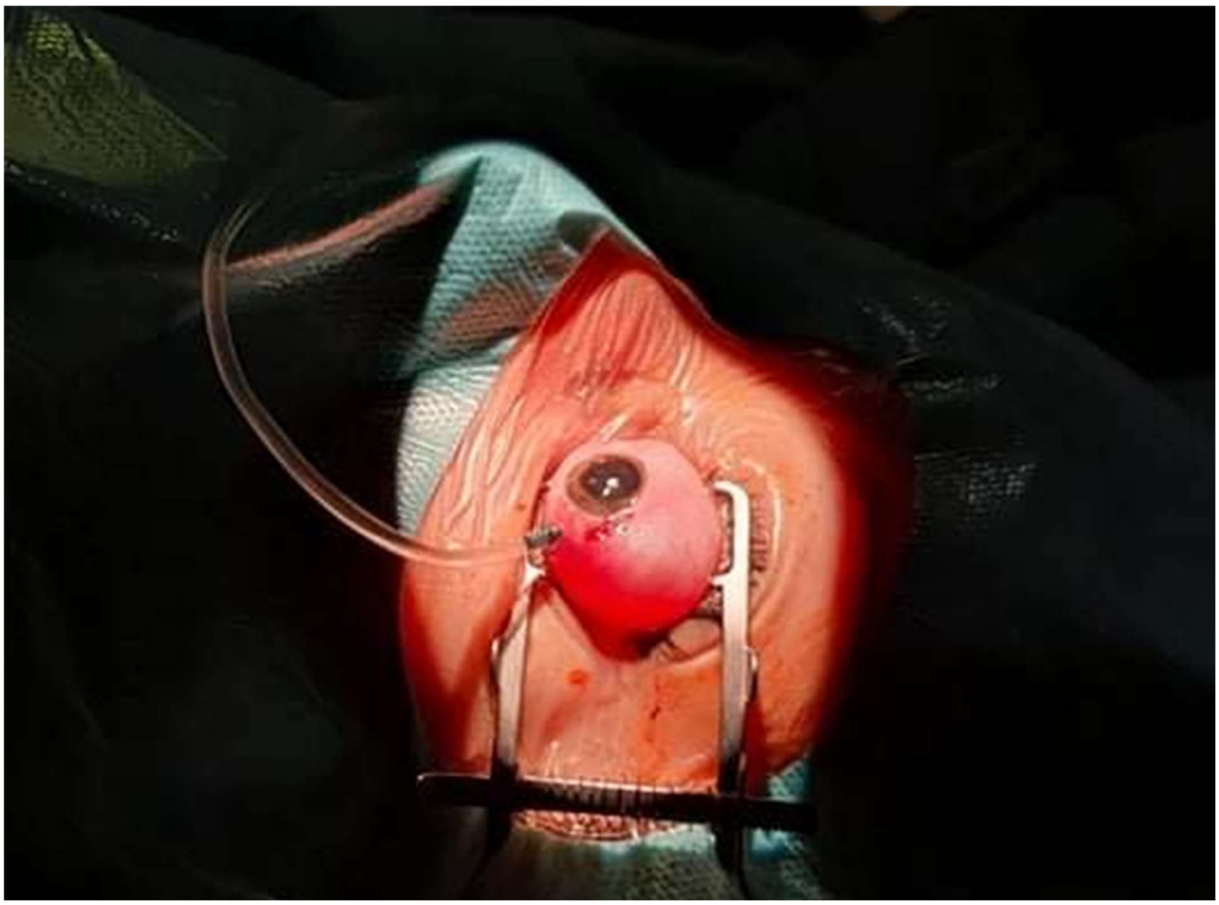

Figure 5. Proptosis and deformation of globe.

Reoperation was scheduled with removal of the traumatic cataract in the bag implantation of intraocular lenses and peeling of both the posterior vitreous face and the ILM was performed via repeated vitrectomy. Spontaneous scleral wound closure was confirmed and focal retinectomy, diathermy and endolaser treatment were performed. Retinal reattachment was secured with additional endolaser burns and silicone oil (Figure 6.), the latter being removed 7 months after surgery (Figure 7.).

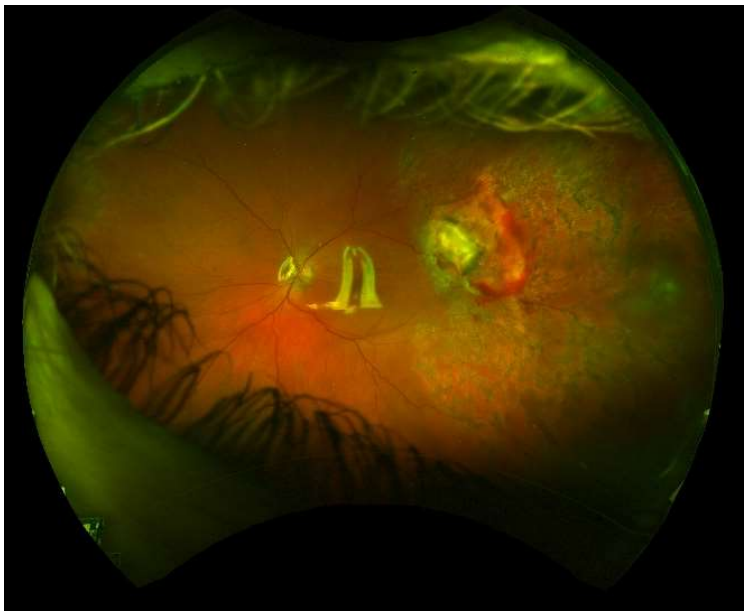

Figure 6. Retinal reattachment is secured with additional endolaser burns and silicone oil.

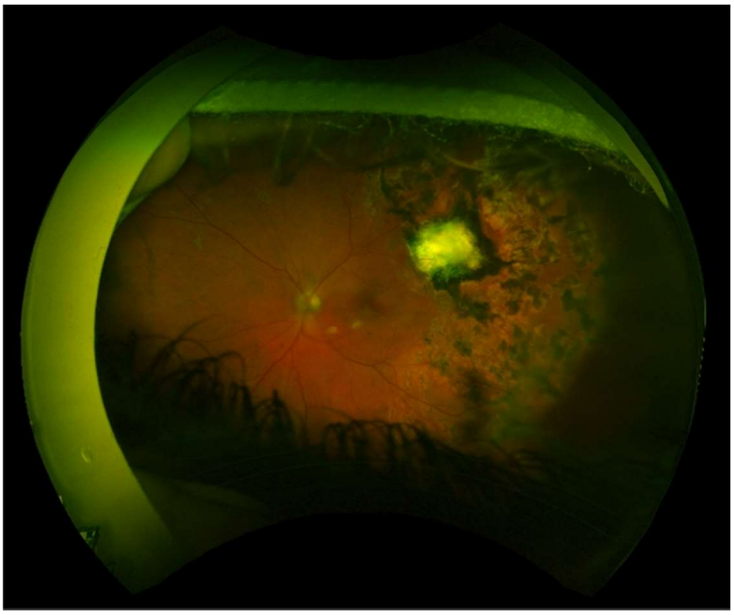

Figure 7. Silicone oil is removed 7 months after surgery.

4.3 Case study 3

A 78-year-old man underwent combined phacoemulsification and vitrectomy with perfluoropropane (C3F8) gas tamponade due to temporal macula off retinal detachment in 2017. Total retinal redetachment was seen three months postoperatively with retinal tear wide open resulting from PVR. Repeated surgery was performed with silicone oil implantation. Persistent lower temporal peripheral detachment remained, the posterior pole showed anatomical restitution. No consent for further surgery was obtained from the patient, so follow-up was scheduled. BCVA was 0.3 at the time. One year postoperatively, IOP rise was treated with tafluprost timolol fixed combination. During the Covid-19 lockdown, the patient failed to report for follow-up for a year. In June 2021, the patient presented with a history of decreasing vision in the previous 6 months. Visual acuity was down to light perception with intraocular pressure exceeding 50 mmHg and loss of corneal transparency induced by emulsified silicone oil present in the anterior chamber (Figure 8.).

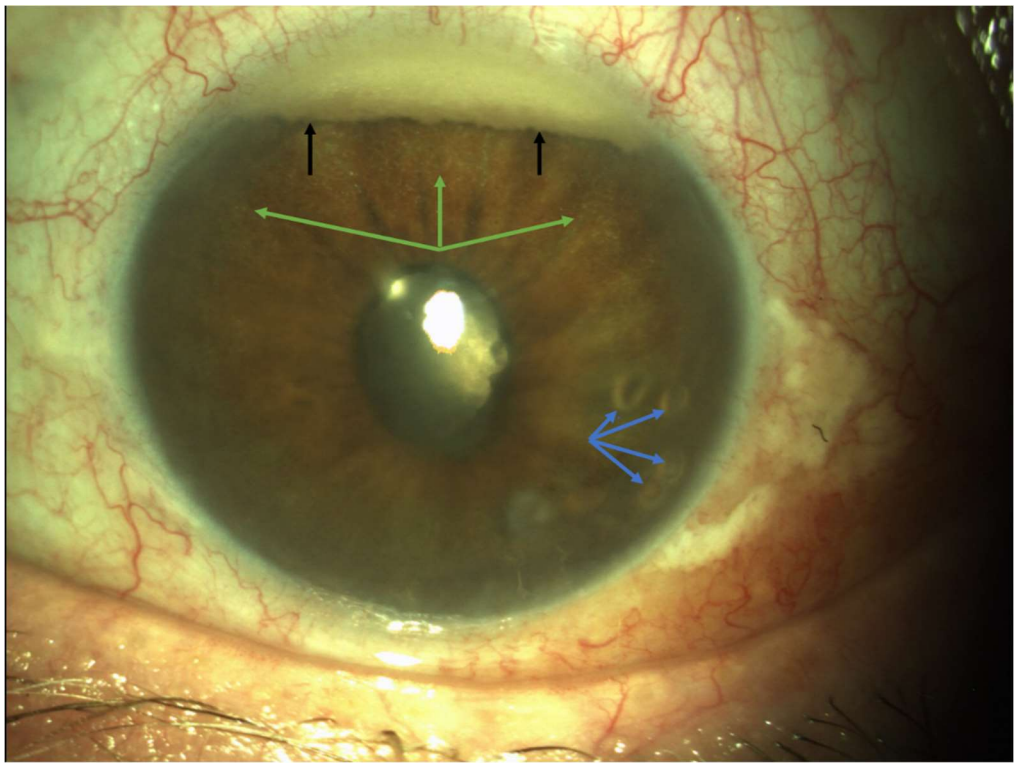

Figure 8. Emulsified silicone oil covering the iris (green arrow) and forming 'inverse hypopyon' (creaming of the emulsified oil droplets) in the anterior chamber (black arrow) accompanying corneal oedema and bullous keratopathy (blue arrow).

4.4 Summary of the case studies

Silicone oils are widely used in vitreoretinal surgery, mainly in complex cases. As can be seen from the clinical case studies presented, their use is problem-free in a significant percentage of cases, but complications also occur. It should also be noted that there is no widely accepted alternative to silicone oils to date, so their use in vitreoretinal surgery is necessary. In my Ph.D. work, I aimed to investigate factors that can be associated with the phenomenon of emulsification during the use of silicone oils, and the elimination of which may reduce the development of complications. Accordingly, an *in vitro* study design was compiled, the details of which are discussed in the next part of the dissertation.

5. *In* vitro experiments: Materials and Methods

5.1 Materials

5.1.1 Silicone oil

Original silicone oil Oxane 1300 (Bausch&Lomb GmbH, Germany) was used.

5.1.2 Hydrophilic phases

For the hydrophilic phase of the emulsions balanced salt solution (BSS, Alcon Laboratories, Inc., USA) and biological aqueous phases such as porcine aqueous humor (AH) and porcine vitreous (VB) were used. Porcine eyes were freshly obtained from a slaughterhouse (Pick Szeged Zrt., Szeged, Hungary) (animals were not sacrificed for the study). The aqueous humor was aspirated through corneal paracentesis and the vitreous was removed with scissors 2–5 hours post-mortem from 100 porcine eyes. The humors were homogenized, chilled and stored at -20 °C. They were melted at room temperature just before the application. Our applied microscopic and zeta potential methods required high optical purity, but the biological fluids often contain pigments and other non-soluble components. Based on our preformulation studies, membrane filters with a pore size of 0.45 and 0.20 μm were not suitable for the separation of the non-soluble components. Therefore, the clear biological aqueous phases were separated from the pigments by centrifugation in Vivaspin 15R 5,000 MWCO Hydrosart tubes (Sartorius, Stonehouse, UK) with Hermle Z323K (HERMLE Labortechnik GmbH, Wehingen, Germany). Centrifugation was performed at 5000 rpm (9000 rcf), for 30 min at 4 °C. This separation method resulted in optically clear biological fluids. Mixtures of the vitreous and BSS were made to model *in vivo* vitrectomized (and silicone oil filled) eyes with residual vitreous.

5.1.3 *In vitro* emulsions

Silicone oil and the hydrophilic phases were mixed with a Vortex stirrer for 5 min. The emulsions/mixtures were measured immediately after preparation. Each emulsion was prepared three times.

5.2 Methods

5.2.1 Surface tension measurements

Silicone oils and aqueous phases were analyzed by means of surface tension measurements by OCA 20 (Automatic Contact Angle Measuring and Contour Analysis System) instrument (Dataphysics Instruments GmbH, Filderstadt, Germany) using the pendant drop method. The instrument detects the contour of the pendant drop and calculates the surface tension. The mean of ten parallel measurements was used for calculations.

5.2.2 Macroscopic investigation

For the macroscopic observations, the hydrophilic phases of the emulsions were dyed with 0.001 % methyl-blue (Sigma-Aldrich GmbH, Germany). The emulsions were photographed, and the clear oil droplet could be seen in the thin layer of the emulsion sample without any magnification.

5.2.3 Microscopic investigation

For the microscopic observations, the hydrophilic phases of the emulsions were stained with 0.001 % Fluorescein sodium (Sigma-Aldrich GmbH, Germany) in a 100:1 ratio. 50 µL of emulsions were put onto microscope slide and covered with cover glass. Microscopic images were made by a Leica DMB6 microscope (Biomarker GmBH, Hungary) in fluorescence mode (Leica DFC7000 T fluorescence camera, Biomarker GmBH, Hungary). The magnification was 200x. Droplet size and droplet size distribution were analyzed on the basis of the black and green contour of the oily and aqueous phase using the LAS X core software of the microscope.

Each composition was prepared three times, and each sample was scanned by microscope. The images where droplets were observed were analyzed by the software. Emulsions with 2:8 and 8:2 oil and aqueous phase ratios were investigated.

5.2.4 Zeta potential measurements

The zeta potential of the *in vitro* emulsion was measured by Zetasizer Nano ZS (Malvern Instrument, UK) with electrophoretic light scattering. Emulsion droplets are surrounded by an electric double layer. This double layer is formed when surface-charge-carrying emulsion droplets are surrounded by counter-ions of charge opposite to that of the droplet surface. A given amount of these counter-ions move together with the droplet, hence a slipping plane beyond the emulsion droplet. The electrical potential at this slipping plane is the zeta potential

(ζ, mV). As a result of increased electrostatic repulsion, the coalescence of the oil droplets is hindered. An emulsion with a high absolute value of zeta potential is more stable in comparison to that with lower zeta potential absolute values [67]. With this method, emulsions with 2:8 and 1:9 oil and aqueous phase ratios were investigated.

5.2.5 Rheology

The viscosity of silicone oil and *in vitro* formed emulsions/mixtures was measured by a Physica MCR 101 rheometer (Anton Paar, Austria). The measuring device was cone and plate type (diameter 25 mm, gap height in the middle of the cone 0.046 mm, cone angle 1°). The flow and the viscosity curves of the samples were plotted. The shear rate was changed from 0.1 to 100 s^{-1}. The viscosity of the samples was evaluated at 100 s^{-1}. The experiments were carried out in triplicate. With this method, emulsions with 2:8 oil and aqueous phase ratio were investigated.

5.2.6 Statistics

Unpaired t-test was performed using GraphPad Prism (GraphPad Software Inc., USA). A level of $p \leq 0.05$ was taken as significant, $p \leq 0.01$ as very significant, and $p \leq 0.001$ as highly significant.

6. Results

6.1 Surface tension of the potential media

All ophthalmic hydrophilic substances had significantly lower surface tension than that of BSS. Aqueous humor had the lowest surface tension (54.99 ± 1.98 mN/m), which was followed by the value of the vitreous (61.16 ± 1.30 mN/m), while BSS had the highest value (72.62 ± 0.08 mN/m) (Figure 9.). When comparing the three hydrophilic media, aqueous humor had the lowest surface tension. The surface tension of silicone oil was 20.64 ± 0.29, to which aqueous humor presented the closest value, indicating the lowest interfacial tension in the case of the aqueous humor and silicone oil mixture.

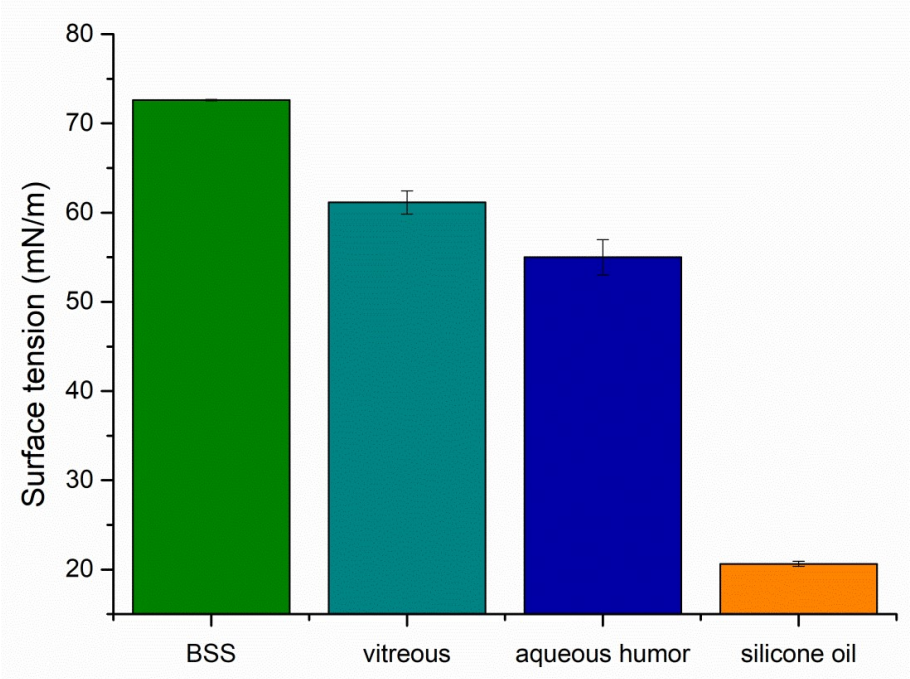

Figure 9. Surface tension of the aqueous phases and silicone oil.

The surface tension of fresh and freeze-thaw vitreous was also compared in order to investigate the effect of freezing. Our results indicated that the surface tension remained the same (Figure 10.).

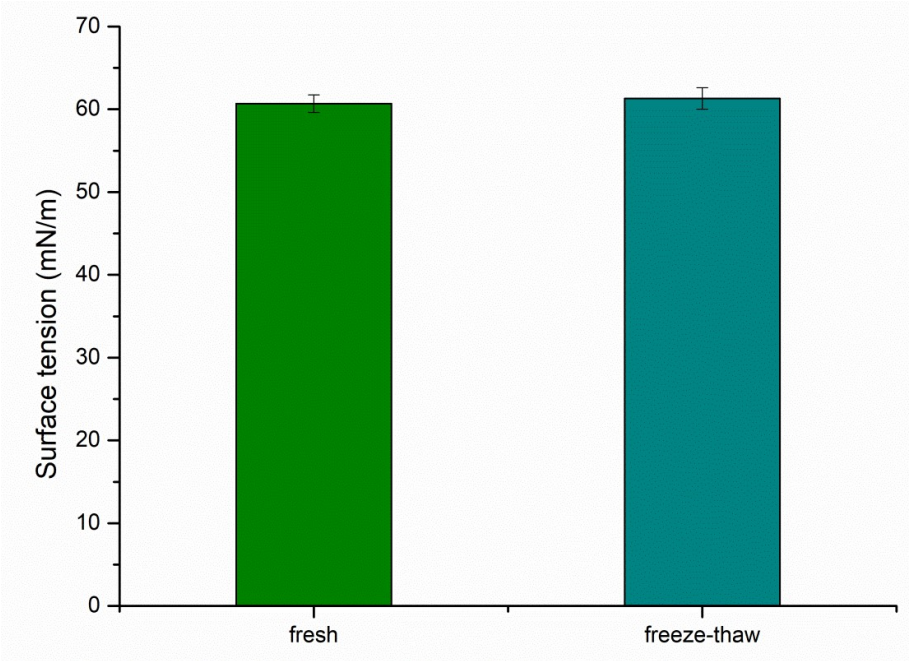

Figure 10. Surface tension of fresh and freeze-thaw vitreous.

6.2 Comparison of different ophthalmic media with different methods

6.2.1 Microscopic investigation

For easy distinction of hydrophilic and lipophilic phases, a fluorescent dye was applied during the microscopic measurements. Green fluorescein sodium can dye the aqueous phases of the emulsions, while silicone oil can be seen in black color in the pictures. A few large and many small black oil droplets can be observed in the green aqueous phase in the microscopic images (Figure 11. A, B and C) when a high amount of aqueous phase (80 %) was applied. At this oil:water ratio an oil-in-water type (o/w) emulsion was formed.

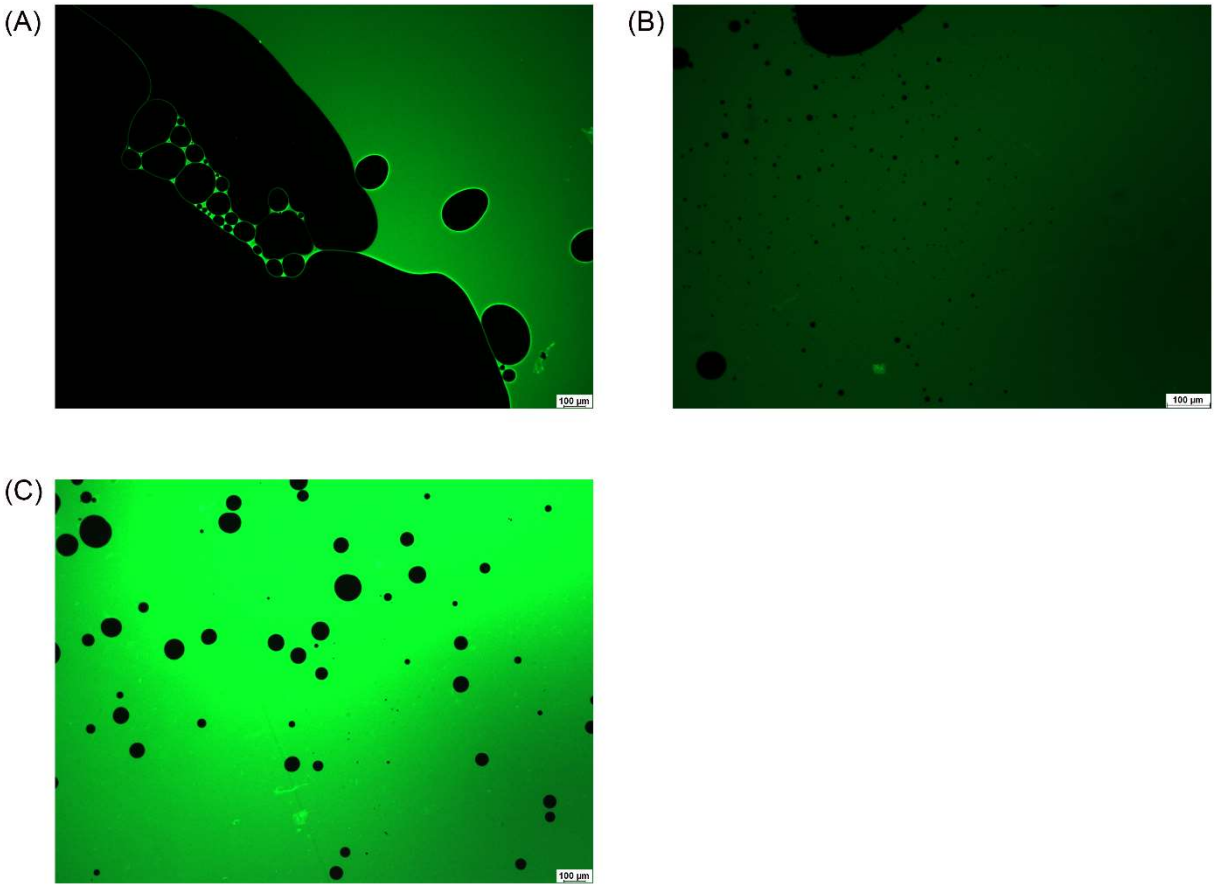

Figure 11. Microscopic pictures of emulsions containing BSS (A), VB (B) or AH (C) at 2:8 oil and aqueous phase ratio.

The sharp contour of the droplets made it possible to analyze droplet size and droplet size distribution using the software of the microscope (Figure 12.). The emulsions containing BSS proved to have greater droplet frequency at bigger droplet size (above 100 μm). The droplet size distributions of the emulsions containing VB or AH were almost similar, but in the case of VB a higher amount of smaller droplets could be observed (more remarkable frequency below 20 μm).

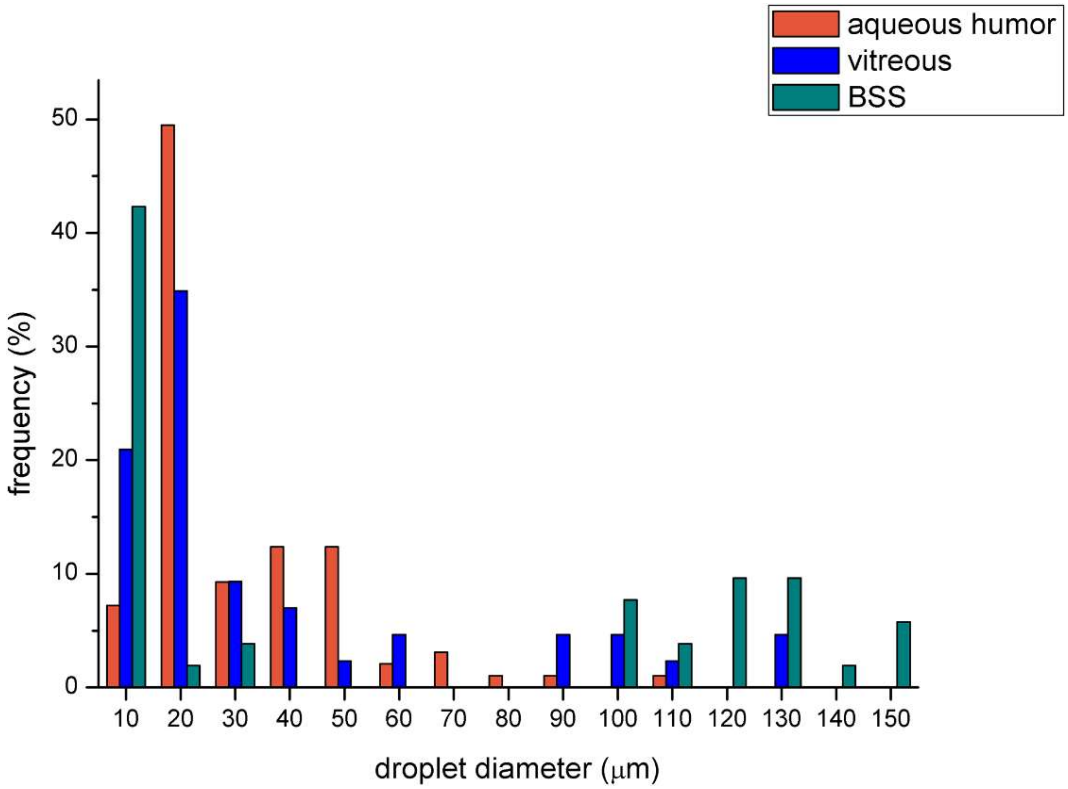

Figure 12. Droplet size distribution of emulsions containing different aqueous media at 2:8 oil and aqueous phase ratio (the number of analyzed droplets for BSS, VB and AH compositions were 52, 43 and 97, respectively).

6.2.2 Droplet size and zeta potential

Zetasizer was used to measure droplet size from nanometer to several micrometers using dynamic light scattering, and zeta potential using electrophoretic light scattering. This type of droplet size measurement can avoid the errors from microscope resolution and from the selection of the photo imaging.

In our work the droplet size and the zeta potentials of emulsions containing 20 % of silicone oil were only measured (Figure 13.), since a larger percent of oil could not be measured by this method.

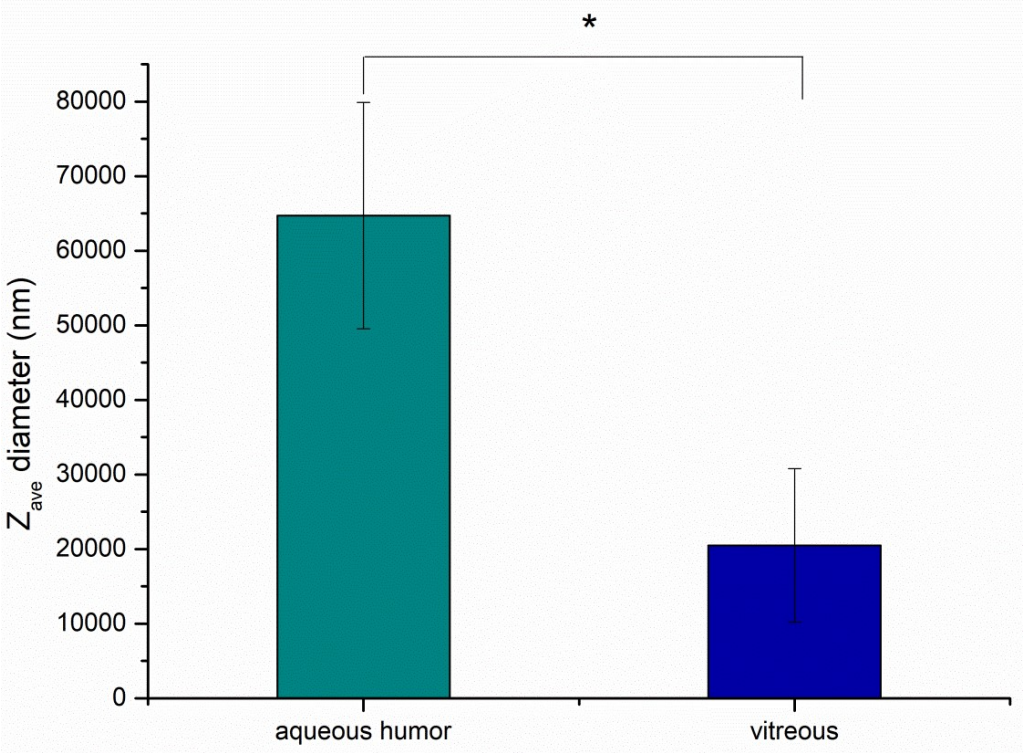

Figure 13. Diameter of the emulsion droplets at 2:8 oil and aqueous phase ratio ($p \leq 0.05$).*

The average droplet size (Zave) of emulsions containing VB was significantly smaller (about 20 μm) ($p = 0.018$), while that of emulsions containing AH was bigger (over 60 μm). This observation correlates with the results of the droplet size distribution presented in the microscopic investigations (Figure 12.).

The absolute zeta potential of the emulsion with VB was remarkably higher than that of AH (Figure 14.). The zeta potential value can indicate the formation of an electric double layer surrounding the oil droplets stabilizing the emulsion droplets. The increase of the absolute values can predict an improvement in the stability of the dispersed system.

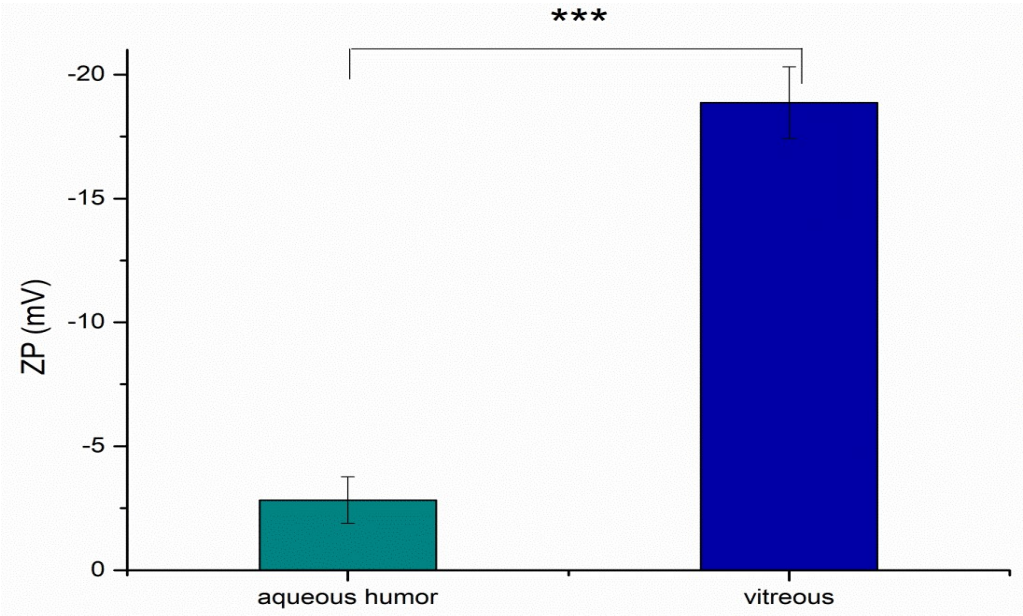

*Figure 14. Zeta potential of the emulsions at 2:8 oil and aqueous phase ratio (*** $p \leq 0.001$).*

6.2.3 Rheology

The viscosity of the original oil, Oxane 1300, was measured to be 1313.3 ± 5.8 mPa·s (mean ± SD). Emulsions were prepared on the basis of the "*In vitro* emulsion" section, where the oil concentration was 20 %, while the aqueous phase (AH or VB) concentration was 80 %. Each emulsion was prepared in triplicate.

In the case of emulsions containing AH, the viscosity values were lower than those of Oxane 1300, and the standard deviation (SD) was very high. Contrarily, the emulsions containing VB revealed elevated viscosity values with moderate standard deviation (Table 2.).

Table 2 Viscosity value of Oxane 1300 and the emulsions in 2:8 oil and aqueous phase ratio.

	Viscosity (mPa·s)				
	Sample 1	Sample 2	Sample 3	Mean	SD
Oxane 1300	1310	1320	1310	1313.3	5.8
Emulsion with AH	1000	702	65	589.0	477.6
Emulsion with VB	1490	1540	1320	1450.0	115.3

6.3 Evaluation of the emulsification effect of the vitreous

6.3.1 Effect of the vitreous concentration

In order to analyze the effect of the vitreous on emulsification, the surface tension of the vitreous and the vitreous diluted with BSS was measured. In composites of the vitreous and BSS, we could observe a remarkable fall in surface tension between 1 % and 10 % vitreous concentrations. Surface tension decreased further when adding more vitreous, but remarkable changes could not be observed between 50 % and 100 % vitreous contents (Figure 15.).

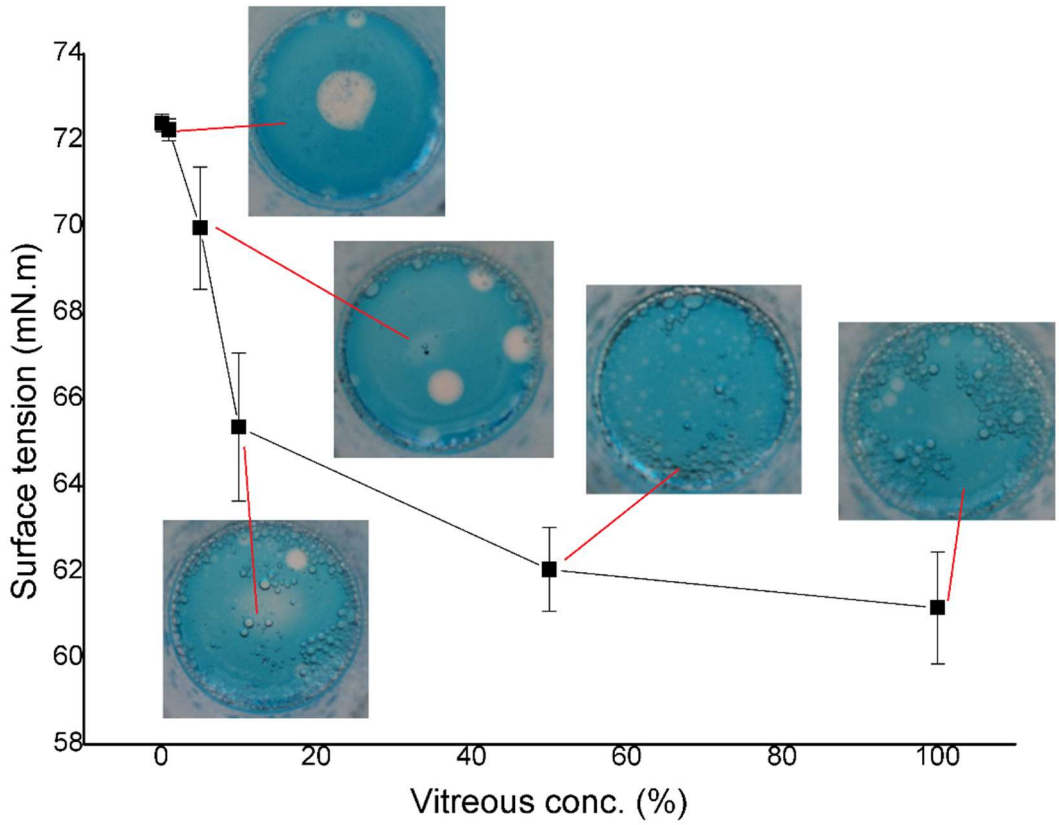

Figure 15. Surface tension of the vitreous-BSS composites with increasing the vitreous concentration, and the picture of the emulsion containing 10 % oil.

The emulsification ability of the diluted vitreous was also investigated. The vitreous and its dilutions were mixed with silicone oil. In the range of 0.01-10 % vitreous concentration in the aqueous phase, a remarkable change can be observed in the emulsification ability. More and more and smaller and smaller emulsified oil droplets can be seen with increasing vitreous concentration in the aqueous phase, while over 10 % vitreous concentration, the number of

emulsified droplets did not increase remarkably, as can be observed in the photos of the emulsion.

As regards the zeta potential of the emulsion containing 10 % silicone oil, a remarkable decrease in zeta potential could be detected with increasing vitreous humor concentration in the hydrophilic phase (Figure 16.). The increased absolute zeta potential value indicates the increased stability of the interfacial layer between the oil and the aqueous phase. Zeta potential measurements were possible only for emulsions with low oil concentrations.

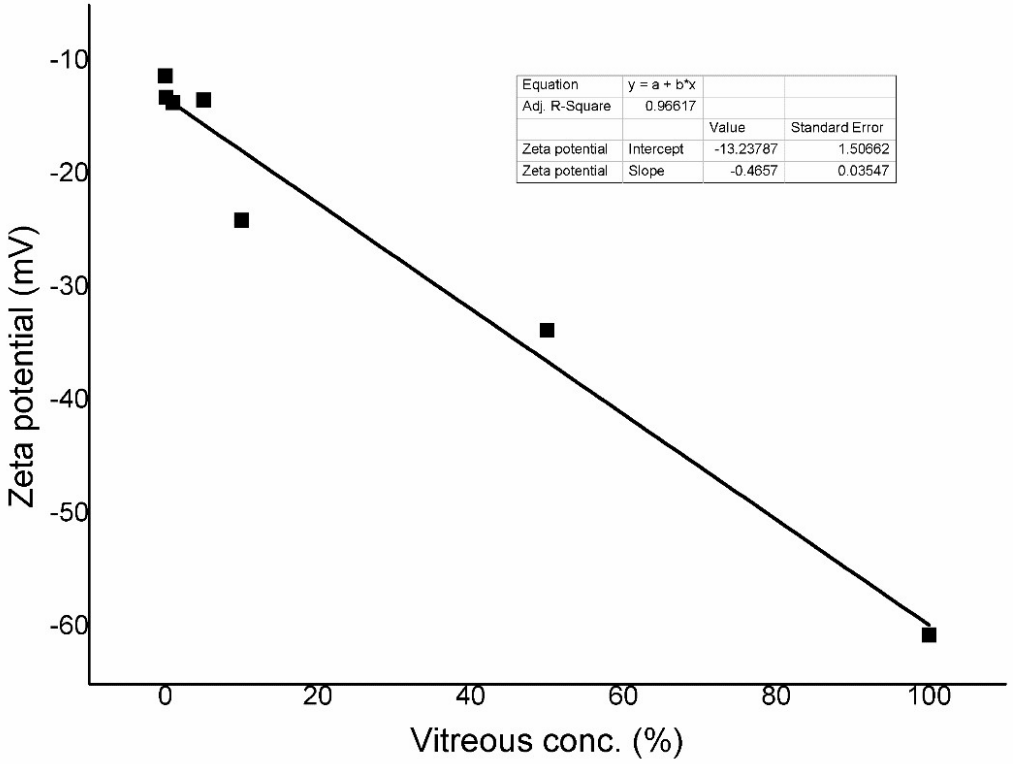

Figure 16. Zeta potential of emulsions containing 10 % silicone oil and 90 % vitreous-BSS composites with increasing the vitreous concentration.

6.3.2 Effect of the oil concentration

Mixtures were prepared from silicone oil and BSS or vitreous in order to demonstrate the formation of emulsions (Figure 17.). The mixtures containing BSS did not show an emulsion structure, the phases remained separated at 10-50 % oil concentrations. At higher oil contents, emulsified BSS droplets could be observed in the continuous oil phase (blue droplets in the transparent oil phase). This phenomenon can be explained by the steric stabilization effect of the viscous oil phase.

Using the vitreous as the aqueous phase, oil-in-water type emulsions were obtained up to 80 % of oil concentration (transparent oil droplets in the blue colored vitreous). At high oil contents, the vitreous formed droplets in the oil phase similarly to the BSS mixture with 90 % silicone oil, but the vitreous presented smaller droplets.

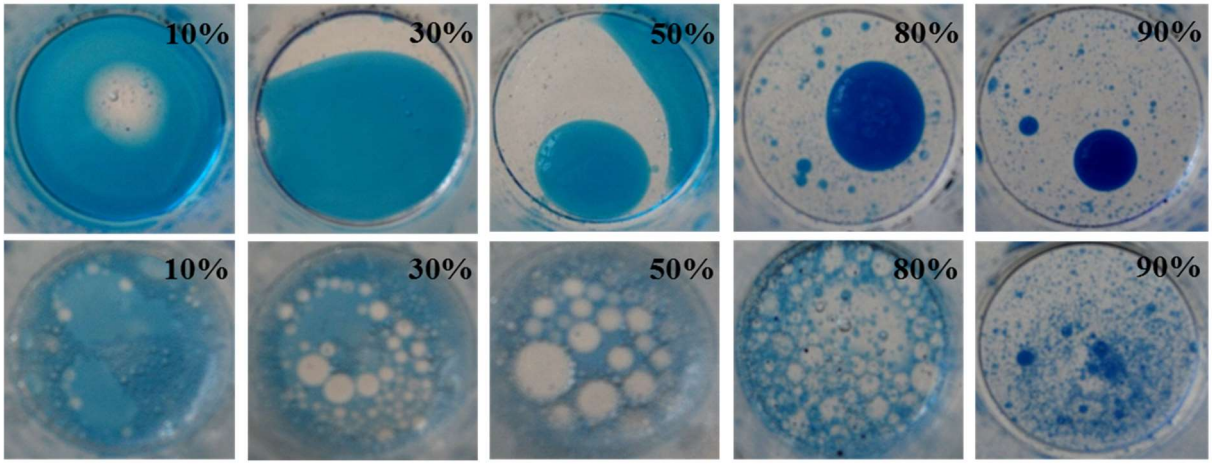

Figure 17. Macroscopic pictures of the formulation prepared from Oxane 1300 (transparent liquid) and from different aqueous media (color blue). In the top right corner of the figures the concentration of Oxane 1300 is shown. In row 1 BSS, and in row 2 vitreous as aqueous media.

Mixing the vitreous with silicone oil resulted in an emulsion system. Figure 18. illustrates the stability of the emulsions formed, each system remained stable one week after the mixing process (Figure 18., row 2) regardless of the oil concentration. The type of the emulsion and the appearance of the droplets (size and number) were the same after one week.

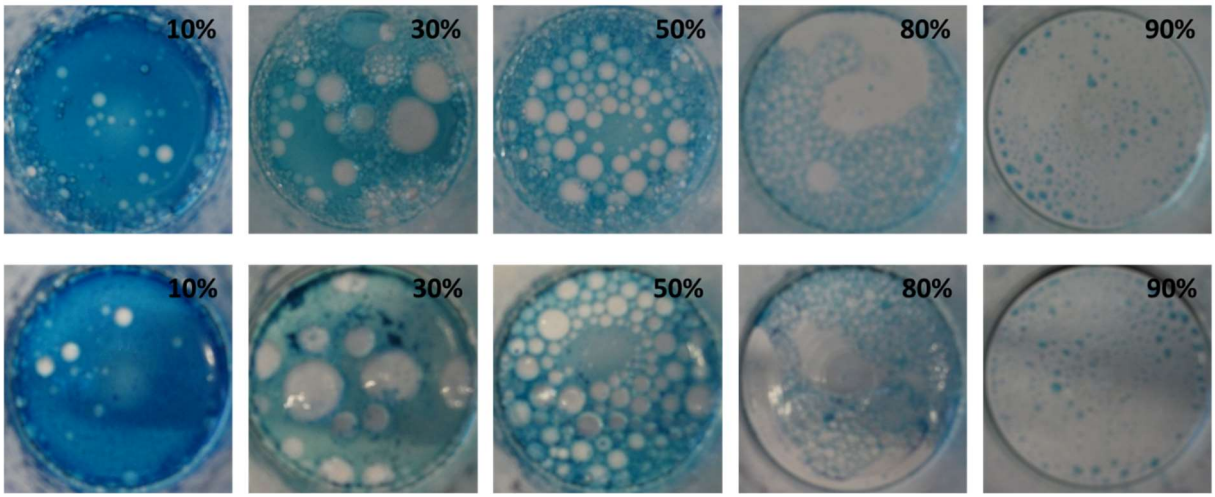

Figure 18. Macroscopic pictures of the formulation prepared from silicone oil (transparent liquid) and vitreous (color blue). In the top right corner of the figures the concentration of silicone oil is shown. In row 1 the state after mixing, and in row 2 the state 1 week after mixing.

7. Discussion

The implanted silicone oil forms a giant continuous oil droplet in the vitreous cavity, which is surrounded by the thin layer of the hydrophilic phase remaining in the eye. This hydrophilic phase can be a leftover vitreous, aqueous humor secreted by the ciliary body, or BSS applied during vitrectomy. Silicone oils produce high interfacial tension opposite to the hydrophilic phases, and this behaviour enables these materials to exert an intraocular endotamponade effect in the vitreous body. Despite the high surface tension, in practice silicone oils undergo emulsification, which can lead to several complications including cataract, keratopathy, glaucoma and silicone retinopathy [58]. Silicone oils have excellent chemical stability, but many studies proved that they are not inert biologically, some impurities such as retinol [68], low-molecular-weight components (LMWC) [9], residual catalysts [69], OH-endgroups [70], and cholesterol [71] were detected in silicone oils, which can probably improve emulsification due to the modification of the surface tension of the oil. The other possible cause of emulsification is the mechanical effect, therefore, some literature analyzed the effect of different agitators and eye movement on emulsification using special eye chambers, which mimic the eye movement *in vitro* [51,72].

A new mechanism of silicone oil emulsification was also proposed, where break-ups of silicone oil led to its adherence to ocular tissue and thus forming emulsified droplets. This mechanism can decrease with the application of high-molecular-weight silicone oils [73,74] and preactivated silicone oils, the latter is able to form a cohesive gel in the vitreous cavity [75].

Impurities and mechanical effects can be largely patient-dependent because these factors can be disease or surgical action related. In our work we focused on different biological and surgical hydrophilic phases which can be present during vitrectomy and can be responsible for the early development of emulsification. These hydrophilic phases can be in contact with silicone oil and are able to form an interface. When the surface tension at the interface is low and a mechanical effect occurs, it promotes the formation of droplets. In our work the surface tensions of the possible hydrophilic phase were investigated, and it was found that the biological media have significantly lower surface tension compared to the non-biological ones (BSS) (about 61 and 54 mN/m compared with 72 mN/m). On the basis of Antonov's rule and its later corrections and modifications [76], these lower surface tension values can mean lower interfacial

tension between silicone oils and biological aqueous media, and the lower interfacial tension can result in increased emulsification.

7.1 Comparison of different ophthalmic media with different methods

In our work we formulated *in vitro* emulsions with silicone oil and the possible hydrophilic phases in order to investigate the structure and stability of the emulsions, and also to compare the emulsification ability of the different hydrophilic phases.

Two types of emulsion can develop after silicone oil implantation, both of them can be observed in clinical practice. In the case of the first emulsion type, the oil droplets get loose from the giant continuous oil droplet and form an oil-in-water emulsion (o/w emulsion). These emulsified oil droplets are able to move easily in the hydrophilic phase, thus they can be present in the aqueous humor, which can be seen in the anterior chamber in the human eye after undergoing vitrectomy with silicone oil endotamponade. On the other hand, the migration of small oil droplets can lead to secondary silicomacrophagocytic open-angle glaucoma [77]. In our study this type of emulsion could be observed when we applied a high amount of biological hydrophilic phase. It had already been established in earlier studies that the components of the biological medium have a strong effect on emulsification. The role of fibrinogen, fibrin, γ-globulin, very low-density lipoprotein, alfa-1-glycoprotein in emulsification had already been clarified *in vitro* [46].

In our work the complex hydrophilic phases were compared concerning the emulsification tendency. The aqueous phases were indicated by fluorescent dye, thus black oil droplets were perceptible in the green aqueous phase on the microscope slide of our *in vitro* emulsions (Figure 11). The number of the oil droplets was higher, and the droplet size was smaller in the case of VB compared with the BSS and AH containing systems. This observation was confirmed by the droplet size and zeta potential measurements. Zeta potential is a sensitive indicator of the stability of disperse systems such as emulsions. Higher absolute Zeta potential means an effective stabilizing layer around the droplets, which hinders the adhesion and the fusion of the droplets. In contrast with microscopic investigations, where the presented and analyzed slides are chosen by the investigator, these types of measurements are more investigator-independent, a bigger amount of the sample can be analyzed using dynamic and electrophoretic light scattering, which results in more representative data from the sample. In

this measurement, similarly to the microscopic investigation, smaller oil droplets were present in the case of VB, furthermore the absolute Zeta potential of the emulsions containing VB was also higher compared with emulsions containing AH (Figure 14.). These statements can mean that the residual vitreous body, which can form an interface with the silicone oil droplet, has more emulsification potential, and it can be the starting point of further emulsification. On the other hand, the formed oil droplets are more stable in the vitreous (indicated by the higher absolute Zeta potential value), predicting that the oil droplet will remain and will not fuse with the continuous oil droplet later.

Earlier studies established the role of the properties of silicone oil and surface-active agents in the biological environment in emulsification. The presence of very small amounts of emulsifier can facilitate the formation of an emulsion. Various biological surface-active agents can accumulate in haemorrhagic and inflammatory situations in the aqueous biological media, such as fibrin, fibrinogen, gamma globulins, acidic alpha 1-glycoprotein, very low-density lipoprotein [16]. In our investigation biological media were collected from healthy animals and were investigated as a complex fluid without specifying of the components, but we can clearly see that the normal biological fluids have a remarkable emulsifying effect. In addition, it is also clear from the results that the residual vitreous can have a much greater effect on subsequent emulsification than aqueous.

As for the second emulsion type, the hydrophilic phase can enter the oil phase resulting in a water-in-oil type emulsion (w/o emulsion). This type of emulsion can be observed in the vitreous cavity, when the opalescence of silicone oil is noticed in clinical practice. In our study this type of emulsion was obtained when lower hydrophilic phase was applied (20 %). The microscopic investigation indicated hydrophilic droplets in the continuous oil phase in all cases, but the biological media showed remarkable emulsification tendency. In order to compare the emulsification ability of the two biological media as a water-in-oil emulsion, rheological investigation was performed, and the viscosity of the systems was evaluated. In our work the viscosities of the w/o emulsions containing biological media were compared with the original oil viscosity data. When a nearly stable w/o emulsion is formed, the emulsified droplets increase the viscosity of the continuous phase, in our case this phase is silicone oil. Considering the viscosity data of the formulated *in vitro* w/o emulsions, mixtures with AH exhibited varying viscosity data indicating an unstable system, while the viscosity of the emulsion containing VB

showed an elevated value, which predicts a real, stable emulsion formation. The latter suggests the VB droplets are more stable in the continuous silicone phase.

7.2 Evaluation of the emulsification effect of the vitreous

In the first part of our *in vitro* study we presented that biological ophthalmic media (aqueous humor and vitreous) had remarkable emulsification abilities. In this section, we prepared *in vitro* emulsions with silicone oil and vitreous in order to investigate the emulsification ability of the vitreous and its dilutions. During vitrectomy, the vitreous is removed from the vitreous cavity and an infusion (BSS) is used to maintain the closed system. The complete removal of the vitreous body is impossible, some residuals may remain. In the lower segment of the vitreous cavity, silicone oil almost never fills the space completely, so in a standing position the aqueous phase (possibly residual vitreous body, infiltrate) meets the oil phase here.

We demonstrated the emulsification and emulsion stabilization ability of the vitreous in an intact and in diluted state. After the insertion of silicone oil into the vitreous cavity, an interfacial layer evolves between the inserted oil and the hydrophilic aqueous phases of the eye (such as aqueous and/or residual vitreous). Due to mechanical energy, droplets can pass from one phase into the other. Besides mechanical energy, low interfacial tension can also promote this process. If interfacial tension is low, emulsification occurs more easily, smaller mechanical energy is needed for the formation of droplets. We measured the surface tension of the vitreous and its dilutions. Dilution was found to increase the surface tension and thus the interfacial tension at the oil-vitreous type interface, but the values of the intact (100 %) vitreous and the 50 % diluted vitreous are very close to each other, while a remarkable increase in the surface tension occurred just after 10-fold dilution (less than 10 % vitreous). This finding predicts that dilution up to 10 times does not change surface tension remarkably, thus from this point of view the emulsification ability of the vitreous can be retained even after 10-fold dilutions.

This emulsification ability in the diluted state was confirmed with macroscopic investigations. When increasing the vitreous content, more and more droplets form in the aqueous phase up to 10 % vitreous content, which is in correlation with the increase of viscosity in this range. Over this concentration, the number and size of the droplets are more similar (Figure 17.).

It could be clearly seen that the vitreous and its dilutions can easily form emulsions with silicone oil, but the additional emerging question is the stability of the emulsion system formed. If they can be considered as stable emulsions, the phases will not separate, thus the original state will not be restored and the emulsified state will remain. The stability of the emulsions formed from the vitreous or its dilutions was demonstrated with zeta potential measurements. Zeta potential gives important information on the long-term stability of emulsion droplets and their tendency to agglomerate and coagulate. High negative or positive potential results in the repulsion of droplets, decreasing the tendency of droplets to aggregate or coagulate. A higher absolute zeta potential value indicates higher stability for the dispersed (emulsion) system, thus the phases will not separate and return to the original state. In our measurements, the increase in the vitreous concentration increased the absolute value of zeta potential, and a linear relationship was found between the two factors. This finding means the presence of the vitreous increases the stability of the emulsion. In general, in the case of electrostatically stabilized nanodispersions, a zeta potential of $\pm$ 30 mV and $\pm$ 60 mV indicates good and excellent stability, respectively. Minimum $\pm$ 20 mV is required for steric and electrostatically stabilized systems. Our vitreous containing emulsions showed very high absolute zeta potential values, the sample which contained 10 % vitreous in the aqueous phase demonstrated a value of -25 mV, which can already provide good stability for the dispersed droplets, and at higher vitreous concentrations this value decreased continuously, indicating a higher absolute value and higher stability. This observation may mean that at 10-fold dilution the vitreous can emulsify silicone oil and the system remains stable.

In our work, the emulsification ability of the vitreous was demonstrated at different silicone oil contents as well, in these experiments the hydrophilic phase was 100 % vitreous or 100 % BSS. The zeta potential measurements could not be performed over 20 % oil concentration, this was the upper limit of the measurements, and therefore, we used macroscopic observations.

The systems which contained BSS did not show the formation of oil-in-water type emulsions, while the vitreous containing mixtures presented an emulsion structure at each oil-water ratio (Figure 17., row 1). In the case of the vitreous, oil-in-water type emulsions could be observed up to 80 % oil concentration (Figure 17., row 2), while at a high oil content (90 %) the water-in-oil type form could be seen. This phenomenon could also be detected in BSS

containing systems over 80 % oil concentration. The similar behaviour at a higher oil content is explained by the higher viscosity of oil compared to the aqueous phase, where mechanical energy resulted in droplet formation, and the higher viscosity of oil prevents the coagulation of droplets. The stability of the emulsions formed was also evaluated, the systems were stored for 1 week, and checked again (Figure 18.). It could be clearly seen that the emulsion structure was retained in each case, the droplet size and number did not change. This finding is in correlation with the zeta potential measurements, where we found that the electrokinetic potential around the droplet can ensure the long-term stability of the systems.

8. Conclusion

The aim of my Ph.D. work was to investigate the emulsification process of silicone oil, used as endotamponade, in the presence of hydrophilic phases obtained from porcine eyes and BSS. Our new findings are as follows.

- The vitreous increases the risk of the emulsification of silicone oil.
- The vitreous has remarkable emulsification potential compared with aqueous humor and BSS.
- The vitreous retains its emulsification ability even in 10-fold dilution.
- The vitreous-silicone oil *in vitro* emulsions are very stable, which means that phases of the already formed emulsion will not separate over time. The emulsified silicone oil does not return to its original spherical form.
- Both emulsion types (w/o and o/w) can be developed depending on the silicone oil concentration.

The results of our *in-vitro* studies raise the possibility that the remaining vitreous after vitrectomy may have an emulsifying effect. In case of incomplete oil filling, the oil-water interface increases, which can further enhance the emulsification process.

9. References

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ANNEX I.