JACOBS UNIVERSITY

New dyes for ocular surgery
Synthesis and preclinical testing

by

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To my parents, Teresa and Jerzy

Summary:

In cases of vitreoretinal interface diseases, such as macular hole or macular pucker, the inner limiting membrane (ILM) or epiretinal membranes (ERM) need to be surgically removed. The challenging step in the surgery is the transparency of ILM and ERM that makes the membranes difficult to identify and to peel without risk of damaging the retina. Only a few vital dyes have been proposed to stain the ILM and ERM and no perfect dye is available that would stain the tissue of interest sufficiently, while being non-toxic and stable in aqueous solution.

During cataract surgery a dye to stain outer part of a capsule of the lens is used when the red reflex from the fundus is absent. Dyes used for capsulorhexis need to be safe for corneal endothelial cells.

Phototoxicity needs to be tested for dyes intended to be used in vitreoretinal surgery. A very strong source of light is present during the surgery, that can cause the dyes to become toxic while illuminated. A set-up for testing phototoxicity on a 96-well plate has been developed.

New models for staining ability of dyes for ILM and ERM are suggested, as porcine eye was proved not reliable for those studies. Instead, a model for ILM and ERM was developed with good correlation to staining of human ILM and human ERM. Pig eyes can, however, serve as a useful model for capsule staining when cataract is induced in them.

For proper dye formulation viscosity, density, and osmolarity of the solution need to be considered. In order for the dye to reach the membrane in a buffer-filled eye it needs to sink to its bottom. Different agents were investigated to test their usefulness in solution preparations and safety of their use.

A new dye for capsulorhexis is presented. No safe, green dye that would stain the capsule had been suggested before. The new dye is an alternative to the currently used Trypan Blue and provides as strong staining while being slightly less toxic to corneal endothelial cells.

Fourteen new dyes, that belong to the cyanine family, were synthesized and investigated for staining ability, toxicity, and stability in aqueous solution. Two of the new substances were found to stain the model for ILM in green, while being safe to retinal epithelial cells. Their stability in aqueous solution can be achieved with additives.

List of Papers

This thesis is based on the following papers:

I. Awad D, **Wilińska J**, Gousia D, Shi X, Eddous J, Müller A, Wagner V, Hillner C, Brannath W, Mohr A, Gabel D (2018) Toxicity and phototoxicity in human ARPE-19 retinal pigment epithelium cells of dyes commonly used in retinal surgery. *Eur J Ophthalmol.* 28(4):433–440. ***first joint author**

II. **Wilińska J**, Stefanoska D, Apostu M, Apostu E, Stanciu A, Begaj B, Mohr A, Gabel D (2018) Models for testing staining for the Internal Limiting Membrane ILM and Epiretinal Membrane ERM in vitreoretinal surgery. *Manuscript.*

III. **Wilińska J**, Awad D, Gousia D, Stefanoska D, Apostu M, Apostu E, Bhardwaj N, Mohr A, Gabel D (2018) Additives for improving staining in retinal surgery. *Manuscript*.

IV. **Wilińska J**, Mocanu B, Awad D, Gousia D, Hillner C, Brannath W, Mohr A, Gabel D (2018) New stains for anterior capsule surgery. *Manuscript accepted for publication in Journal of Cataract and Refractive Surgery.*

V. **Wilińska J**, Apostu M, Apostu E, Griffith A, Mohr A, Gabel D (2018) New cyanine dyes as vital stains in vitreoretinal surgery. *Manuscript.*

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Abbreviations

AV	Acid violet 17
ARPE	Human retinal pigment epithelial cells
BB	Bromophenol blue
BBG	Brilliant blue G
BIP	2-[5-[3,3-dimethyl-1-(4-sulfobutyl)-1,3-dihydro-indol-2-ylidene]-penta-1,3-dienyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium sodium
CCC	Collagen Cell Carrier membrane
DG	Diglycerol
DME	Diabetic macular edema
DMI	Dimethyl isosorbide
ERM	Epiretinal membrane
Gdp	Gadopentetate
HCEC	Human corneal endothelial cells
HeLa	Human adenocarcinoma
ICG	Indocyanine green
ILM	Inner limiting membrane
ISO	Isosorbide
MH	Macular hole
MP	Macular pucker
OP	Optiprep, iodixanol
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PVDF	Polyvinylidene difluoride
TB	Trypan blue
WST-1	Cell proliferation reagent tetrazolium salt WST-1

Introduction

Ocular surgeries

Ocular surgery is a surgery performed on the eye, eyebrow, eyelids or lacrimal apparatus. The first mention of ophthalmic surgery was contained in the Hammurabi's Code of Law (1800 BC).[1] The law described that any doctor who would restore or improve someone's eyesight would receive payment, while in the event of a failure like causing blindness or death his hands would be cut off. There are many types of ocular surgeries including refractive surgery (using conventional tools i.e. keratomileusis, or laser i.e. in-situ keratomileusis), corneal surgery (i.e. corneal transplant), vitreoretinal surgery (i.e. vitrectomy or macular hole (MH) repair), eye muscle surgery.

Cataract surgery

Treatment of cataract was first mentioned 500 years BC in Indian Sanskrit manuscript.[2] It describes treatment of cataract by removing the lens either with a needle or by hard blow to the eye. Cataract surgery is now the most frequently performed procedure not only in the group of ocular surgeries, but all surgeries. In 2010 in United States more than 24.4 million Americans had cataract surgery. Approximately 50% of Americans had it by the age of 75.[3] In the same year in Germany 1027.7 cataract surgeries per 100 000 inhabitants were performed.[4] Cataract is mainly caused by aging but can also be induced by diabetes or head trauma. It forms when lens proteins clump together and the passage of light through the lens becomes disturbed. It results in distorted, blurry vision. Additionally, light transmission is also hindered by the accumulation of pigment associated with the aging process.[5] Surgical removal of the cataract is the only effective treatment. The opaque lens is removed in a process of phacoemulsification and subsequently replaced with an artificial one. To remove the lens, first the outer part of the capsule needs to be opened by a technique called capsulorhexis. In the absence of red fundus reflex due to the opacity of the vitreous body or the lens, the capsule needs to be stained with a dye to facilitate its visualization.

ILM and ERM peeling - Vitreoretinal surgery

Vitreoretinal surgery, as the name implies, takes place in the inside of the eye, where vitreous and retina are found. For surgery, the eye surgeon places 3 ports in the pars plana of the eye, which serve as the openings for light pipe, vitrector (to cut and remove the vitreous), and cannula, which enables liquid exchange and maintaining the right pressure in the eye throughout the surgery.

In cases of macular hole (MH), macular pucker (MP), and diabetic macular edema (DME), vitreoretinal surgery is performed in order to peel the inner limiting membrane (ILM). Removing of the ILM successfully closes the macular holes in 95% of the cases.[6,7] The ILM is a membrane of a few micrometers thickness found on the retina-vitreous connecting surface. The ILM is also frequently removed when the epiretinal membrane (ERM) is peeled, as it has been reported to improve surgery outcome. [8,9] The ERM is an idiopathic fibrocellular membrane that grows on the retina. Its origin is unknown, but it has been reported to be age-dependent and related to posterior vitreous detachment.[10] In case of microscopic ERM residue staying on ILM, the latter can serve as a scaffold for ERM re-growth and increases the risk of ERM recurrence. Shimada et

al. report that ERM re-grew in 40% of cases where the ILM was not peeled while the ERM was removed.[8] No recurrence was observed in the eyes that underwent double peeling.[8,9] This surgical procedure is challenging even for experienced surgeons due to transparency and thinness of ERM and ILM. Staining the tissue of interest was suggested to make the peeling easier, to ensure complete membrane removal, and to decrease the risk of damaging the retina during the procedure. Few dyes have been proposed so far for chromovitrectomy including: trypan blue (TB), brilliant blue G (BBG)[8], bromophenol blue (BB), indocyanine green (ICG) [9], and acid violet 17 (AV)[11]. However, still not a perfect dye was found that would stain the desirable structures, while being non-toxic and form a stable solution.

Preclinical investigation of dyes

Along with research for new compounds that could serve as potential dyes for chromovitrectomy, there are ongoing efforts made towards improvement of testing procedure. Preclinical investigation of new substances should include their staining ability, toxicity, and stability in aqueous solutions.

Toxicity

Toxicity *in vitro* on human retinal pigment epithelium cell line (ARPE) had commonly been investigated, however usually without photo-toxicity being examined. Only few studies examined consequences of illumination. The effects of light exposure were tested mainly for TB[12-15], ICG[13,15-18], BBG[12,13,18,19], and BB[12,13,19]. During the surgery there is a strong source of light that the dye and the tissue is exposed to. We found out that exposure to dye and light simultaneously can have a crucial influence on ARPE cells survival. We developed a protocol for cyto- and phototoxicity tests, including survival investigation under four different conditions, with and without light exposure mimicking surgery conditions, at two time points, right after exposure to the dye and with a further 24 hours delay. Our findings have been published in a manuscript [20] and are presented in Paper I. It is important to note, that AV-17, that had been commercially available as medical device for vitreoretinal surgery, caused sight defects in patients that underwent surgery in which this dye was used. [21] We report in our study, that AV-17 is highly phototoxic, and we stress that effects of light illumination on cells exposed to dyes are crucial and need to be examined before patient application.

In vivo studies for substances with a potential use in eye diseases had been conducted on animal models like rodents, rabbits, cats, dogs, and pigs, but all of those models have their limitations. Rats and mice do not have a macula, but they serve well for investigating mechanisms of ophthalmological diseases, due to analogical retinal vasculature and layers in comparison to humans. Rabbits are used for studies of substances injected intravitreally, although they have smaller vitreous volume, do not have a macula (just as rodents) and their retina has fewer blood vessels. Anatomically, the most similar to the human eye is the pig eye. It is complementary not only in terms of size, but also thickness of retinal layers, and photoreceptors. Porcine eyes are much easier accessible than eyes from non-human primates, and only these would be the ones more alike. [22]

Staining

The next step in our research was screening commercially available dyes, to test their staining potential in vitreoretinal surgeries. There are not many models available for staining, the most common being enucleated porcine eyes. BIP (2-[5-[3,3-dimethyl-1-(4-sulfobutyl)-1,3-dihydro-indol-2-ylidene]-penta-1,3-dienyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium sodium) stained the (what we assumed was the) porcine ILM very strongly in green under laboratory conditions, but it did not stain the human ILM during the surgery. Consequently, a demand for developing new, more reliable staining model has arisen. We proposed models consisting of coats of proteins that are found in the membranes, as well as non-biological material that shows strong correlation with staining of the human ILM. Our findings are described in Paper II.

It is difficult to screen for new dyes without a good staining model to determine which of dyes have a potential to stain tissue of interest sufficiently. We experienced that even an animal model of an eye that is very much alike human eye can fail. It is difficult to mechanically remove the vitreous completely from ILM surface in enucleated pig eyes. We did not observe staining with BBG in laboratory conditions, although we know that BBG stains the ILM in humans. This might be to vitreous remnants on the ILM making it impossible for the dye to reach it. Enzymatic disruption of the vitreous was suggested,[23] but it is quite expensive and hard to optimize.

Tissue from a patient is not an easily accessible material. The number of donor eyes donated for research decreases.[24] ILM removed from a patient during a surgery without dye application can be stained *ex vivo*,[25] but it is unknown which surface, inner vitreal or outer retinal, comes in contact with a dye during an experiment.[26]

Sinking/additives

In order for the dye to reach the ILM and ERM, if present, it needs to be heavier than the buffer the eye is filled with. Commercially available dye for ILM and ERM simultaneous staining (Membrane-Blue Dual from DORC International, Zuidland, the Netherlands) contains 4% of polyethylene glycol (PEG).[27] Other additives suggested were heavy water (D_2O), glucose, mannitol, and polyvinylpyrrolidone. [28] Additives can prevent dye dispersion and facilitate its sinking to the bottom of the buffer-filled globe. Additionally, they allow for viscosity, density and osmolarity adjustment. Any additive considered for dye formulation needs to be tested for toxicity and reported safe.

Dyes

In order for an organic compound to be colored several requirements have to be met: the molecule, which has at least one chromophore with a conjugated bond system, needs to absorb light in the visible spectrum (400-700 nm). Staining of the substrate depends on dye-substrate affinity. Dyes used to stain living cells or tissues are called vital dyes and can be arranged into groups depending on their chemical structure. Some of the dyes already used in chromovitrectomy are: azo dyes, arylmethane dyes, cyanine dyes, xanthene dyes, colored corticosteroids. [29]

Dyes suggested so far

Azo dyes

Trypan Blue

Trypan Blue was reported to weakly stain the ILM, but it stains ERM strongly in blue. This prominent staining occurs due to high affinity of this dye to collagen I and dead glial cells contained in the ERM. TB is widely used for capsulorhexis during cataract surgery and is commercially available in Vision Blue (DORC International, Zuidland, Netherlands) and in Treq-Blue (Vitreq, Vierpolders, Netherlands) at 0.06% concentration, as well as in MembraneBlue-Dual® (DORC International, Zuidland, Netherlands) at 0.15% concentration along with 0.025% BBG for staining of ERM and ILM simultaneously. TB was reported to be safe to use at low concentrations but shows moderate toxicity at higher concentrations.

Cyanine dyes

Indocyanine green

Indocyanine green is primarily used for choroidal angiography and its use in vitreoretinal surgery is off-label. ICG is an amphiphilic dye that binds to cellular and acellular elements in tissues and stains the ILM in green. ICG exhibits high affinity to collagen IV, laminin and fibronectin [29-31] that are all contained in the ILM. ICG has been reported to have toxic effects by some of the researchers, while being advised safe by others (more in SI of Paper I). The mechanism of any damage that ICG causes is unknown but damage to the retina, changes in RPE cells, and visual field defects were reported.[32] The observed toxicity might be linked to the osmolarity of the ICG solution that needs to be adjusted, for example by glucose or mannitol addition. ICG is soluble in water, but not in PBS. It is not stable in aqueous solution. Due to this problem its solution must be prepared freshly in the clinic before every surgery. A stable, ready-to-use, dye would be preferred.

Triarylmethane dyes

Brilliant Blue G

BBG is known as Coomassie Blue stain for proteins in SDS-PAGE gel electrophoresis. It was suggested as a good alternative to ICG for ILM staining. Staining in patients is sometimes reported to be patchy. BBG is considered safe in concentrations sufficient for reasonable staining. It is available at 0.025% concentration along with 0.15% TB in MembraneBlue-Dual® and as alone dye in ILM-BLUE® (DORC International, Zuidland, Netherlands).

Bromophenol Blue

Bromophenol Blue was suggested for ILM and ERM staining alone and in combination with BBG. We observed nice staining in blue in porcine eyes. Staining of the models for ILM and ERM was not so good. We would not recommend this dye for clinical use, as our phototoxicity studies revealed delayed cell death after exposure to this dye and light. [20]

Acid Violet-17

This dye, similar in structure to BBG, and reported to be safe at concentrations below 0.125 mg /ml,[33] was commercially available at 1.5 mg/ml contained in ala@purple (no longer available). Gerding[21] reports that this dye, which had received CE certification, was tested and found to be safe in much lower concentrations that how it was commercialized. Gerding's studies revealed toxic effect on ARPE and ganglion cells viability, along to Müller cell activation caused by AV.

Steel at al.[34] report no bad effects were observed during clinical studies – but the dye was not applied as it is described in the label. Gerding reports transient discoloration of hydrophilic intraocular lenses and degeneration of the RPE in cases of macular holes. In none of the reported test settings, the dye had a composition that was put to the market. Tests conducted on superfused retina were carried out in 30°C, without obvious explanation, tests carried out on rabbit eyes included use of much lower concentrated dye compared to that in the commercially available product. The terrifying effects of using this dye revealed in clinical use should be a warning and motivation for a better organized and systematic study in the process of developing new medical products.

Aim

The aim of this thesis was to develop an investigation method for new potential dyes for vitreoretinal surgery, and in the end to propose new dyes for staining of internal limiting membrane and epiretinal membranes.

More specifically:

- To develop a method for toxicity and phototoxicity investigation, where light to which tissues and dyes are exposed during a surgery could be mimicked.

- To develop new models for testing stains for ILM and ERM.

- To investigate additives for increasing stability of dyes in aqueous solution, and for improving staining.

- To design, synthesize and suggest new compounds to be used for ILM and ERM staining, that:
  i. Would stain the tissue of interest specifically and sufficiently
  ii. Would be non-toxic and non-phototoxic to surrounding cells
  iii. Would be stable in aqueous solution

The present study

Methods

Toxicity and phototoxicity measurements

Human corneal endothelial cells (HCEC), human retinal pigment epithelium cells (ARPE), and human adenocarcinoma (HeLa) cells were the cell lines used in the presented study.

Cells (10000 per well) were seeded in 96-well plates 24 hours before the experiment. Before the incubation with dyes the medium was removed, and the cells were washed with 100 µl of phosphate buffer saline (PBS). Cells were incubated with 50 µl of tested dye for 30 min at 37°C. After the incubation, cells were washed 3 times with 200 µl PBS each. For phototoxicity studies, after incubation with dyes, cells in 100 µl PBS were exposed to a strong source of light for 15 minutes by being placed above the floodlight. Cells that were not incubated with the dyes, but with PBS, were used as a control. Viability was assayed either immediately after dye/light incubation or with a 24 hours delay (cells were further left to grow in fresh medium for 24 h).

To determine cell survival, the WST-1 assay was used. Cells were incubated with 10 µl/well of WST-1 diluted up to 100 µl with fresh medium. After 3 hours, the absorbance was measured in a plate reader at 450 nm and its intensity was taken as proportional to quantity of alive cells, cells incubated with PBS being 100% living.

Staining investigation

Porcine eyes

Initially, enucleated porcine eyes were used as model for ILM staining. Eyes obtained in the slaughterhouse were put on ice and used no later than 6 hours after enucleation. Residual eye muscles were removed with scissors and the eyeball was placed in a 3-cm Büchner funnel filled with small glass beads to 2/3 of the height. On the glass beads household wrap was put and few holes were made in it. The eye was kept in the funnel with dynamic vacuum applied to the stem of the funnel. The eyeball was cut slightly above the equator with a scalpel and then the upper half of the sclera was separated with scissors. Vitreous was removed by eye inversion and the fundus was washed three times with PBS. A glass tube (ø 10 mm), open on both sides, was placed on the retina over the optic nerve. 200 µl of the dye solution was applied in the tube, while the surrounding eye cup was filled with 1 ml PBS. After 30 s incubation with a dye, the tube was lifted, resulting in immediate dye dilution. The eye cup was subsequently washed 3 times with PBS or until the dye was completely washed away. Part of the retina including dyed and unstained area was cut with a 10 mm biopsy punch and moved to a coverslip for photography.

PVDF filter and CCC membrane staining

New models for ILM and ERM are suggested in this thesis, For ILM, a 96-well MSBVN1210 MultiScreen BV Filter Plate (Merck-Millipore, Darmstadt, Germany) can serve as a model, and for ERM, Collagen Cell Carrier (CCC) membrane (Viscofan, Weinheim, Germany) might be used. Punches of 4 mm diameter were cut out of the CCC membrane and placed on the filter with a hypodermic needle. Membranes and filters

were soaked in 100 µl PBS for 30 min. Afterwards, PBS was removed by vacuum filtration and membranes and filters were incubated with 50 µl of dyes for 30 seconds. Dyes were aspirated by vacuum filtration and wells were washed three times with 100 µl of PBS. Membranes were transferred to 96-well flat bottom plates and 20 µl of PBS was added to each well to keep the membranes moist. Pictures of filters and membranes were taken.

Protein coated plates

Clear flat-bottom 96-well plate was coated overnight with following proteins and their combinations: collagen I (3.2 µg/well in 0.25% acetic acid), collagen IV (3.2 µg/well in 0.25% acetic acid), fibronectin (1.6 µg/well), and laminin (0.64 µg/well). Protein solutions were removed, and coats were incubated with 50 µl of dye per well for 30 s. Subsequently dyes were removed, and protein coats were washed 3 times with 100 µl of PBS. Absorbance spectrum of protein coats was recorded with a plate reader.

Stability investigation

Solutions of dyes, with or without additives, were prepared and sterilized either by autoclaving or syringe filter (0.2 µm pores ø) filtration and kept in 4°C or RT. On the first day solutions were further diluted to final absorbance <1 and absorbance scans were taken. After various time points another part of solution was diluted the same way and absorbance spectra were measured and then the maximum absorbance was compared to that recorded on the day of sample preparation. Solutions were centrifuged after different time points and checked for precipitates.

Lens capsule staining

To check the staining ability of the dye to stain the lens capsule, porcine eyes were used. Cataract was induced by heating the eye in the microwave for 8 seconds at 800 W preceded by dipping the cornea in glycerol. After cataract induction the eye was placed in a Büchner funnel holder mentioned before, and kept in place by dynamic vacuum. The cornea was removed with a 10 mm diameter biopsy punch and separated completely with scissors. The anterior chamber was washed with PBS and 50 µl of dye was applied. The dye was washed away after 10 s. A cut with a sharp needle was made in order to see the contrast between the capsule and lens color. Staining was recorded photographically.

Results

Toxicity and phototoxicity (Paper I)

In paper I, we put emphasis on the importance of toxicity measurements not only in the ambient light, but also after light illumination that would mimic the strong source of light placed in the eye during vitreoretinal surgery. Our study revealed a potential problem that was overlooked when developing a new dye (acid violet) for ILM staining and that caused several defects in patient that underwent surgery with this dye. Viability of cells after incubation with AV dropped from 79% to 23% already immediately after exposure when exposed to dye and light. Cells exposed to dye and light would not recover after further 24 hours incubation with fresh medium (survival=9%). We also reported that toxicity should be investigated at more than one time point. In our case we assayed the cells for survival immediately after exposure and after 24 hours incubation with fresh medium. One of the dyes tested, bromophenol blue, could still be regarded as considerably safe to use when investigated immediately after dye, or dye and light exposure (survival= 68% in ambient light, 65% when exposed to strong light), but more cells were dying in the next 24 hours after they were exposed to dye and light (survival=23%). In this paper we present results that confirm how important well-planned toxicity investigations are. We strongly recommend complex toxicity and phototoxicity assessment of dyes *in vitro* before clinical use.

Staining models (Paper II)

In paper II we suggest two new models for investigating the staining ability of new potential dyes for ILM and ERM. During our study we initially used enucleated porcine eyes obtained from the slaughterhouse. When screening commercially available dyes, one of the dyes, BIP, seemed to strongly stain what we thought was ILM in the porcine eye, but it did not stain human ILM when applied to patients. Further tests revealed that what was stained by BIP in the pig eye was the remnant of the vitreous on the ILM. As porcine eyes are obtained from young animals, attachment of the vitreous is still very strong in those eyes and the vitreous cannot be completely removed mechanically. Enzymatic disruption of the vitreous would be needed to ensure complete removal of any residue from the ILM. Therefore, we investigated if there is any other model or non-biological material that could serve well as a model for stains. We suggest protein coats (including collagen I, collagen IV, laminin, and fibronectin) on 96-well plates that after incubation with a dye can be subjected to absorbance measurement. We also report on two materials (PVDF filter and CCC membrane) that show strong correlation with staining observed for ILM and ERM respectively in human.

Improving staining with excipients (Paper III)

When dye is applied in the buffer filled eye during vitreoretinal surgery it needs to sink to the bottom to get in contact with ILM and ERM. Some agents i.e. polymers can be used to increase the density and viscosity of dye solutions and thus facilitate sinking. We have found that some of the tested substances can prevent the staining of porcine ILM or models for ILM and ERM described in Paper II. In order to find suitable agent for adjusting density and viscosity without preventing staining, the following substances were studied: polyethylene glycol (PEG), isosorbide, dimethyl isosorbide, diglycerol, Kolliphors (RH40, HS15, EL, P188), Kollidon VA, Gadopentetate, Gadoterate,

and Gadovist, and iodixanol. Gadopentetate increases only the density without increasing the viscosity, which is in contrast to Kollidon and Kolliphors tested. These increase greatly the viscosity with very little density increase. For iodixanol viscosity and density increase concurrently. Differences reported indicate the possibility to design a combination of agents for desired properties. We found out that PEG prevents the staining of ERM model with TB and that weakening of the staining is concentration dependent. PEG did not prevent the staining of ILM model with TB+BBG. Kolliphors and Kollidon excipients prevent staining of the models completely (ERM model for TB and ILM model for ICG). We also report on toxicity of additives considered for improvement of dye solutions. Isosorbide and dimethyl isosorbide turned out to be toxic to ARPE cells and could not be used for ophthalmic solutions. Mannitol and diglycerol did not lead to reduction in cell survival. We suggest diglycerol as a helpful additive considering its lack of toxicity, lack of staining reduction, and ability to adjust density and viscosity of the formulation depending on its concentration.

New dye for cataract surgery (Paper IV)

In paper IV we investigated if there are any new dyes or dye combinations that could stain the capsule of the lens that is removed during cataract surgery. We explored if any of the dyes could give stronger staining than TB and/or demonstrate lower toxicity towards corneal endothelial cells compared to this presently available dye. A cyanine dye, BIP (2-[5-[3,3-dimethyl-1-(4-sulfobutyl)-1,3-dihydro-indol-2-ylidene]-penta-1,3-dienyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium sodium) that we had looked into before for its ability to stain ILM, turned out to be a good alternative to trypan blue for capsulorhexis. Trypan blue is the gold standard used in cataract surgery at the concentration of 0.06%. We report that BIP can be used in combination with lower concentration of TB resulting in stronger staining of the capsule, while maintaining the same or better cell survival parameters. The color of the stain achieved with this combination shifts from green to blue, depending on the ratio of the dyes. BIP alone results in strong green staining of the capsule and is also characterized by lower toxicity towards HCEC cells. In this paper we also investigated toxicity of all dyes and dye combinations on two other cell lines: ARPE, as those cells are frequently used for toxicity assessment of dyes intended for ocular surgeries, and HeLa, a cell line we found is very sensitive to dyes.

New cyanine dyes suggested as potential stains for ILM (Paper V)

There is an ongoing search for new potential dyes to be used in vitreoretinal surgery. In paper V we present the results of search for a new cyanine dye that would stain the ILM. As none of the commercially available dyes met the requirements of desired new medical device, fourteen new molecules were designed and synthesized in our working group. Chemical formulas of new substances were based on ICG and BIP structures. We present an investigation of new compounds that includes solubility, staining, stability and toxicity experiments. We have found that even small alterations in the chemical structure can result in different dye characteristics. Out of all the dyes tested, two were characterized with good staining ability of ILM model and high ARPE cell survival, out of which one is stable in aqueous solution with no other additives than diglycerol.

Structure of this dye will be patented, and the substance will be further investigated for the best solution composition and subsequently taken to clinical trials.

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Paper I

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Toxicity and phototoxicity in human ARPE-19 retinal pigment epithelium cells of dyes commonly used in retinal surgery

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Abstract

Purpose: To compare, for the first time, systematically toxicity and phototoxicity of dyes and dye combinations used in vitreoretinal surgery. The dyes were trypan blue, brilliant blue G, trypan blue+brilliant blue G, indocyanine green, bromophenol blue, bromophenol blue+brilliant blue G, and acid violet 17, in clinically used concentrations.

Methods: Human ARPE retinal pigment epithelium cells were exposed to the dyes for 30 min. For phototoxicity, the cells were exposed for 15 min to high-intensity light from a light emitting diode source with an intensity similar to surgical conditions. Toxicity was assayed either directly after exposure to either dye alone or dye and light, or with a delay of 24 h.

Results: None of the dyes and their combinations was toxic when cells were exposed to them at ambient light. Acid violet led to a reduction viability by 90% already immediately after light exposure. Bromophenol blue and its combination with brilliant blue G showed strong phototoxicity (reduction of viability by 83%) when assayed with delay. Indocyanine green with different agents to adjust osmolarity (balanced salt solution, glucose, mannitol) was not found to be toxic.

Conclusion: The strong immediate phototoxicity of acid violet reflects its clinical toxicity. Bromophenol blue might also be disadvantageous for patient outcome because of its delayed phototoxicity. The other dyes (trypan blue, brilliant blue G, and indocyanine green) were not found to be toxic neither with exposure to ambient light nor after exposure to light of intensities used in surgery.

Introduction

The internal limiting membrane (ILM) is a very thin (1–10 μm) and semi-transparent acellular membrane on the surface of the retina, composed of collagen type IV. It is involved in the pathogenesis of vitreoretinal interface diseases, such as vitreomacular traction syndrome, macular hole (MH), and epiretinal membrane (ERM). The removal (peeling) of the ILM improves the anatomical and functional outcome in macular surgeries.[1] Removal is challenging, due to the transparency of the ILM, ERM, and vitreous cortex.

Several vital dyes have been used to highlight the ILM. Indocyanine green (ICG) at concentrations around 0.1% was used first.[2] Its absorption peaks in the near infrared range, and solutions are not stable. Trypan blue (TB) was introduced later. Many commercial dyes contain TB at up to 0.15%.

Several alternative dyes such as brilliant blue G (BBG), acid violet (AV)-17, and bromophenol blue (BB) have been commercialized as staining agents.

BBG is present at 0.025%–0.05% in Brilliant Peel® and Ocublue Plus. TB with BBG is present, among others, in Bio-Blue DUO and Membrane Blue-Dual® at 0.15% and 0.025%, respectively, and in Doubledyne™ at 0.15% and 0.025%, respectively, together with 2% lutein.

AV, a trityl dye like BBG, is present in ala®purple (no longer available) at 0.15%. It has been reported to be detrimental to the vision in patients.[3]

BB and BBG are present in Brilliant Peel® Dual Dye at 0.13% and 0.025%, respectively.

Dyes used for surgery must stain the tissues of interest. At the same time, they must not be toxic. Toxic effects can be caused by the dye alone or the dye in combination with the strong illumination during surgery.

Safety studies, with conflicting results, for ICG[1,4–18] and TB[7,9,10,12,14,19–23] on retinal pigment epithelium (RPE) cells in the dark have been published. Also for BB alone, safety in cells has been studied.[15,18,23–25] While combinations of TB and BBG have been investigated for safety in cells,[22] combinations of BB and BBG appear not to have been investigated. AV has been studied for toxicity cell in the dark.[26] Only a few studies have investigated the effect of illumination, that too only with single dyes.[4,12,15,17,21,23,25,27] A literature survey on the viability of RPE cells exposed to the dyes is found in the Supplementary Table S1.

Here, we present the results for the first comparative study of toxicity and phototoxicity for dyes in clinical use for chromovitrectomy. We used the permanent cell line ARPE-19 (ARPE), used in the majority of other studies, and the WST-1 assay for assessing cell viability and complemented it with analysis of apoptosis markers and morphological changes in cells for immediate cytotoxicity. We used light exposure with intensities and spectra similar to those of the clinical setting. We measured the cell viability not only immediately after exposure but also after a recovery period of 24 h.

Materials and methods

Dyes

BBG, AV, and BB were from Sigma-Aldrich (Steinheim, Germany); TB from Carl Roth (Karlsruhe, Germany); and ICG from PULSION (Feldkirchen, Germany). Balanced salt solution (BSS) was from Alcon Laboratories (Fort Worth, USA). Phosphate-buffered saline (PBS) was made from (g/L) NaCl 8.0, KCl 0.2, Na2HPO4 1.42, and KH2PO4 0.24 and adjusted to pH = 7.2 with HCl. Polyethylene glycol (PEG) MW 3350 (50% solution in water) and D2O (99% D) were from Sigma-Aldrich. All dyes (except ICG) were dissolved in PBS. ICG was dissolved in distilled water, producing a 0.5% stock solution; further dilution of the stock was with BSS, or with mannitol or glucose solutions, to a final concentration of 0.1% ICG in either BSS or 5% mannitol or 5% glucose, representing the clinically used dilutions of ICG.[28] The composition of the dye solutions tested is shown in Table 1.

Table 1. List of solutions tested.

Abbreviation	Composition
PBS	Phosphate buffered saline
BBG	Brilliant blue G 0.025% in PBS
TB	Trypan blue 0.15% in PBS
TB-BBG	Trypan blue 0.15%, brilliant blue G 0.025% in PBS
TB-BBG-PEG	Trypan blue 0.15%, brilliant blue G 0.025%, PEG 4% in PBS
BB	Bromophenol blue 0.13% in PBS
BB-BBG	Bromophenol blue 0.13%, brilliant blue G 0.025%, D_2O 10% in PBS
AV	Acid violet 17 0.15% in PBS
ICG-BSS	Indocyanine green 0.1% in balanced salt solution
ICG-glucose	Indocyanine green 0.1%, glucose 5% in water
ICG-mannitol	Indocyanine green 0.1%, mannitol 5% in water

Light sources

The clinical light sources Xenon BrightStar and LED Star (both from the Dutch Ophthalmic Research Center (DORC), The Netherlands) were used for characterization of spectra and intensity. Light was extracted from the light sources with a 23G optical fiber (DORC).

For phototoxicity experiments, a 300 W light emitting diode (LED) floodlight (TechBox, Hong Kong) with 360 LED lamps on an area of 7.5 × 17.5 cm^2 was used. Cells were irradiated in 96-well plates placed 75 mm above the protecting glass of the floodlight.

Measurement of light intensity

The spectral irradiance of the light source was recorded with a calibrated absolute irradiance (NIST traceable) Mightex Systems HRS-BD1-025 spectrometer. Light was collected with a multimode fiber with a cosine corrector, with a distance between floodlight and entrance of the fiber of 75 mm. Xenon BrightStar and LED Star were measured in distances of 10 and 25 mm, the maximum and minimum working distances during surgery. A dark measurement was subtracted from the light measurement. The different refractive indices of air (calibration for the light sources) and water (application in the eye) were corrected for.

The light intensity in the sterile bench, where cells are manipulated before and after exposure to the dye, was measured with the same instrument.

Measurement of temperature increase

A 10-cm Petri dish filled with PBS at room temperature to the same height as in the 96-well plates was exposed to the floodlight for 15 min. Temperature increase was measured with a digital thermometer.

Cell culture and toxicity

ARPE cells were from latent growth curve (LGC), Wesel, Germany. Cells were used at passages between 30 and 50. Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) and WST-1 cell proliferation reagent were from Sigma-Aldrich. The cells were grown in DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2-humidified air.

Exposure to dyes and light

Cells were seeded at 10,000 cells per well in 96-well, flat-bottom tissue culture plates and grown for 48 h. The culture medium was removed, the cells were washed once with PBS, and incubated with the dye solutions (see Table 1) for 30 min, then washed three times with 200 µL PBS. Cells incubated with PBS were used as a control.

For phototoxicity, 100 µL of PBS was added to each well after the removal of the dye solution, and the plate was illuminate for 15 min by the floodlight. Experiments with cells exposed only to the ambient light of the laboratory are called "in the dark," although technically, they were exposed to low levels of light (only 0.4% of the intensity seen by the cells on the flood panel). Also, manipulation times were shorter (usually only 5‑10 min) than the exposure to the light panel.

Toxicity assay

After exposure, cells were incubated under culture conditions either immediately with 10 µL of WST-1, diluted 10 times with the cell culture medium or with cell culture medium for 24 h before adding WST-1. Absorbance was measured at 450 nm in a plate reader (TECAN M1000; Mannedorf, Switzerland). The intensity of developed color was taken as proportional to the number of viable cells. Percentage of cell viability was calculated by relating the measured absorbance to that of the control, treated identical with the exception of the exposure to the dye.

Other assays

For morphology, cells were cultivated in 24-well plates and exposed as above. Pictures were taken immediately after exposure and after 24 h in fresh medium using Evolution QEi monochrome camera (Media Cybernetics Inc., Silver Spring, USA) on an Axiovert 200 microscope (Carl Zeiss, Göttingen, Germany) at 20x magnification.

For apoptosis and necrosis, the Apoptosis/Necrosis Detection Kit from Enzo Life Sciences (Lörrach, Germany) was used. Cells cultivated in 24-well plates (for cytometry) or 4-well ibidi plates (for microscopy) were exposed as above and treated with the kit according to the manufacturer's instructions. The apoptosis inducer staurosporine (2 µM) served as positive control. After trypsinization, the suspension was read with a CyFlow Space apparatus (Sysmex Partec, Münster, Germany). Gating was adjusted to a control (cells not treated with the dye and incubated with the detection reagent) to subtract necrotic cell death caused by sample preparation (trypsinization and centrifugation). Images were taken with an AxioCam HRc camera on an LSM 510 Meta confocal laser scanning microscope (Carl Zeiss) at 20x magnification. For all pictures, identical settings of laser and gain were used.

Lactate dehydrogenase (LDH) leakage was measured with the Cytotoxicity Detection KitPLUS (LDH) (Sigma-Aldrich). For each experiment, six wells per plate were exposed to the same conditions. All experiments were run at least in triplicate.

Reactive oxygen species

Reactive oxygen species (ROS) were determined with the Fluorometric Intracellular ROS Kit Deep Red (Sigma- Aldrich) in 96-well black plates with clear flat bottoms. The fluorescence was detected at Ex = 650 nm, Em = 675 nm using a fluorescence microplate reader (Tecan Safire, Männedorf, Switzerland).

ARPE Cells (10,000 cells per well) were cultivated for 48 h and treated with the dye solutions as above. ROS was detected in live cells after 1 h incubation with fluorogenic sensor at 100 µL per well. As positive control, 100 µM of tert-butyl hydroperoxide was used.

Statistical analysis

Viability of ARPE cells exposed to dyes was measured on several 96-well plates after four different types of exposure: immediate dark, immediate light, delayed dark, and delayed light.

Since measurements for each dye were from different plates, it can be assumed that the correlation of two dyes on the same plate is higher than the correlation across two plates. Using a linear-mixed model, this effect is accounted for by means of a plate-specific intercept-only random effect in addition to a dye-specific fixed-effect model. For each type of exposure, the mean viability of cells exposed to TB-BBG-PEG (which might be the most frequently used dye in vitreoretinal surgery) was compared to the mean viability of all other dyes, respectively, resulting in a Dunnett's test to adjust for the multiplicity that arises from simultaneously testing more than one hypothesis. Moreover, we applied the Bonferroni procedure to account for multiplicity with regard to the two time points (immediate and delayed). Since our main interest was the

simultaneous effect of light and dye, we only conducted the above procedure using data from cells exposed to light.

Using a significance level of $\alpha = 5\%$, the multiplicity-adjusted p-values are reported for both types of light exposure. For descriptive purposes, a similar comparison was done with the TB group as reference.

The data analysis was conducted in R using the lme4 package for linear-mixed models and the multcomp package for multiple comparison procedures.

Results

Light sources

The most intense light source was Xenon BrightStar at 10 mm distance from the spectrometer (Figure 1). At 25 mm distance, it is as bright as sunlight. The floodlight has an intensity of about 40% of that of Xenon BrightStar at 10 mm and about 50% higher than LED Star at 10 mm. All three surpass the intensity of sunlight.

The floodlight thus mimics closely the light levels used in surgery. It allows to expose all cells of a 96-well plate simultaneously, in contrast to sequential exposure of individual wells by others.[4,7,9,12,15,23,27,31–33] Sequential exposure leads to a considerable lag between exposure to light and assessment of toxic effects. Sixty wells would require a total exposure time of 10 h with exposure of 10 min per well.

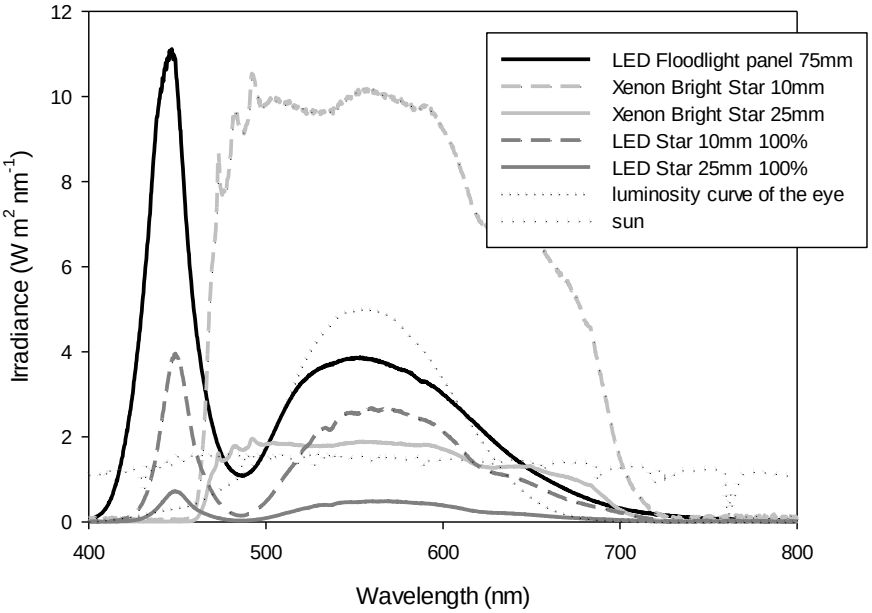

Figure 1. Spectra of light sources. The spectrum of the LED floodlight was measured at a distance of 75 mm from the protecting plate. For Xenon BrightStar and LED Star, the end of the light guide was positioned at 10 and 25 mm, respectively, from the entrance to the photometer. For Xenon BrightStar, a cut-off filter of 475 nm and maximum possible power (92%) was used. The solar spectrum is that of the global tilt (on the surface of the earth, facing toward the equator, and tilted 37° from horizontal). The solar spectrum was taken from Renewable Resource Data Center (RREDC)[29] and the luminous efficiency function of the human eye from Sharpe et al.[30]

Toxicity

Toxicity data are found in Table 2 and in Figure 2.

For cells without exposure to light, neither immediately after exposure to dye nor after 24 h, no statistically significant viability differences between TB-BBG-PEG and the other dyes were found. Immediately after exposure to light, there are statistically significant mean differences between TB-BBG-PEG and ICG, ICG-mannitol, and AV. In the delayed light group, there are, in addition to the significant differences already found in the immediate light group, also significant differences to BB, BBG, BB-BBG, and ICG-glucose.

With a few exceptions, the results for TB are similar with somewhat less statistically significant differences, but similar trends. For TB and TB-BBG-PEG, the survival data agree with our previous data.[22,34]

The temperature increase caused by the exposure to light was about 3.4°C±0.8°C (from 22°C to around 25°C) after 15 min, which should not influence cell viability (Table 3).

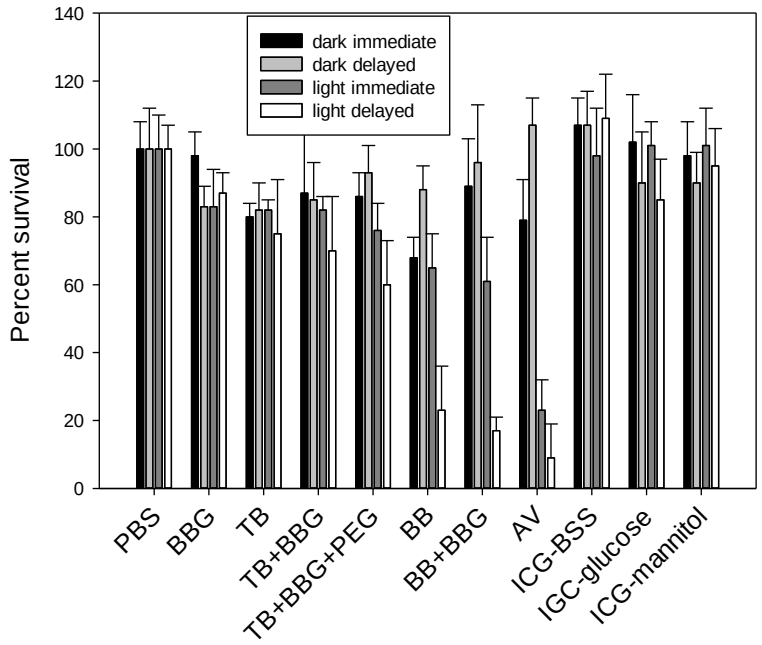

Figure 2. Toxicity of dyes on ARPE cells after exposure. The average and the standard deviation are shown. Cells were exposed to the dyes for 30 min, washed, and where indicated exposed to light for 15 min. Toxicity was measured immediately after exposure or delayed, following incubation in growth medium for 24 hours at 37°C. Cell viability was set to 100% for cells not exposed to the dye, but exposed to light and/or assayed with delay where applicable. A representative set of data is shown as „Control". For statistical differences between the data, consult Table 3. The standard deviations for PBS (the control for all experiments) are those for the six wells of an individual experiment, whereas the other standard deviations were derived from three or more individual experiments, each comprising six wells.

Table 2. Data for viability of ARPE cells after different exposures. Percent viability ± standard error of the mean (SEM) is given.

Dye	Mean±SEM			
	immediate dark	immediate light	delayed dark	delayed light
TB-BBG-PEG	86±7	76±8	93±8	60±13
TB	80±4	82±3	82±8	75±16
BBG	98±7	83±11	83±6	87±6
TB-BBG	87±22	82±4	85±11	70±16
BB	68±6	65±10	88±7	23±13
BB-BBG	89±14	61±13	96±17	17±4
AV	79±12	23±9	107±8	9±10
ICG-BSS	107±8	98±14	107±10	109±13
ICG-glucose	102±14	101±7	90±15	85±12
ICG-mannitol	98±10	101±11	90±9	95±11

Table 3. Comparison of toxicity. Mean differences and SEM TB-BBG-PEG (TB) and all other dyes. A negative value indicates that the mean of TB-BBG-PEG (TB) is lower than that of the respective other dye. p-values from the Bonferroni-adjusted Dunnett's tests are given in the brackets.

	Mean difference ± SEM (Bonferroni-adjusted Dunnett's test p-values)							
	Immediate light				Delayed light			
Dye	TB-BBG-PEG (reference)		TB (reference)		TB-BBG-PEG (reference)		TB (reference)	
TB-BBG-PEG	-		7±4.7	(1.0)	-		16±12.1	(1.0)
TB	-7±4.7	(1.0)	-		-16±12.1	(1.0)	-	
BBG	-7±7.7	(1.0)	0±6.6	(1.0)	-28±8.4	(4e-10)	-12±9.9	(1.3e-04)
TB-BBG	-6±5.1	(1.0)	1±3.1	(1.0)	-11±11.1	(1.0)	5±12.3	(1.0)
BB	11±7.3	(1.0)	18±6.1	(0.38)	36±10.8	(9.8e-06)	52±12.0	(4.4e-16)
BB-BBG	15±8.6	(0.18)	22±7.6	(0.02)	42±8.0	(6e-08)	58±9.6	(4e-16)
AV	53±6.9	(14e-14)	60±5.6	(2.2e-16)	50±9.6	(5.6e-10)	66±11.0	(4e-16)
ICG-BSS	-22±9.2	(0.02)	-15±8.2	(0.06)	-50±10.4	(1.3e-08)	-34±12.6	(1.7e-08)
ICG-glucose	-25±5.7	(0.08)	-18±4.1	(0.2)	-26±8.8	(0.02)	-10±10.3	(0.2)
ICG-mannitol	-25±6.9	(0.04)	-18±5.6	(0.12)	-34±11.0	(1.9e-03)	-18±12.2	(0.02)

Morphological changes

Microphotographs of cells after incubation are shown in Supplementary Figure S1. The most drastic change in morphology was caused by AV, already in the dark. The nuclei became very pronounced while the cell body shrank. Very few cells recovered after 24 h and only when not exposed to light. While TB did not influence the cell morphology, a slight cell loss was observed after 24 h. TB-BBG-PEG caused shrinking of cells after light exposure. BB in the dark did not influence the cells, but after light exposure, multiple smaller vesicles were observed, a hint to apoptotic processes (see below). BB-BBG caused cell-body shrinkage. For ICG-BSS, we did not observe any morphological changes.

Apoptosis/necrosis and LDH leakage

For all dyes, except TB, very few cells showed only apoptosis or only necrosis signals (see Table 4). Cells frequently showed both signals (for both exposure in dark as in light), which according to the manufacturer's protocol indicates late apoptosis.

TB was the only dye with a strong signal in the necrosis channel. We had observed before that propidium iodide binds strongly to the surface of cells exposed to TB but not to the nucleus.[34] This could also be true for the DNA-intercalating dye of the apoptosis/necrosis kit (whose nature is unknown to us), making the necrosis values for TB is highly unreliable.

After exposure to AV in the dark, around 38% of all cells were stained with either or both of the two detection agents, much more than for any other dye, which unexpectedly was not the case for light exposure. There was, however, no correlation between cell viability and staining for apoptosis/necrosis markers.

Table 4. Results of staining for apoptosis and necrosis. Dk: dark, Lt: light.

Dye		Apoptosis(%)	Necrosis(%)	Late apoptosis (%)	Viability (%)		Apoptosis(%)	Necrosis(%)	Late apoptosis (%)	Viability (%)
PBS	Dk	0.9	0.1	3.8	100±8	Lt	0.9	0.3	9.2	100±10
TB	Dk	0.4	58.4[a]	10.6	80±3	Lt	1.6	26.5[a]	33.1	83±2
TB-BBG-PEG	Dk	1.8	1.0	4.4	86±4	Lt	2.2	1.0	25.7	76±4
BB	Dk	1.2	2.2	4.7	68±4	Lt	1.2	2.4	15.4	65±6
BB-BBG	Dk	1.8	1.2	5.1	89±8	Lt	2.6	2.4	22.8	61±7
AV	Dk	4.2	3.9	30.3	79±7	Lt	3.0	2.1	22.1	23±5
ICG-BSS	Dk	1.3	0.7	4.8	107±5	Lt	2.9	1.0	16.5	98±8

[a]See text for these values. The viability data are from WST-1 assay immediate (Table 2).

In microscopy, staining for apoptosis was generally weak and that for necrosis was much stronger (Supplementary Figure S2).

Incubation with TB led to strong fluorescence from necrosis detection reagent for most cells, probably by the association of the necrosis dye with cell-adsorbed TB.[34] With TB-BBG-PEG, binding of the necrosis dye to cells is prevented. Staining with both stains was observed for cells incubated with BB, BB-BBG, and AV, and for most of the cells they colocalized. Some nuclei of cells incubated with ICG-BSS were stained with necrosis detection reagent. Due to the possible interaction of the fluorescent-detection dyes with adsorbed dyes, the interpretation of the pictures is not obvious.

No correlation between staining for necrosis/apoptosis and viability was found.

We also tried to measure LDH leakage, a well-known assay for cellular integrity. The presence of the dyes prevented the measurement of color developed in the LDH assay. Leakage of LDH occurs during incubation; therefore, washing of the cells to remove the dyes will also remove the leaked LDH. In addition, we observed precipitates of the LDH leakage reagent with dyes.

ROS production

We speculated whether the phototoxicity of the dyes (especially of BB and AV) was correlated to the production of ROS by the dyes remaining in the cells after extensive washing. The amount of ROS was measured both in the dark and after light exposure. Results are shown in Figure 3.

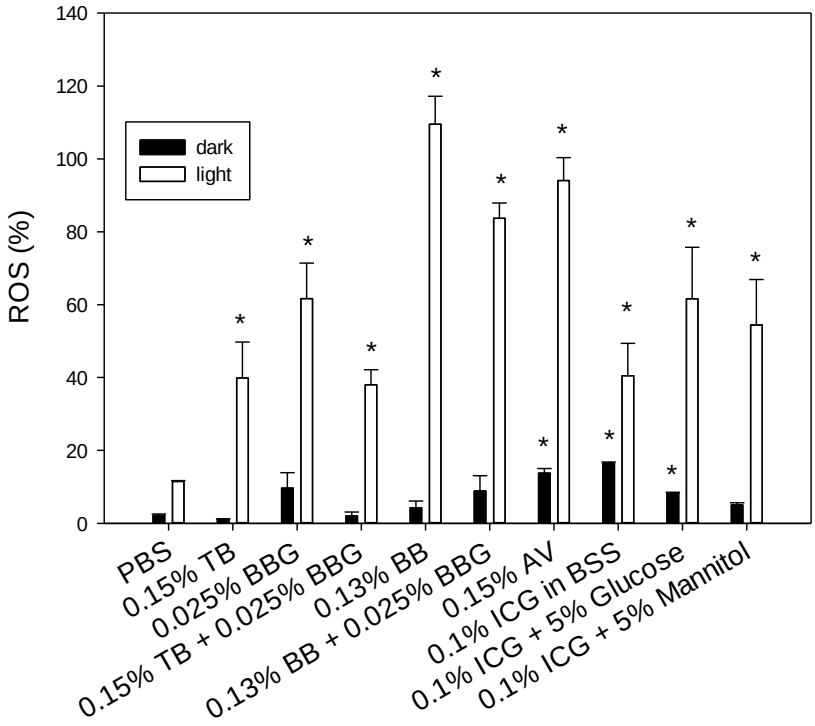

Figure 3. ROS production induced in ARPE after exposure to dyes in the dark and after 15 min illumination. The value for 100% was set for the production of ROS by tert-butyl hydroperoxide. Values significantly higher ($P < 0.05$) than the PBS control for dark or light are indicated with *.

In the dark, AV as well as ICG in BSS and 5% glucose showed a small but significant increase of ROS. After light exposure, all dyes induced significantly more signal in the ROS measurements than in cells kept in PBS. Although BB, BB + BBG, and AV, with the highest phototoxicity, show the highest production of ROS, it is not possible to state that this is also the reason for the phototoxicity, as BBG and ICG, which show no phototoxicity, also have high rates of ROS production.

Discussion

This study is, to our knowledge, the first to investigate toxicity of dyes for vitreoretinal surgery under identical conditions. As a model, we chose ARPE cells. This cell line has been used by many other investigators for assessing biocompatibility of dyes in vitreoretinal surgery.[9,12,14,16,20–23,34,35] Although the cell line might not be the ideal model, it has the advantage of being permanent, thus avoiding problems with primary cell lines which cannot be standardized.[5,10,27] The ISO standard 10933 for biological evaluation of medical devices prefers established cell lines over primary cell lines and states that the latter "shall only be used if reproducibility and accuracy of the response can be demonstrated."[36]

Cell-based assays are not the only type of assays used to investigate detrimental effects of dyes. As an alternative to toxicity in cell culture, histological changes[37–39] and electroretinogram (ERG) changes in rat eyes[40–42] have been used. Also, changes in ERG of perfused bovine retina after exposure to dyes have been investigated.[43] For both cell viability and other test systems, correlation with clinical outcome in patients still

remains to be established. Also, dyes might be retained by structures in the eye and causeproblems, which might go unnoticed in a cell model.[44]

Evaluation of toxicity was immediate (right after exposure) or delayed (24 h after exposure), following other authors in similar studies.[8,9,12,15,20,23,27] Most studies had looked at immediate toxicity only. The delayed toxicity measurement allows for recovery of sublethal damage but has the disadvantage that cells can regrow and thereby repopulate, giving a false impression of safety. RPE cells in the eye will not regrow easily.[45] We did not find an indication of recovery from damage for the dyes which we investigated.

ICG in isoosmolar preparations was not toxic to the ARPE cells, and the toxicity of TB-BBG-PEG was significantly higher. The toxicity of ICG in cell culture and in patients has been discussed quite controversially in the literature.[1,5–8,11,13,18,46,47] Kiilgaard et al.[11] have attributed the possible toxicity of ICG to hypoosmolarity of the solutions and we would tend to agree with their conclusion.

BB at 0.05% was not phototoxic to ARPE cells immediately after exposure to light,[31] in agreement with our results. Giansanti et al.[23] found phototoxicity of BB on ARPE cells when assayed 24 h after exposure, in line with our results. BB should, therefore, be considered less suitable as a dye in vitreoretinal surgery. It remains unclear whether the effect of BB + BBG on the visual outcome of patients has been investigated.

The dye which, in combination with light, induced most damage to the viability of cells was AV. AV at low concentrations (0.0125%) in the dark was found not to lead to damage of RPE cells isolated from bovine retina; this was, however, different when the concentration was increased to 0.025% (still below a clinically useful concentration).[48] Penha et al.[1] had found similar toxicity levels as ours, while Hurst et al.[26] found lower values. A recent survey of the literature by Gerding[3] shows that toxic effects of AV are found at concentrations well below those that were used clinically.

Our results show that not only toxicity but also phototoxicity of dyes must be assessed in vitro before a clinical use can be envisaged. These experiments must include not only toxicity assessed immediately after exposure but also after a time delay. It is only with the exposure to light that the full detrimental effect of AV on cells was found. We, therefore, strongly advocate that future investigations on toxicity of dyes for ocular surgery also take into account the phototoxicity of the dyes.

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Supplementary material

A file containing material on compilation of toxicity experiments carried out on retinal pigment epithelium cells, morphology of ARPE cells exposed to dyes, and apoptosis and necrosis detection in cells.

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Toxicity and phototoxicity in human ARPE-19 retinal pigment epithelium cells of dyes commonly used in retinal surgery

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Supporting Information

Supplementary Table S1.
Compilation of toxicity experiments carried out on retinal pigment epithelium cells.

Supplementary Figure S1.
Morphology of ARPE cells exposed to dyes.

Supplementary Figure F2.
Apoptosis and Necrosis detection in cells.

Supplementary Table S1. Compilation of toxicity experiments carried out on retinal pigment epithelium cells.

Dye	Test object	Test method	Concentration	Conditions	Outcome	Reference
ICG	primary human RPE cells	cell survival by WST-1 assay	0.1% (hypoosmolar)	20 min exposure to dye in dark 10 min exposure to light	reduction to 75% survival in dark, 63% in light	(Sippy et al. 2001)
ICG	primary human RPE cells	live-dead staining with calcein-AM and ethidium homodimer-1	not given (both hypo-osmolar and isoosmolar)	up to 20 min exposure to dye in dark	reduction to 80% survival for isoosmolar solution after 20 min reduction to 16% survival for hypoosmolar solution after 5 min	(Stalmans et al. 2002)
ICG	ARPE-19 cell line	cell survival by MTS assay	up to 0.05% (hypoosmolar)	exposure in dark up to 3 h	reduction to 50% survival after 30 min at 0.05%	(Ho et al. 2003)
ICG	ARPE-19 cell line	cell survival by MTT assay	up to 0.5% (hypoosmolar)	exposure in dark up to 30 min	reduction to 30% survival after 30 min	(Gale et al. 2004)
ICG	primary human RPE cells	cell survival by MTS assay	up to 0.24% (probably hypoosmolar)	exposure in dark for 3 min toxicity measured 24 h after exposure	reduction to 40% survival at 0.24%	(Hsu et al. 2004)
ICG	ARPE-19 cell line	cell survival by MTT assay	up to 0.5% (slightly hypo-osmolar)	exposure in dark for up to 30 min 1 min light exposure toxicity measured 24 h after exposure	no toxicity	(Jackson et al. 2004)

Dye	Test object	Test method	Concentration	Conditions	Outcome	Reference
ICG	primary human RPE cells	propidium iodide exclusion	up to 0.05% (both hypo-osmolar and isoosmolar)	exposure to dye for 5 min in the dark measurement of PI uptake time dependent	toxicity recorded after following the cells for 2 h	(Kodjikian et al. 2005)
ICG	ARPE-19 cell line	cell survival by MTT assay	up to 0.5% (for some dye brands hypo-osmolar at highest con-centration)	exposure to dye for 10 min in the dark	toxicity only for hypoosmolar conditions	(Kiilgaard et al. 2006)
ICG	ARPE-19 cell line	live-dead staining with DAPI and propidium iodide	up to 0.5% (slightly hypo-osmolar)	up to 20 min exposure to dye in dark up to 5 min exposure to light	0% survival at highest ICG concentration, with and without light no significant effect of light	(Peters et al. 2007)
ICG	ARPE-19 cell line	cell survival by MTT assay	0.05%	3 min exposure to dye in dark	no toxicity	(Proulx and Gonder 2007)
ICG	ARPE-19 cell line	cell survival by MTT assay	0.01%	30 min exposure to dye in dark	no toxicity	(Yuen et al. 2009)
ICG	ARPE-19 cell line	cell survival by MTT assay	up to 2% (probably hypoosmolar at high concentration)	3 min exposure to dye in dark or in light	at 2%, 50% survival	(Morales et al. 2010)
ICG	ARPE-19 cell line	neutral red uptake	up to 0.05%	up to 30 min exposure to dye in dark	reduction to 50% cell integrity	(Balaiya et al. 2011)
ICG	ARPE-19 cell line	cell survival by MTS assay	0.5%	3 min exposure to dye in dark continuous exposure to light	no toxicity in dark reduction to 45% cell survival with light	(Takayama et al. 2012)

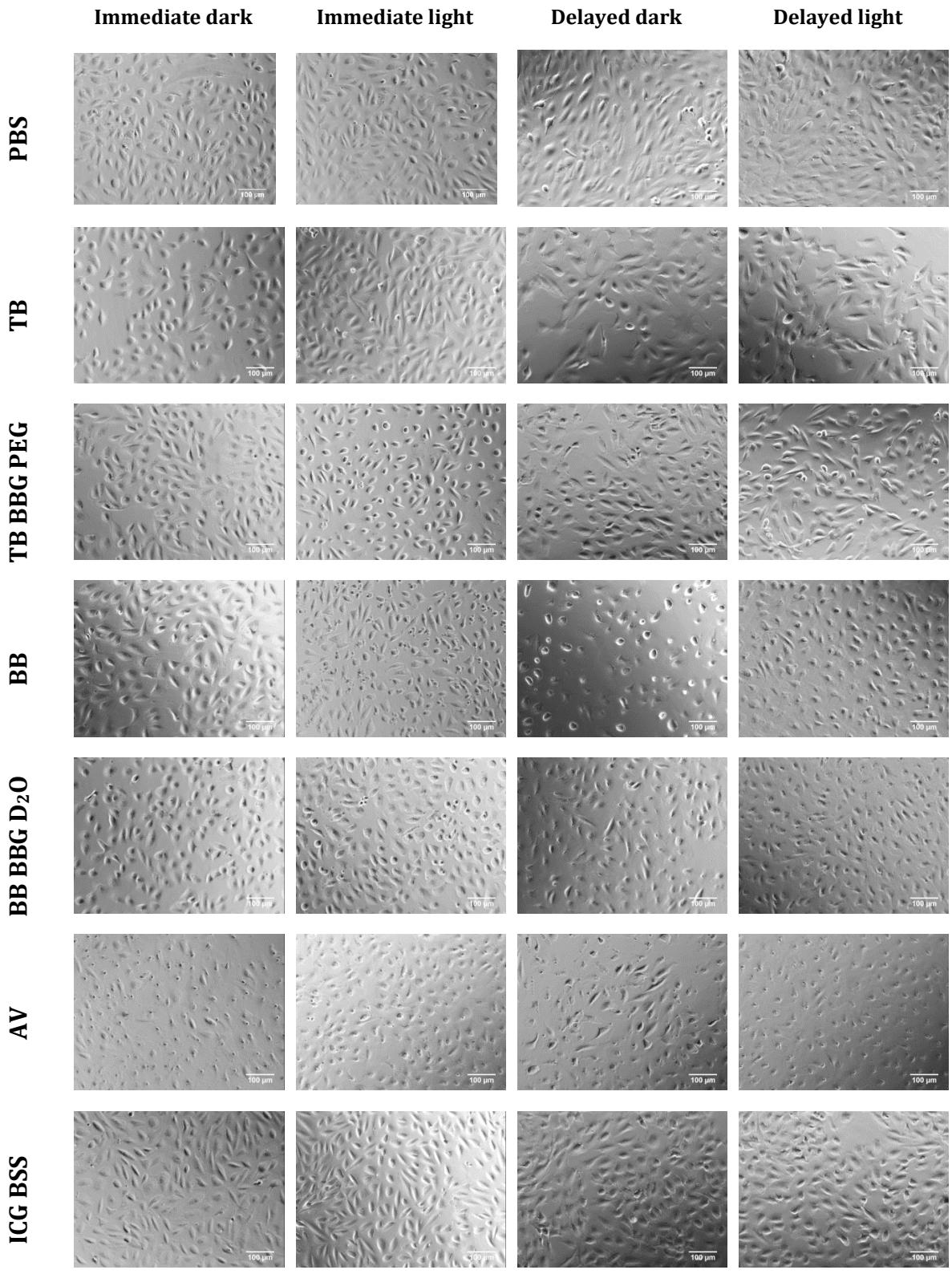
Dye	Test object	Test method	Concentration	Conditions	Outcome	Reference
ICG	ARPE-19 cell line	cell survival by MTS assay	up to 0.1% (probably hypoosmolar at high concentration)	up to 30 min exposure to dye in dark	reduction to 35% cell survival, independent of concentration	(Penha et al. 2013a)
ICG	ARPE-19 cell line	cell survival by MTS assay	up to 0.1% (probably hypoosmolar at high concentration)	up to 30 min exposure to dye in dark	reduction to 35% cell survival, independent of concentration (data identical to (Penha et al. 2013a)	(Penha et al. 2013b)
TB	primary human RPE cells	live-dead staining with calcein-AM and ethidium homodimer-1	up to 0.3%	up to 20 min exposure to dye in dark	no toxicity	(Stalmans et al. 2003)
TB	ARPE-19 cell line	cell survival by MTT assay	up to 0.15%	30 min exposure to dye in dark	no toxicity	(Gale et al. 2004)
TB	ARPE-19 cell line	cell survival by MTT assay	up to 0.2%	exposure in dark for up to 30 min toxicity measured 24 h after exposure	no toxicity	(Jackson et al. 2004)
TB	ARPE-19 cell line	cell survival by MTT assay	up to 0.4%	1 min exposure to dye in dark	no toxicity with immediate assay toxicity independent of concentration when assayed 2 or more days after exposure	(Kwok et al. 2004)

Dye	Test object	Test method	Concentration	Conditions	Outcome	Reference
TB	primary human RPE cells	propidium iodide exclusion	up to 0.5%	exposure to dye for 5 min in the dark measurement of PI uptake time dependent	no toxicity	(Kodjikian et al. 2005)
TB	ARPE-19 cell line	cell survival by WST-1 assay	up to 0.1%	exposure to dye for 2 min in the dark up to 10 min exposure to light	no toxicity	(Narayanan et al. 2005)
TB	ARPE-19 cell line	live-dead staining with DAPI and propidium iodide	up to 0.15%	up to 20 min exposure to dye in dark up to 5 min exposure to light	0% survival at highest TB concentration, with and without light after 20 min exposure no effect of light	(Peters et al. 2007)
TB	ARPE-19 cell line	cell survival by MTT assay	up to 0.15%	30 min exposure to dye in dark	reduction to 80% cell survival at 0.15%	(Yuen et al. 2009)
TB	ARPE-19 cell line	cell survival by WST-1 assay	up to 0.25%	up to 30 min exposure to dye in dark	reduction to 80% cell survival at 0.25%	(Awad et al. 2011)
TB	ARPE-19 cell line	cell survival by WST-1 assay	up to 1.0%	up to 60 min exposure to dye in dark	reduction to 40% cell survival at 1.0% and 60 min	(Awad et al. 2013)
TB	ARPE-19 cell line	cell survival by WST-1 assay	0.5%	5 min exposure to dye in dark 5 min exposure to light	no toxicity no effect of light	(Giansanti et al. 2014)

Dye	Test object	Test method	Concentration	Conditions	Outcome	Reference
BB	primary human RPE cells	cell survival by MTT assay	up to 0.2%	10 min exposure to dye in dark	no toxicity at 0.2% reduction of cell survival to 90% at 0.02%	(Haritoglou et al. 2005)
BB	ARPE-19 cell line	cell survival by MTT assay	up to 1%	3 min exposure to dye in dark or in light	reduction to 22% cell survival at 1% dye, to 64% at 0.25% dye reduction with light exposure to 16% at 1% dye, to 47% at 0.25% dye	(Morales et al. 2010)
BB	ARPE-19 cell line	cell survival by MTT assay	0.05% (dark) 0.005% (light)	10 min exposure to dye in dark or in light	no toxicity in dark reduction to 90% cell survival in light	(Costa et al. 2013)
BB	ARPE-19 cell line	cell survival by MTS assay	up to 0.1%	up to 30 min exposure to dye in dark	reduction to 737% cell survival	(Penha et al. 2013a)
BB	ARPE-19 cell line	cell survival by WST-1 assay	0.5%	5 min exposure to dye in dark 5 min exposure to light	no toxicity in dark reduction with light exposure to 80% cell survival	(Giansanti et al. 2014)
AV	ARPE-19 cell line	cell survival by MTS assay	up to 0.1%	up to 30 min exposure to dye in dark	reduction to 48% cell survival	(Penha et al. 2013b)
BBG	ARPE-19 cell line	cell survival by MTS assay	up to 0.1%	up to 30 min exposure to dye in dark	reduction to 60% cell survival	(Penha et al. 2013b)
BBG	ARPE-19 cell line	cell survival by WST-1 assay	up to 0.2%	up to 60 min exposure to dye in dark	reduction to 60% cell survival at 0.2% and 60 min exposure	(Awad et al. 2013)

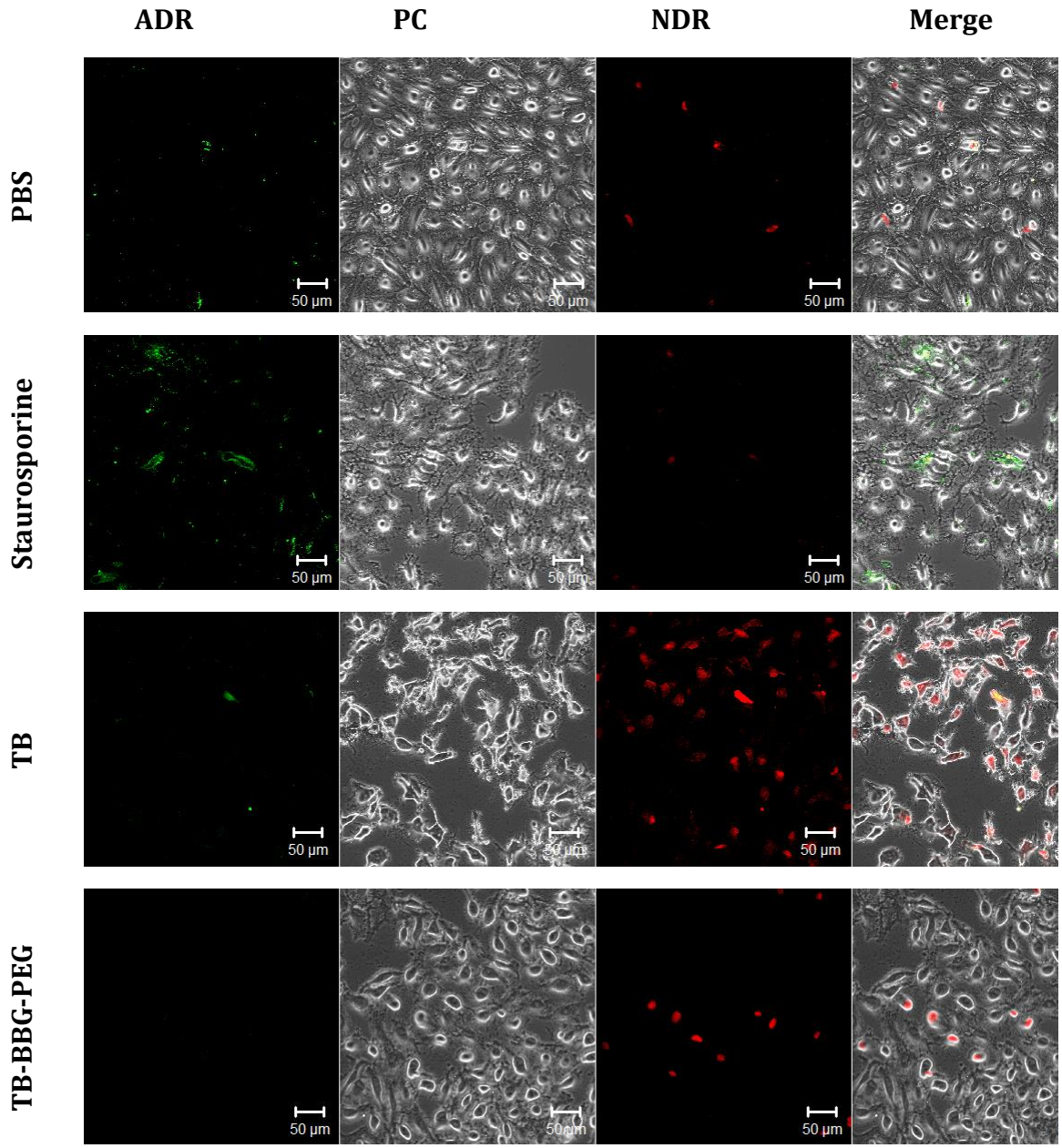
Supplementary Figure S1. Morphology of ARPE cells exposed to dyes.

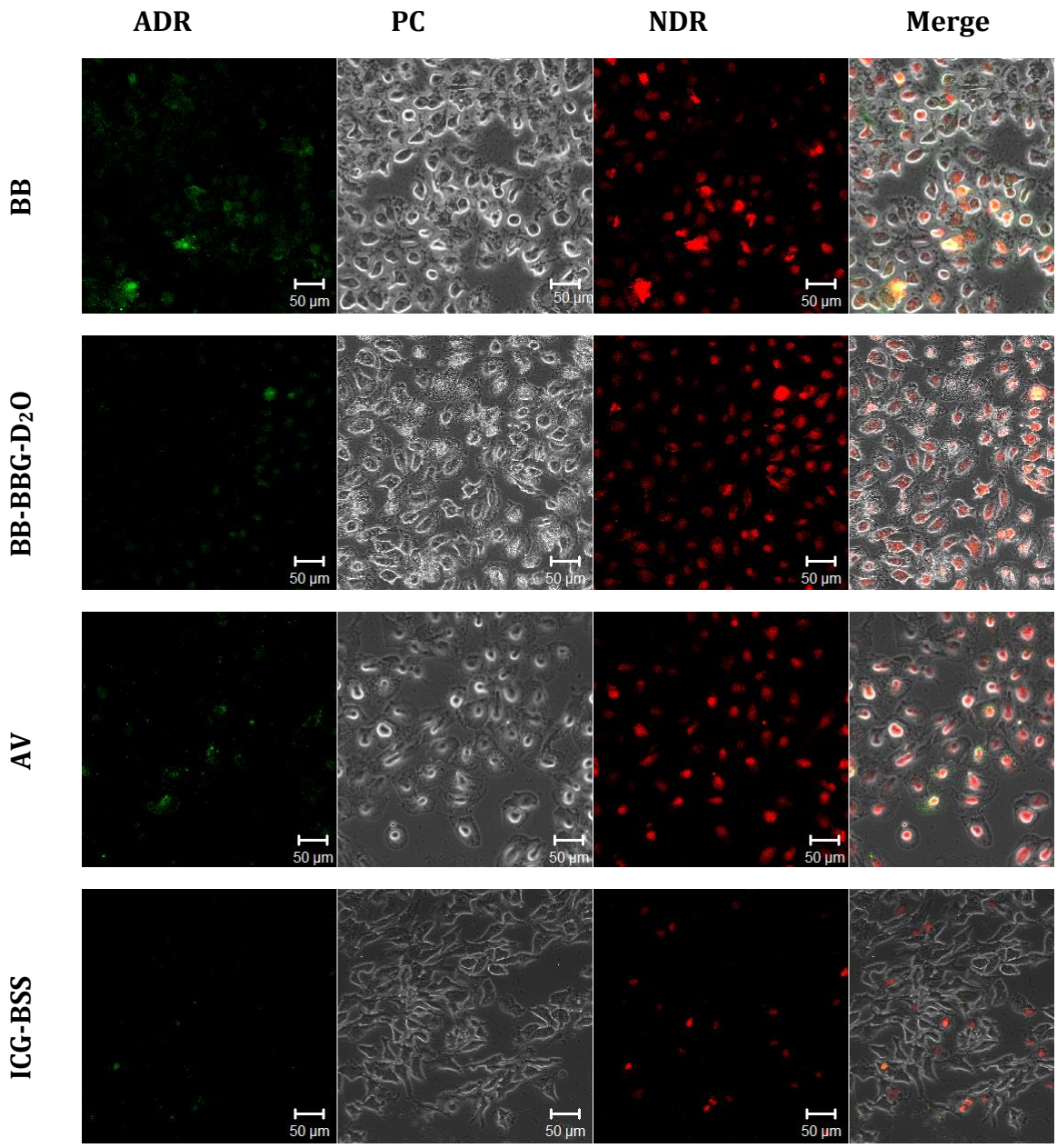
Pictures of ARPE after incubation with dyes for 30 min and 15 min light exposure where indicated, taken immediately after exposure (immediate), or after further 24 h incubation with fresh medium (delayed).



Supplementary Figure S2. Apoptosis and Necrosis detection in cells.

Cells incubated with dyes for 30 min and exposed to light for 15 min incubated with Double Detection Reagent from Apoptosis/Necrosis Detection Kit. ADR: signal from Apoptosis Detection Reagent, NDR: signal from Necrosis Detection Reagent, PC: phase contrast. Staurosporin induces apoptosis, and was used as a positive control for the ADR response.





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Paper II

Models for testing stains for the Internal Limiting Membrane ILM and Epiretinal Membrane ERM in vitreoretinal surgery

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Abstract

Purpose: To investigate whether porcine eye is a reliable model for ILM staining and if there are better models available. To examine and suggest new model for ERM.

Methods: Staining of pig eyes was compared with and without enzymatic vitreous disruption. Porcine eyes were collected post mortem. Globe was cut at the equator and its upper half with vitreous attached was removed. Staining was performed, and punch of retina was photographed. Polyvinylidene filter and collagen membrane were incubated with dyes, washed with PBS and documented by photography. Staining was graded 0-3 and correlated with results for staining of human ILM and ERM.

Results: Staining observed in porcine eyes can be staining of the vitreous, and not ILM. Two new models, polyvinylidene difluoride (PVDF) filter and Collagen I membrane were suggested as suitable models for ILM and ERM respectively. They correlate strongly with staining of human membranes.

Conclusion: PVDF filter and Collagen I membrane are useful tools for investigating the staining ability of dyes with a potential use for vitreoretinal surgery. Their staining is strongly correlated with the staining observed for ILM and ERM, respectively, in patients.

Introduction

The development of dyes for vitreoretinal surgery is still an active area of research. While trypan blue (TB) has been found to be a good stain for the epiretinal membranes (ERM), brilliant blue G (BBG) and indocyanine green (ICG) have been found to stain the internal limiting membrane (ILM). BBG has the same color as TB, and differentiation is not easy; in addition, staining has been reported as patchy or absent in a non-negligible number of patients.[1] ICG has been reported to have poor affinity for the ERM.[2] In addition, its solutions are not stable and need to be prepared freshly in the clinic. A stable green dye with affinity for the ILM would be highly desirable.

For developing such dyes, staining as well as toxicity must be evaluated. While toxicity is usually assessed in cell culture models (see e.g. [3-6]), pig eyes as well as cadaveric human eyes have been used as models for the ILM.[7,8] In an effort to find green dyes for vitreoretinal surgery, we located a cyanine dye, sodium 4-((E)-2-((2E,4E)-5-(3,3-dimethyl-1-(4-sulfonatobutyl)-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene)-3,3-dimethylindolin-1-yl)butane-1-sulfonate (BIP) with excellent staining ability in what we considered pig ILM. This dye, which is non-toxic at the concentrations where very good green staining in pig eye retinas was observed, did not stain the human ILM during surgery.

Therefore, we have investigated what the reason for the discrepancy between human and pig eyes could be, and which alternatives to pig eyes could exist for the preclinical testing of dyes. As human ILM and human ERM consists of different proteins commonly found in connective tissue [9-11] we used different proteins adsorbed to plastic as models. We found that a non-natural material might be a very reliable model of the human ILM.

Materials and methods

Dyes

The following dyes were used. All were from Sigma-Aldrich, except where indicated: Brilliant blue G (BBG), bromophenol blue (BB), crystal violet (CV), Evans blue (EB), fast green (FG), indigo carmine (IC), indocyanine green (ICG) , light green SF (LGSF), methyl blue (MeB), methyl violet (MV), methylene blue (MB), patent blue (PB), toluidine blue (TolB) , trypan blue (TB) (Carl Roth), BIP (FEW Chemicals).

Models investigated

The following models were used:

Filter and membrane: A 96-well filter plate (MSBVN1210 MultiScreen BV Filter Plate, Merck-Millipore, Darmstadt, Germany) was used as ILM model. The filter material is hydrophilic polyvinylidene difluoride (PVDF). A Collagen Cell Carrier membrane (CCC) (Viscofan, Weinheim, Germany) was used as model for the ERM. CCC consists of long collagen fibers of type I. Punches of the membrane (5 mm diameter) were fixed to the filter with a hypodermic needle and soaked in PBS for 30 minutes. The PBS was removed by vacuum filtration, and 50 µL of the dye solution was pipetted into the filter well. After 30 s, the dye was removed by vacuum filtration, and membrane and filter were washed three times with PBS. The membrane was removed and put into a 96-well plate for spectrum recording, using a Tecan Spark 20M (Tecan Group Ltd.,

Männedorf, Switzerland) plate reader. The staining of filter and membrane was recorded photographically. The intensity of staining was graded in four steps (0 to 3), as by Rodrigues et al. [7]

Proteins on plastic: A 96-well flat-bottom plate was coated with 3.2 µg/well of collagen IV, 3.2 µg/well of collagen I, 1.6 µg/well of fibronectin, or 0.64 µg/well of laminin and combinations of those. The plate was incubated overnight with the protein solutions. On the next day the solutions were removed, and the protein-coated wells were incubated with dyes for 30 s. After removing the dyes, the plate was washed 3 times with 100 µl of PBS and absorbance spectrum was recorded using a Tecan Spark 20M plate reader.

Pig eye retina: Pig eyes were obtained post mortem from the local slaughterhouse. They were freed from muscles and the extruding optic nerve and kept on ice until used.

For access to the retina, the eye was kept in place by vacuum in a holder. The holder consisted of a 3-cm Büchner funnel, filled to about two thirds with small glass beads, which were covered by a piece of household wrap into which a few holes were cut. Dynamic vacuum was applied. With a scalpel, the sclera was incised circularly slightly above the equator, and separated completely with scissors. The anterior chamber (usually with adherent vitreous) was removed, and the exposed surface of the fundus was rinsed three times with PBS. The eye cup was placed on a flat surface.

A glass tube (diameter 10 mm) was placed over the optic nerve and the fovea and was filled with the dye solution (200 µL); the eye cup outside the glass tube was then filled with 1 mL PBS. After 30 s, the glass tube was removed, and the eye cup was washed repeatedly with PBS until the solution ran clear. With a 10-mm biopsy punch, a part of the stained area (not including the optic nerve) and a part of the unstained area was cut and placed on a coverslip for photography.

For microscopic examination retina was placed on a cover slip and observed under LSM 510 Meta confocal laser scanning microscope (Carl Zeiss). Z-stack pictures were taken with an AxioCam HRc camera (height of the stack: 54 µm, excitation at 542 nm, long-pass filter 560 nm).

Enzymatic liquification of the vitreous:

Our approach was similar to the experiments described in the literature for plasmin. [12,13] Plasminogen (15 U) was activated with 0.25 ml Alteplase (0.05 mg/0.1ml) for 5 min in 37°C, then kept on ice. Eyes were incubated for 15 min in 37°C before the injection of activated plasminogen, and then were injected with 100 µl (2U) activated plasminogen or 100 µl PBS and incubated in 37°C for 1 hour. Staining was performed as described above.

Results

Staining had been tried before in pig eyes, with correlation of the staining intensity to that observed in human patients by Rodrigues et al. [7] We plotted the data from Rodrigues et al. in a correlation plot (Fig. 1). For a perfect correlation, a slope of 1 would be expected, and an r^2 value close to 1. As can be seen, there is a negative correlation

of the staining of porcine ILM and human ERM, and not a very good correlation between the staining of the human ILM and the porcine ILM.

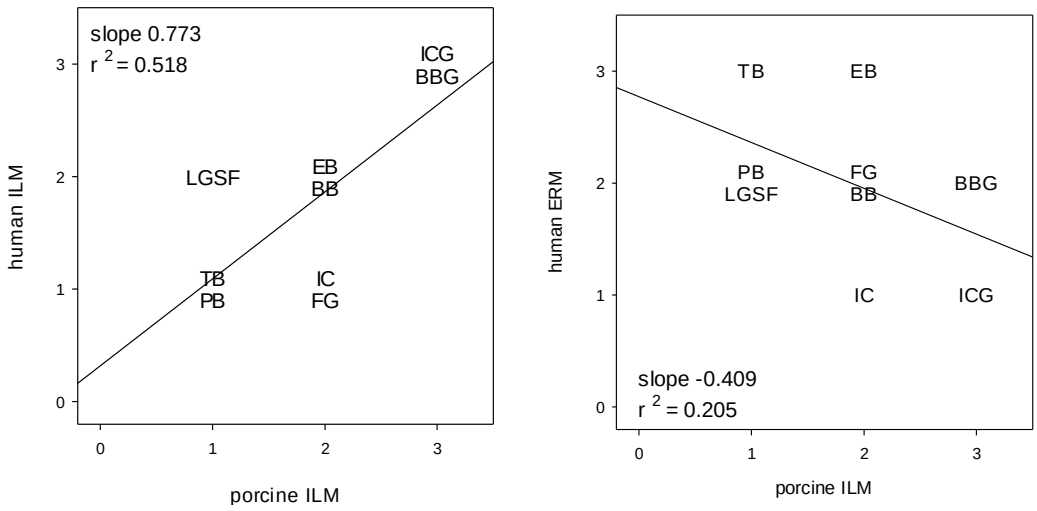


Figure 1. Correlation of staining intensity between porcine ILM and human ILM and human ERM, respectively. Data replotted from Rodrigues et al.[7]

There is no animal model available for staining of the ERM, as this scar-like tissue is a pathological condition that does not occur in young animals which the eyes are obtained from.

The correlation between the staining of the CCC membrane and human ERM data from Rodrigues et al. is shown in Fig. 2. A good correlation is found for the staining of human ERM and the CCC membrane. Both porcine ILM and human ILM show positive correlation of staining with the PVDF filter. It is important to note, that in a study of Rodrigues et al.[7] several dyes stated to be dissolved in BSS (ICG, SB, CV, MV, CR) do not dissolve in this buffer, which means that the solutions used for the experiments were in fact suspensions. This can affect the results of staining, making the data less reliable. For the above dyes which were not soluble in BSS we used solvents the dyes were dissolving in (PBS or water). It is important to remember that the evaluation of the color is done in a subjective way on a non-standardized scale.

It is remarkable that BBG and ICG, the two dyes used most frequently for ILM staining, also stain very strongly the filter.[2]

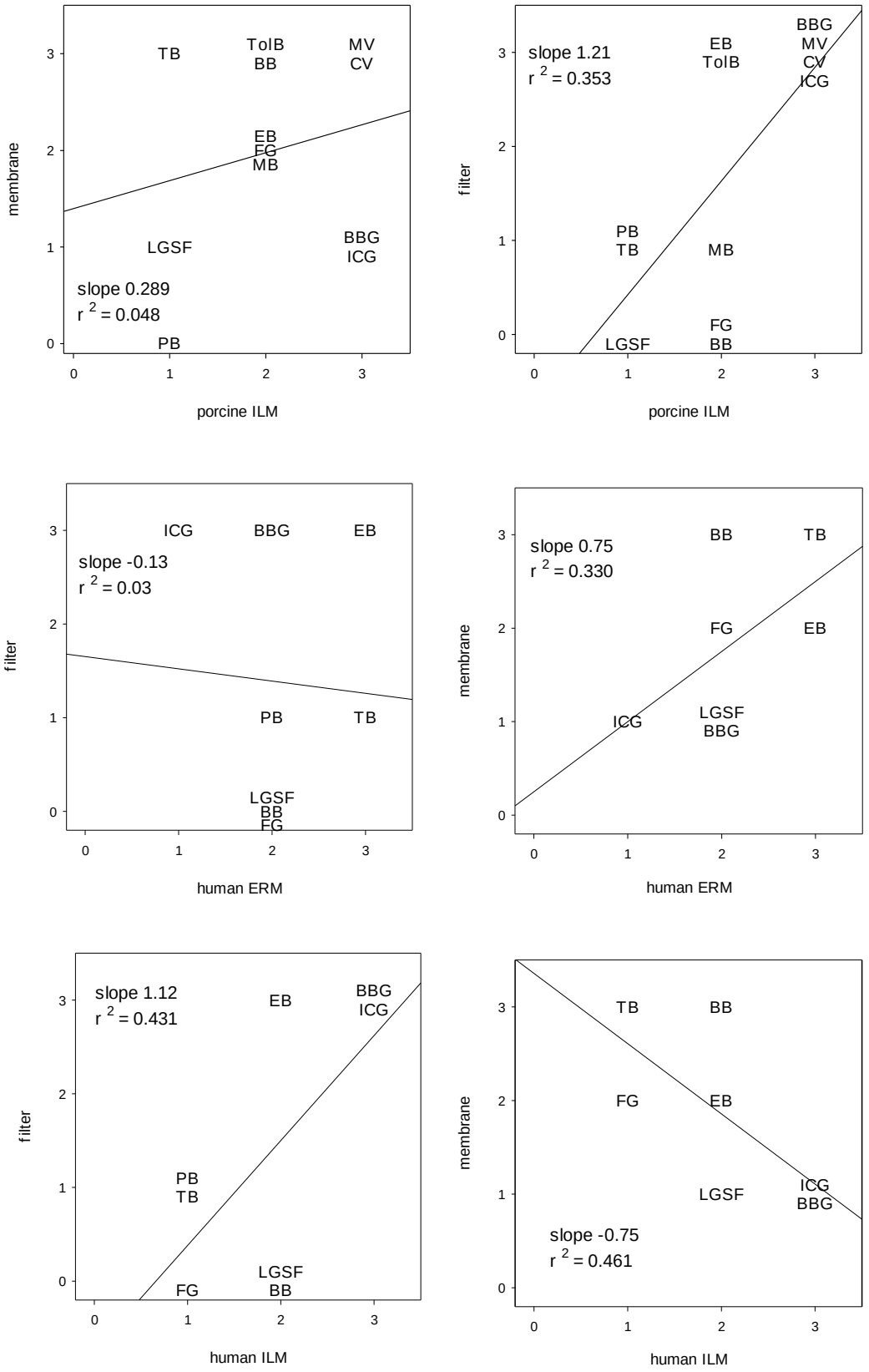


Figure 2. Correlation of staining between CCC membrane, PVDF filter, and biological tissues.

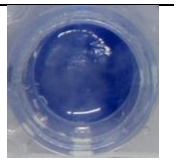
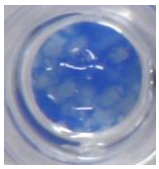
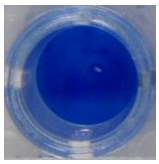
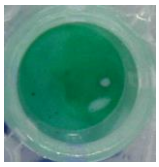
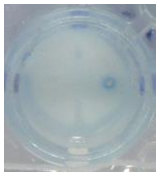
Dye	Membrane	Filter
TB		
BBG		
ICG		
BIP		

Table 1. Results of filter membrane staining with TB, BBG, ICG and BIP.

Enzymatic disruption of the vitreous

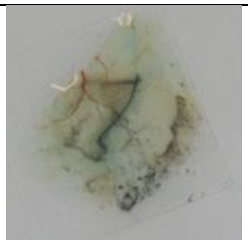
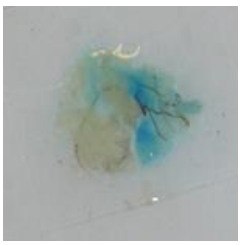
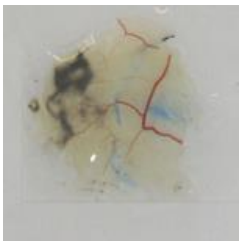
Dye	PBS	Activated plasminogen
BIP 0.1		
BIP 0.1 BBG 0.05		
BBG 0.05		

Table 2. Staining of pig retina with treatment or with treatment by activated plasminogen.

Staining of the pig retina with BIP is strong and clearly visible, whereas in the enzyme-treated eyes, staining is much reduced as mentioned above, there is no staining of the ILM with BIP in older patients,

BBG is known to stain the human ILM, although not necessarily homogeneously.[1] After incubation with Alteplase activated plasminogen we see patchy staining of the pig eye retina with BBG. Staining with BIP is weaker after Alteplase activated plasminogen treatment. We would conclude that BIP is not able to stain the ILM, but that it is able to stain parts of the vitreous.

Staining of pig eye retina preparations with BIP

In order to locate and identify the structures stained with BIP in the pig eye retina preparations, we made use of its fluorescent properties. A punch from the retina stained with TB 0.1% BIP 0.1% was placed on a cover slip and observed under a laser scanning microscope. Results are shown in Fig. 3.

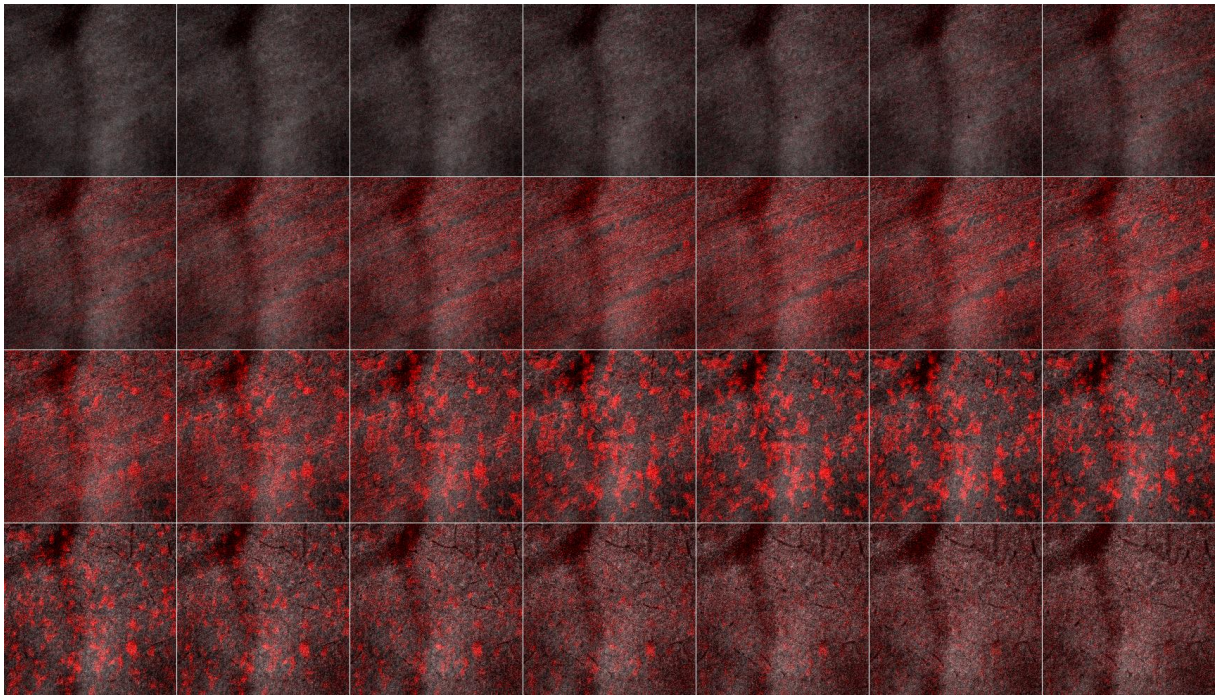


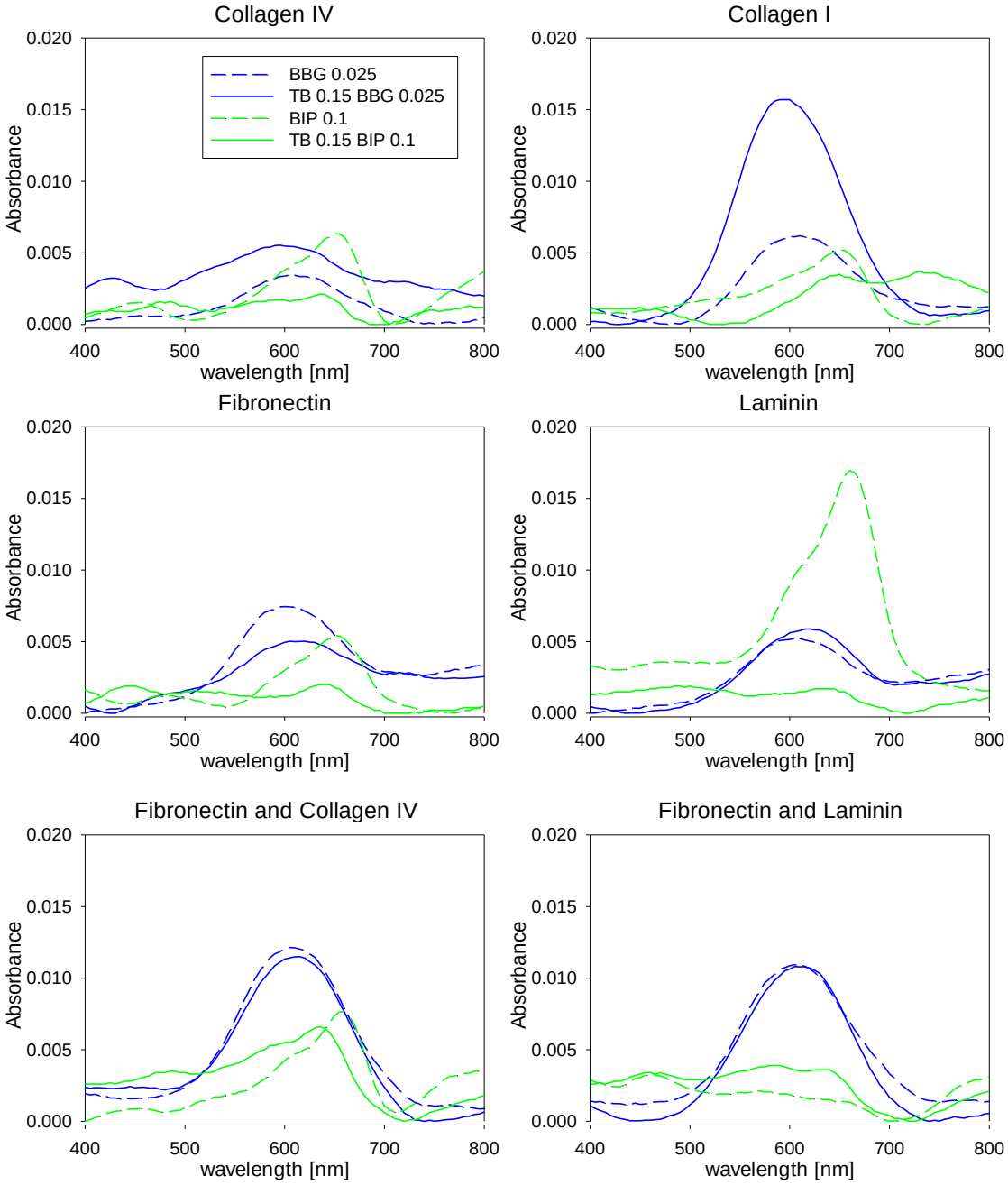
Figure 3. Staining of pig eye retina with TB 0.1% BIP 0.1%. From top left to bottom right increasing depth from the surface of the retina punch. The dark shadow observed is a blood vessel in the retina. Height of the stack: 54 µm.

Two different patterns stained by BIP are distinguishable: parallel bundles and patches. Such structures are known for the vitreo-retinal interface.[14] In the last picture blood vessel is almost, but not yet in focus. The total height of the stack was 54 µm. Assuming that the visible blood vessel is located in the choroid, patchy staining is observed around 10 µm above it. Considering that the staining is observed throughout 32 µm and the ILM thickness is at most a few microns in thickness, a different structure is stained with BIP.

We therefore assume that BIP does not stain the ILM, but rather the remnants of the vitreous (probably collagen fibers of an unspecified type) in the pig eyes.

Staining of proteins on surfaces

As the ILM consist of proteins and we presume that these proteins are stained, we checked whether proteins adsorbed to the plastic surface of 96-well plates could be stained. For this, individual proteins and protein mixtures were tested. Results are shown in Fig. 4. Absorbance peaking at around 600 nm is due to the blue dyes BBG and TB, whereas absorbance peaking at around 650 nm is due to BIP.



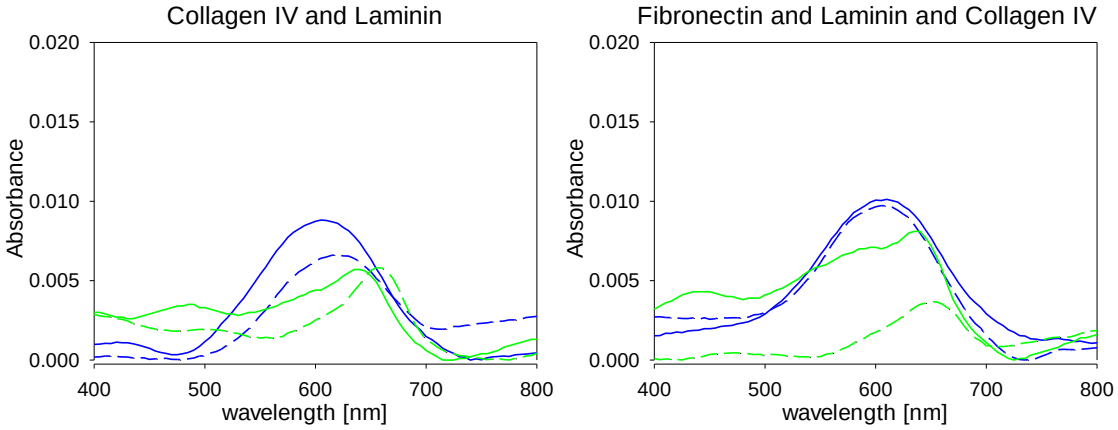


Figure 4. Spectra of the protein coated wells incubated with the dyes.

Consistent staining (with the exception of collagen IV) is seen for BBG. The addition of TB seems to have a limited effect on the adsorbed blue color, with the exception of collagen I.

Combination of TB and BBG seems to stain the protein coats very similarly to BBG alone, with the exception of collagen I, which is stained much more by TB+BBG than by BBG alone. TB is known to stain the ERM well and it is reported that ERM contains newly formed collagen type I. [15]

For BIP, strongest adsorption is seen to laminin. TB does not to enhance the staining much for any of the proteins, with the exception of the mixture of fibronectin, laminin, and collagen IV. Remarkably, the strong staining by BIP of laminin is abolished by the addition of TB. The combination of laminin, fibronectin and collagen IV is stained very weakly by BIP alone, which could explain why this dye did not stain human ILM in a patient.

Discussion

Vitreoretinal surgery is performed most frequently in elderly patients. The perceived most realistic, readily available model for staining of the ILM has been eyes from pigs obtained from slaughterhouses. As these animals are at a rather young age, the attachment of the vitreous to the posterior pole is still very strong. Any staining therefore is most probably caused by staining of the remaining vitreous.

The use of plasmin to partially digest collagens has been suggested before to facilitate the separation of vitreous from the ILM.[12] We found that treatment with plasminogen activated with Alteplase did not lead to complete detachment of the vitreous, and staining results still reflect most often the staining of the remaining vitreous.

One model for the ILM has been proposed in the literature, which consists of a lipid membrane with incorporated proteins.[16] The surface area of the lipid and the surface pressure were investigated for pure dipalmitoyl phosphatidylcholine, and after addition of retidyne (a mixture of lutein and BBG). The dye seems to have a considerable influence on the surface pressure, and therefore might be surface active. As no other dye has been tested, it is not clear how the response of the model system relates to successful staining of the ILM.

The ILM is a rather homogeneous, non-pathological structure. Its major proteins are type IV, VI and XVIII collagens, laminin, nidogen, agrin, and perlecan.[10,17] Collagen IV is the major protein in humans (reaching over 50% in adult human ILM).[17,18] In older human eyes, fibronectin and laminin are more pronounced than in younger eyes.[19] Age-related changes include increase of collagen IV and agrin relative concentration. Additionally, ILM steadily becomes thicker with aging, varying from 70 nm in fetus to few microns at the age of 90. [17]

The different epiretinal membranes have a higher degree of diversity.[9,15] The cellophane macular reflex membrane contains little collagen type I and type II, whereas the preretinal macular fibrosis membrane is rich in either collagen I or collagen II. Both types contain collagen IV, fibronectin, and laminin. ERM from idiopathic macular holes contain collagens I, III, and V.[15] Collagen I protein coat is stained strongly by TB+BBG, but not with TB+BIP. TB+BIP stains all the coats weaker than TB+BBG. TB+BBG stains all the coats similarly except for fibronectin coat, which is better stained with BBG alone. As ERM differ so much depending on their origin, better understanding of the ratio between the proteins would be needed to design a coat for reliable staining investigation. Surprisingly, despite the diversity of proteins contained in different ERM, CCC membrane that is pure collagen I, shows very good correlation to staining observed in patients.

Fluorescent BIP binds in retinal preparation to a three-dimensional structure that shows two different patterns: parallel bundles and patches, which most likely are parts of the vitreous; the patches would be where the fibers are connected to the retina. We suppose this structure is collagen II fibers, previously observed and reported by Breazzano et al.[20] Collagen II can be present in the vitreous remains left on the retina after mechanical vitreous removal from the pig eye.

We were very surprised that the non-physiological PVDF filter turned out to be a good model for the human ILM. On the basis of the limited number of dyes tested in patients, we are inclined to see this filter as the best model for the human ILM available so far.

Less surprising is the observation that the collagen CCC membrane is a reasonable model for the ERM. The ERM contains collagens I, III, and V[15], and the CCC membrane consists of pure collagen I.

Searching for new potential dyes for vitreoretinal surgery made us aware that there is a great need for new models for staining, as porcine eyes can be deceiving, due to vitreous remnants on the ILM. We therefore propose two different models, strongly believing that they can serve well for this purpose and improve development and investigation of dyes.

Acknowledgments

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Paper III

Additives for improving staining in retinal surgery

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Abstract

Purpose: To investigate viscosity and density adjustments with additives for dye formulation and their relation to sinking behaviour. To study staining prevention by polymeric agents.

Methods: Toxicity of additives was tested on ARPE-19 cells. Staining prevention was tested on polyvinylidene difluoride filter and collagen I membrane, models for Inner Limiting Membrane (ILM) and Epiretinal Membrane (ERM) respectively. Sinking of dyes with additives was recorded when dyes were injected slowly to phosphate buffer saline filled photometric cuvettes. Viscosity, density and osmotic pressure were tested for different additives concentration.

Results: Density and viscosity increase is not simultaneous and depends exclusively on the additive. Sinking of the dyes is more ruled by the density than viscosity of the solution. Polyethylene glycol prevents the staining of model for ERM with Trypan Blue alone, but it does not prevent the staining of ILM and ERM models with Trypan Blue + Brilliant Blue G. Staining of models is reduced for Indocyanine Green with addition of Kolliphor HS-15, Kolliphor RH-40 and Kolliphor EL. Diglycerol and mannitol show no toxicity to ARPE-19 cells up to 20% concentration. Isosorbide and Isosorbide dimethyl ether are cytotoxic to retinal epithelium at 10% and 5% concentration respectively.

Conclusion: Diglycerol was identified as new additive for staining in vitreoretinal surgery. It adds to the viscosity and density of the dye solution and is safe to use.

Introduction

In retinal surgery, dye solutions are injected into the eye after the removal of the vitreous body. With an air-filled eye, the solutions can come into direct contact with the internal limiting membrane (ILM). When the eye has been filled with salt solution after the removal of the vitreous, the dye solution must sink to the bottom of the liquid-filled eye and thus get in contact with the ILM and, if present, the epiretinal membrane (ERM). For this, different suggestions have been made in the literature: 5% glucose,[1] mannitol (Arcadophta), polyethylene glycol (PEG) (DORC),[2,3] heavy water,[3] polyvinylpyrrolidone,[3] iodine-containing contrast agents.[4] While the polymeric agents add little to the osmolarity of the solutions, they increase the viscosity considerably. With low-molecular weight agents, viscosity is increased only moderately, but density can be increased considerably.

In the clinical situation, it is not quite clear how much viscosity and density are interdependent, and which is more effective in aiding staining during surgery.

We have found that some of the agents, especially polymeric agents, can actually decrease the staining with trypan blue (TB) and indocyanine green (ICG). Therefore, we were curious whether this pertains to other polymers as well. Also, we looked into the relative importance of density and viscosity on the sinking behavior. For increasing density and/or viscosity, we used a variety of polymeric and low-molecular weight additives, among them excipients which are used in drug preparations.

Excipients are used in the formulation of drugs, especially when the drug is of limited water solubility. Such excipients are surface active. They have been used to stabilize solutions of ICG. [5]

During this investigation, we also found out that some excipients can reduce or even prevent staining of the ILM and of models for ILM and ERM. We have therefore searched for biocompatible additives to dyes which allow to adjust density or viscosity.

Osmolarity has been considered a major factor with respect to cell toxicity. We had shown before that hyper- or hypo-osmolar solutions do not lead to cell damage within a considerable range.[6] As several of these excipients are known to be potential cryo-protectors, we also investigated the apparent osmolarity, as measured by the depression of the melting point of water, as a function of concentration of several of these additives.

Materials and Methods

Materials

Polyethylene glycol, molecular weight 3350 (PEG) as 50% solution in water, WST-1 reagent, isosorbide (ISO), dimethyl isosorbide (DMI), ICG, and iodixanol (Optiprep, OP, as 30% solution in water) were from Sigma (München, Germany). Trypan Blue (TB) was from Carl Roth, Karlsruhe, Germany, . Diglycerol (DG) was a gift of Solvay (Bruxelles, Belgium). Kolliphor HS15 (HS15), Kolliphor EL (EL), Kolliphor P188 (P188), Kolliphor RH40 (RH40), and Kollidon VA64 (VA) were gifts from BASF (Ludwigshafen, Germany). Gadopentetate (Gdp) as a 1 M solution was from Ratiopharm (Ulm, Germany), Gadoterate and Gadovist from Bayer (Berlin, Germany).

CCC membranes were from Viscofan (Weinheim, Germany). MultiScreen BV Filter Plates were from Merck Millipore (Darmstadt, Germany).

Preparation of solutions

All solutions were prepared in PBS, except where stated, and contained the indicated amount of additive on a weight-per-volume basis. For the Gd-containing agents, the original (clinically used) solution was taken as 100%, and the dilution was on a volume-per-volume basis.

Staining of membranes

Circular punches (5 mm diameter) of CCC membranes were placed into individual wells of a MultiScreen BV Filter Plate and kept in place with a hypodermic needle. The membrane was soaked for 30 min with PBS, then drained by applying vacuum. The dye solution (50 µL) was applied and removed after 30 s by application of vacuum. The filter was washed three times with PBS, the membranes were placed in a new 96-well plate and photographed. Then the filters were cut with a biopsy punch and placed into a 96-well plate. A mixture of DMSO (90%) and PBS (10%) was added to each of the two plates, and spectra of each well were recorded.

Viscosity and density

Viscosity was measured in an Ubbelohde viscometer. Density was measured in a 5-mL pycnometer.

Cell toxicity

Toxicity in ARPE cells was measured after a 30-minute exposure to the additives, as described elsewhere.[6,7]

Sinking of dyes

For recording the sinking behavior, regular 1-cm photometric cuvettes were filled with PBS at room temperature. From a multichannel pipet, the solutions (all containing 0.1% TB for proper visualization) were injected slowly, and the sinking was recorded with a camcorder.

Osmotic pressure

Osmotic pressure was measured with a Gonotec Osmomat 030 (Berlin, Germany).

Results

Viscosity and density

Figure 1 shows the interrelation between viscosity and density for different additives to PBS solutions. Gdp increases solely the density, whereas polymers such as PEG increase mostly viscosity. For OP, both parameters increase simultaneously. While at lower concentrations, one would have expected density and viscosity to increase linearly with concentration, this is not valid for higher concentrations. Deviations are such that viscosity increases more than density. DG alone shows this behavior, and also the mixture of 20% DG with OP. The highest viscosity was found for the Kollidon and Kolliphor polymers: 5% VA gave a very viscous solution with very little increase in density.

By combining a polymeric agent with an agent increasing only the density of a solution, any desirable density-viscosity combination can be obtained.

Of the substances tested, DG and OP were the ones which could increase both viscosity and density at the same time; in comparison to OP, DG was equally effective in increasing density, while viscosity was increased considerably more than with OP.

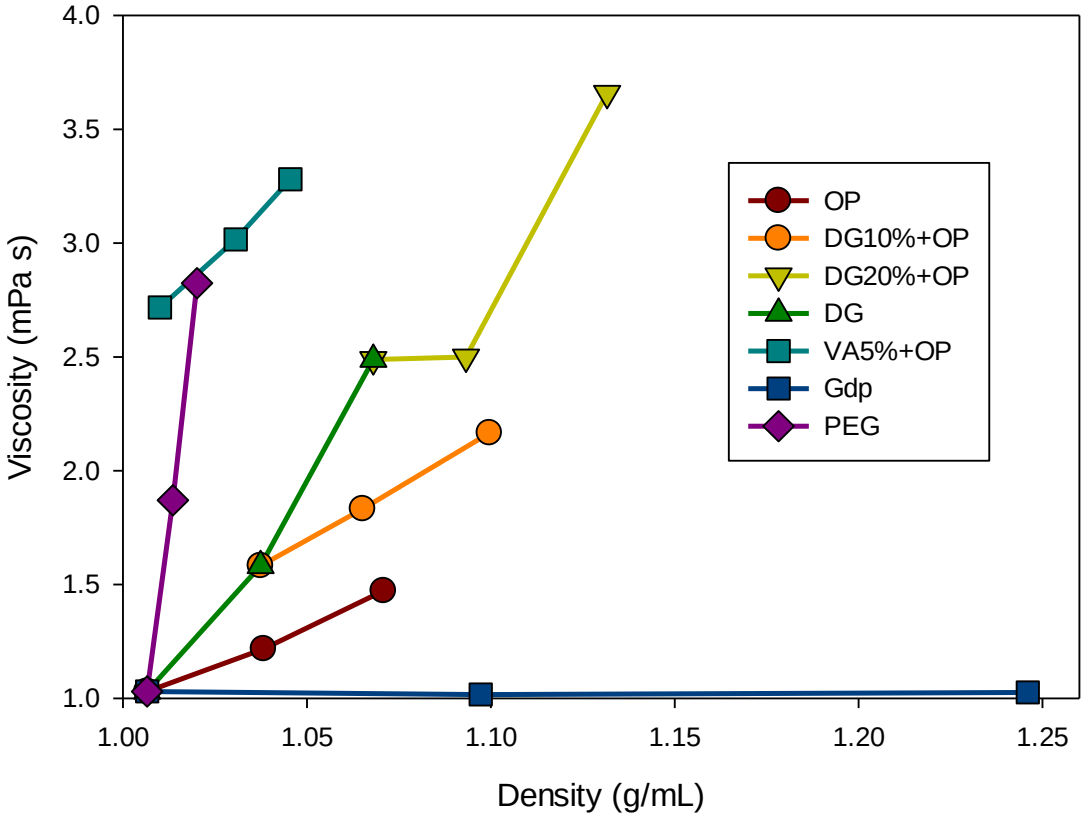


Figure 1. Viscosity and density of PBS solutions containing additives. Data with a percentage given contain the excipient in the given concentration, with varying concentrations of OP (0%, 10%, 20%). Concentrations of individual excipients: DG: 0%, 10%, 20%; Gdp: 5%, 10%; PEG: 5%, 10%.

Sinking of combinations

We found that the sinking of different preparations was more governed by the density of the solution than its viscosity (see Fig. 2). Gadovist, which has a viscosity very similar to PBS sank fastest. However, once the density exceeds about 1.03 g/mL, sinking was sufficiently fast to ensure that the bottom of the cuvette (path of about 15 mm) was reached within about 3-5 seconds.

Viscosity plays some role in how much each droplet would spread on the way to the bottom. Nevertheless, all additives tested allowed the supernatant to be free from dye.

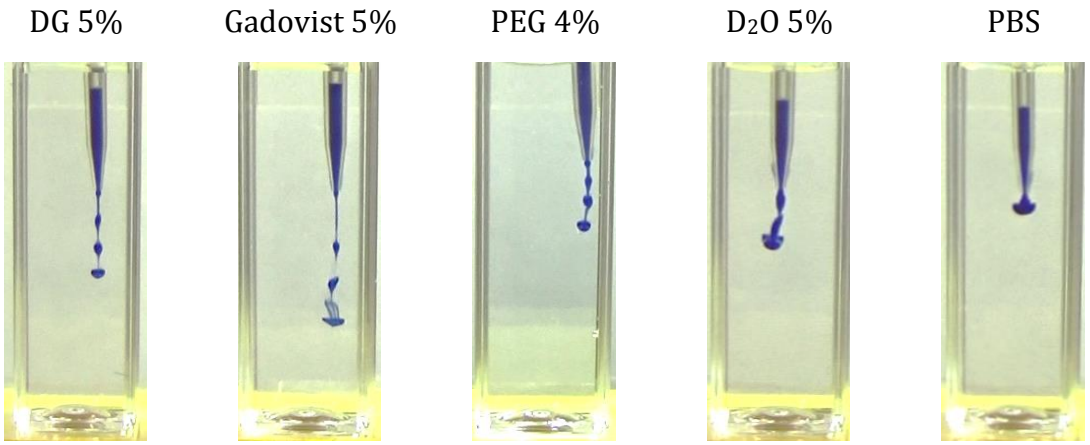


Figure 2. Photos of dye solutions sinking into PBS. The photos are at identical times about 2 s after the start of the injection.

Staining on filter/membrane

PEG reduces staining of the CCC membrane by TB (0.15%) alone and combination with BBG (0.025%) in a concentration-dependent fashion (Fig. 3). BBG led to stronger staining than TB alone. The filter was stained only by the combination of TB+BBG, and its intensity was not influenced by PEG. Numerical values of the staining intensity are found in Fig. 4

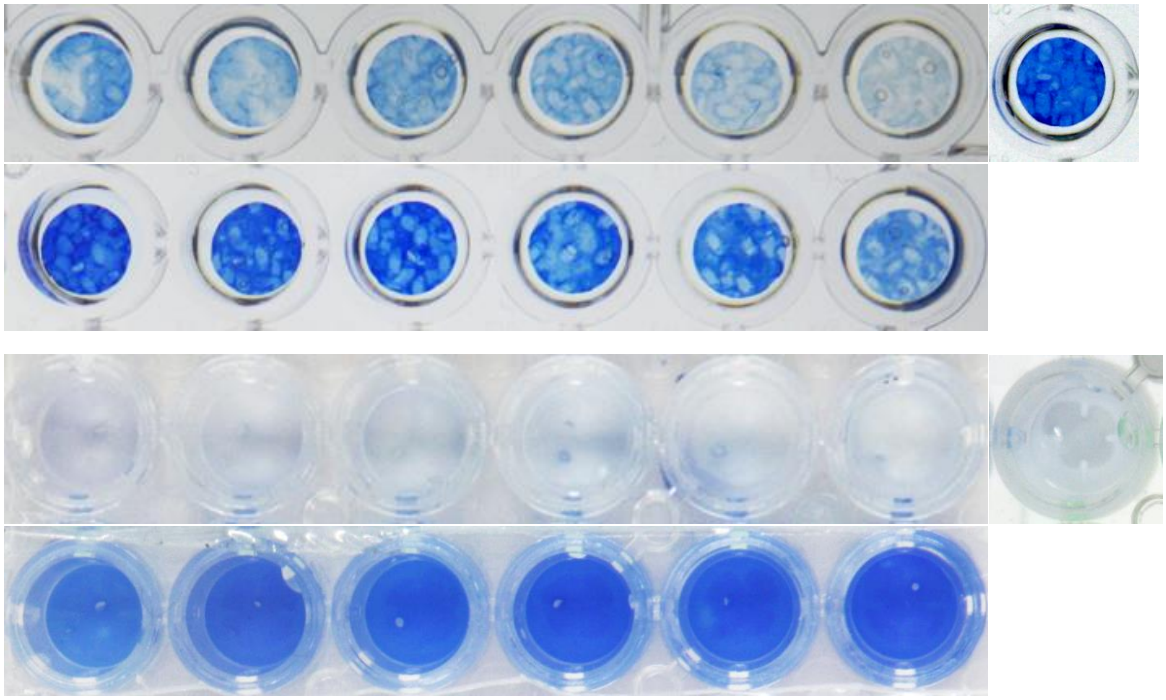


Figure 3. Staining of CCC membranes (top) and filters (bottom) by TB (top part of each picture) and TB+BBG (bottom part of each picture) as a function of PEG concentration (from left to right: PBS, 1%, 2%, 4%, 8%, 16%). For TB alone additionally on the right pictures for Gdp 5%.

With Gdp at 5%, staining of the membrane with TB alone is increased.

The Kolliphors and Kollidon tested, at concentrations of 5%, abolish almost completely staining of the membrane with TB (data not shown). Also the staining of the ERM in

patients by TB was completely lost when these polymeric excipients were present (see below).

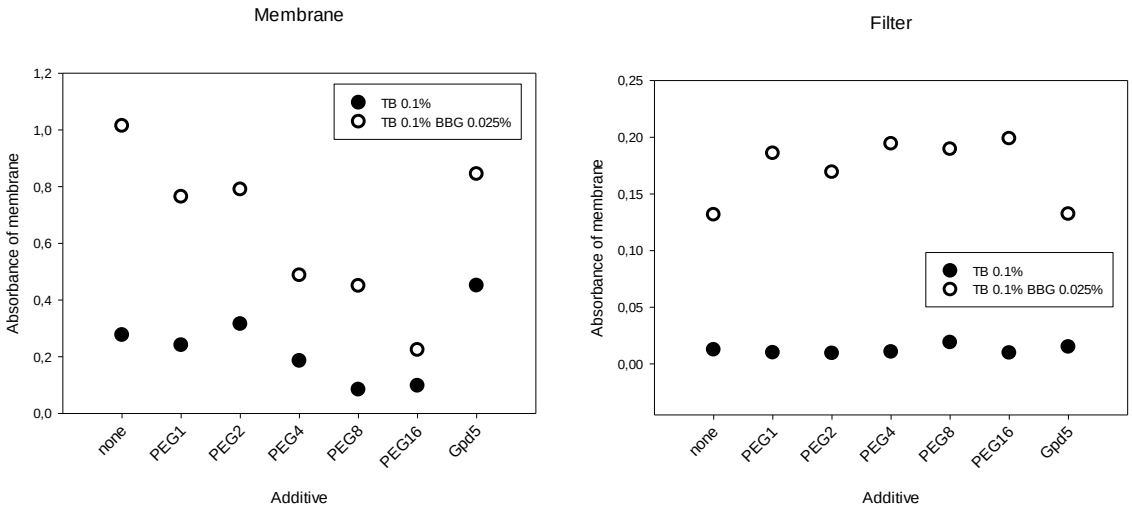


Figure 4. Density of staining of CCC membrane with 0.15% TB alone or with 0.15% TB and 0.025% BBG in the presence of different additives. Note that the values for TB alone and TB+BBG were set to 100%, and the subsequent values are related to these two values.

ICG

Staining of the PVDF filter and CCC membrane by ICG is reduced with the addition of HS15, RH40, and EL for ICG (see Fig. 5).

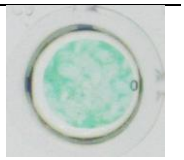
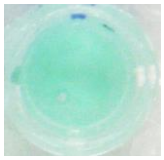
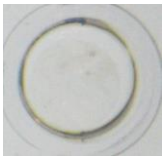
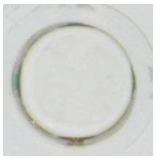
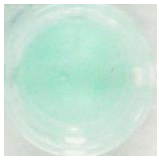
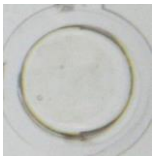
Additive	Filter	Membrane
Glucose		
HS15		
RH40		
EL		

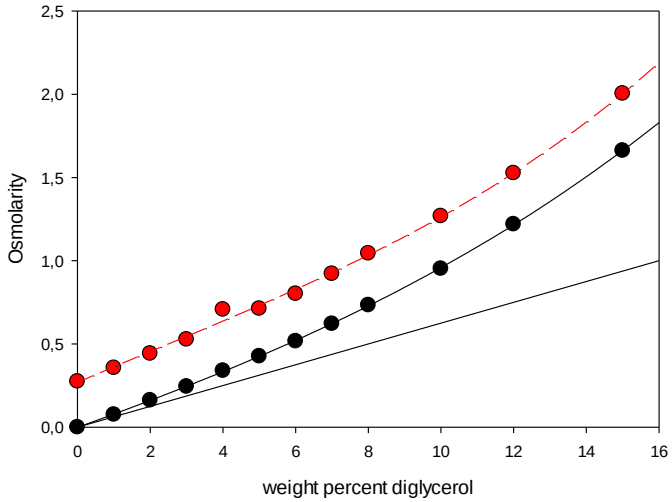
Figure 5. Staining of filter and membrane with ICG (0.1%) with different additives. All additives are present in a concentration of 5%.

While the ICG solutions with the polymeric additives are stable, their staining ability is greatly reduced (to the level of around 15%). In view of the weakly visible staining by ICG solutions without additive,[8] the reduction of the staining by the excipients is too great for a useful application in vitreoretinal surgery. Therefore, other properties (toxicity, viscosity etc.) were not investigated further.

Osmotic pressure

Osmotic pressure has been discussed as a major component of cytotoxicity. Usually it is measured by recording the depression of the freezing point of the aqueous solution. For DG, we found that the depression of the freezing point is not linearly dependent on the concentration of DG, but has a pronounced second virial coefficient both in pure water and in PBS (Fig. 5). The measured osmolality is thus higher than that based on the actual concentration of DG.

For PEG, this is even much more pronounced. Most of the measured increase in osmolality (i.e. decrease of melting point of water) is caused by abnormal behavior. For ISO, although there is a non-zero second virial coefficient, it is too small to be of relevance.



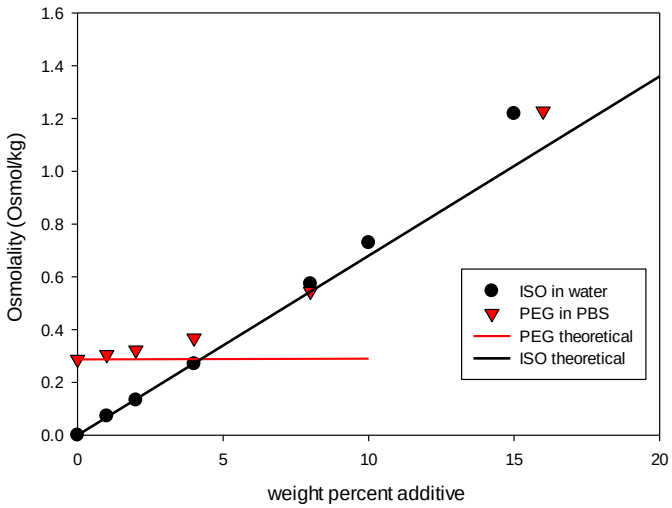


Figure 5. Measured osmolality of diglycerol in water (solid line) and in PBS (dashed line). The theoretical osmolality as a function of diglycerol concentration is given by the straight line.

In ARPE cells (see next page), even extreme hypoosmotic pressure has only a small influence on cell survival.

Cytotoxicity

DG and mannitol have no effect on the cell survival even after exposure of ARPE cells to 20% DG for 30 min (Fig. 7). Glycerol and other small molecules are able to penetrate cell membranes through aquaporins.[9] It is not known whether such porins also allow the passage of diglycerol. Thus, we cannot attribute the lack of cytotoxic effects of DG at high concentrations to the possible penetration of diglycerol through the cell membrane, so that no change in osmotic pressure is sensed by the cell.

Mannitol up to 20% has been tested on ARPE cells by others,[10] and also there no cell toxicity was found. More surprising was that in our study the absence of salt or additives did not lead to significant reduction in cell survival.

Isosorbide and isosorbide dimethyl ether are cytotoxic at concentrations above 10% and 5%, respectively. This observation is surprising, as it the substance "generally recognized as safe" (GRAS) by the FDA. It is, however, an osmotic diureticum.[11] Toxicity is most probably not due to an osmotic effect, as mannitol up the the highest concentration tested (20%) is not cytotoxic.

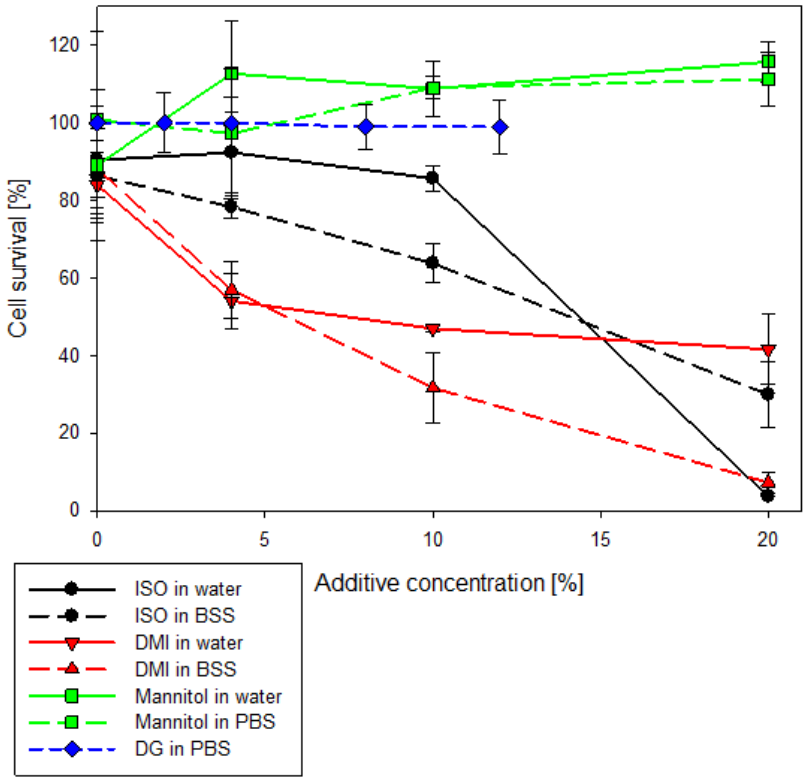


Figure 7. Cell survival as a function of selected additives

Stabilization of dyes by excipients

ICG does not form stable solutions, and just before a treatment it has to be prepared freshly. Excipients have been used to stabilize solutions of ICG.[5] HS15 at 5% concentration stabilized ICG (concentration 0.5%) for long-term storage. This micellar solution did, however, not stain membrane or filter, as shown above. The same was true for solutions of TB and excipients (HS15, EL, VA64, both at 5% concentration).

Discussion

A number of additives have been suggested before for making dyes for vitreoretinal surgery "heavy".[1] Some of the commercially available additives, together with additives suggested in patents, are compared here to several new additives, namely low-molecular weight DG and Gd complexes, and high-molecular weight excipients. Several

of the new additives are used clinically, so they should in principle not present toxicity problems.

In this study, we found that polymeric agents reduce or prevent staining of our test system for ILM and ERM with dyes. The reason for this could be an interaction of the dyes with the polymers, which are known to be surfactants. It is this property which is utilized when hydrophobic drugs require improved solubility. In the case of P188, we could detect this interaction in the visible spectrum of TB in the solution with the polymer, which is caused by a solvatochromic effect.[12] Also the widely used polymer PEG reduced the staining of the ERM model by about 50%.

Low-molecular weight compounds did not exhibit this effect. As, however, their molar concentration for a given weight concentration is higher than that of a polymer, the osmolarity of the solution will increase. Among the tested low-molecular weight compounds, only diglycerol appeared not to contribute to the osmotic pressure felt by the cells, as it might penetrate freely through the cell membrane.[9] It should be noted that DG, as well as the other polymers tested, have a non-negligible second virial coefficient of the concentration-viscosity relation; this should be kept in mind when preparing physiologically compatible solutions with these additives.

Most of the additives were well tolerated by ARPE cells. Surprisingly, ISO and DMI showed considerable cell toxicity, and thus cannot be used in vitreoretinal surgery. This is in stark contrast to the FDA classification, which lists them as GRAS ("generally recognized as safe"). The FDA classification refers to oral ingestion, and not to direct and prolonged (30 min) cellular contact.

For further clinical exploration, DG appears to be the best of the new additives tested here.

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Paper IV

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New stains for anterior capsule surgery

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The authors have no financial interest in the work described. Andreas Mohr and Detlef Gabel have filed a patent on the use of BIP in ocular surgery.

Abstract

Purpose: To investigate whether new dyes and dye combinations can give equivalent or better staining in anterior capsule surgery than existing dyes, with low degree of toxicity on relevant cells.

Setting: University laboratory of Jacobs University Bremen, Germany

Design: Laboratory experimental study

Methods: Pig eyes were collected post mortem. Cataract was induced by microwave irradiation. Access to the lens capsule was through open-sky surgery. Staining was performed and results were documented by photography. Toxicity of the dyes was evaluated in three different cell lines, immediately after exposure and with a delay of 24 h, with exposure in the dark or subsequent strong illumination.

Results: A new cyanine dye, BIP, was found to lead to green staining, with reduced toxicity on corneal endothelial cells. Staining could be further enhanced by combining it with trypan blue. Methylene blue was very toxic, whereas its combination with trypan blue was much less toxic.

Conclusion: With BIP alone or in combination with trypan blue, safe staining of the capsule can be achieved, resulting in a green color.

Introduction

In ocular surgery where the anterior lens capsule requires opening, the usual procedure requires a transparent lens and vitreous. When the lens or the vitreous is not transparent, the red reflex from the fundus required for proper visualization of the capsule is absent. In these cases, staining of the anterior capsule is indicated.

The most widely used stain for this is trypan blue (TB) in concentrations around 0.06% [1]. Other dyes suggested were indocyanine green (ICG) [2], light green SF (LGSF) [3], phthalocyanine tetrasulfonate [3], bromophenol blue (BB) [3], Chicago blue [3], acid violet 17 (AV) [4], and gentian violet [5]. The dye brilliant blue G (BBG), which is widely used for staining the ILM, has not found much application in the staining of the anterior chamber [6]. Only one combination of dyes, lutein or zeaxanthin together with BBG, has been investigated for staining of the anterior capsule [7].

We wanted to investigate whether there are other stains or other combination of stains which could be potentially useful for staining of the anterior chamber for capsulorhexis. We focused especially on dyes giving a color different from blue, and on dyes where lower concentrations could achieve adequate staining.

For use in the anterior chamber, any agent must necessarily have only a minimal impact on the corneal endothelial cells, as damage to these cells would lead to visual problems and eventual necessity of a corneal transplant. We have therefore investigated the toxicity of stains with appropriate staining properties. The toxicity investigation was carried out in an immortalized human corneal endothelial cell line (HCEC), the permanent pigment epithelium cell line ARPE19 (ARPE), and the HeLa cell line. The pigment epithelium cell line was selected as it has been used widely for investigating the toxicity of dyes for use in vitreoretinal surgery, and the HeLa cell line was used for its sensitivity to toxic agents.

Materials and Methods

Dyes

TB was the commercially available product from Carl Roth (Germany), which contains about 80% dye (including some pink impurities). MB (methylene blue) and MeB (Methyl Blue) were from Sigma-Aldrich. BIP (2-[5-[3,3-dimethyl-1-(4-sulfobutyl)-1,3-dihydro-indol-2-ylidene]-penta-1,3-dienyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium sodium, CAS number 120768-44-7) was from FEW, Wolfen, Germany. ICG was from Pulsion (Germany).

The chemical structures of the dyes are shown in Fig. 1.

ICG was dissolved as an 0.5% solution in water before dilution with Balanced Salt Solution (BSS).

All other dye solutions were prepared in PBS, in percent concentrations (w/v) given for the individual experiment (0.06% = 0.6 mg/mL).

Staining

Pig eyes were taken as models for staining. Cataract was induced, the cornea was removed, and the dye was applied to the exposed lens.

Induction of cataract

Eyes from the local slaughterhouse were freed from muscles and the extruding optic nerve. They were kept on ice until used. The cornea of the eye was dipped into glycerol, in order to avoid the cornea getting opaque during the subsequent heating. Cataract was induced by placing each eye individually in a microwave oven for 8 seconds at 800 W, similar to the procedure in the literature [8].

Access for staining

For access, staining, and recording, the eye was kept in place by vacuum in a holder. The holder consisted of a 3-cm Büchner funnel, filled to about two thirds with small glass beads, which were covered by a piece of household wrap into which a few holes were punched. Dynamic vacuum was applied.

Open sky surgery was performed by punching out a circular 10-mm hole with a biopsy punch. The cornea was detached completely with scissors.

Staining of lens capsule

The opened anterior chamber was first rinsed with PBS. A few drops of stain (about 0.05 mL) were applied and washed away with PBS after about 10 seconds. The lens capsule was cut with a sharp needle in order to make visible the contrast between stained capsule and unstained lens.

Setup of documentation

The procedure of access and staining was carried out under a trinocular stereo microscope with a zoom lens (Euromex StereoBlue Trino Zoom) and an LED ring illumination (Euromex EuroLED 144, both from Mikroskop-Shop24, St. Ingbert, Germany); the zoom was set to 0.7 magnification. Pictures were recorded with a microscope camera (C-Mount Digital Kamera Model MC7, OCS.tec, Neuching, Germany) attached to the C-mount of the microscope.

Camera control was through ImageView version 3.7.6701. Fixed white balance was used. White balance was achieved by placing a white piece of paper under the microscope and adjusting automatically the white level. This level was used throughout the day. Exposure was done with a fixed exposure time. This was adjusted manually with a printed pattern.

Toxicity in cell culture

Toxicity of the dyes with staining ability was measured on HCEC cells (DSMZ cell line HCEC-12, ACC 646). Toxicity was also assessed in HeLa cells, as these cells were found to be the most sensitive cells to dyes. As reference, ARPE cells (ATCC, LGC Standards GmbH, Wesel, Germany) were taken, for which toxicity for other dyes has been tested extensively before.

Permanent cell lines were chosen as the ISO norm for medical devices clearly prefers permanent cell lines over primary cell lines [9].

For toxicity measurements, the procedure described before [10] was followed. Cells were grown to confluency in 96-well plates. Medium was removed, and the cells were exposed to the dye solution for 30 minutes at 37°C. The dye was washed away with

PBS, and cells were assayed for survival either immediately or with a delay of 24 hours. For experiments with light exposure, the cells, after having been washed, were exposed to an LED light panel for 15 minutes, and then assayed either immediately or after 24 hours.

WST-1 (Sigma) was used as assay for cell toxicity. The control for all cells was exposure to PBS, and light where indicated. Survival is given as percent of the control cells.

Experiments (each experiment consisted of six wells exposed simultaneously) were carried out in triplicates.

Statistical analysis

Viability of three cell lines exposed to dyes was measured on several 96-well plates after four different types of exposure: immediate dark, immediate light, delayed dark and delayed light.

A linear model with cell survival as dependent variable and dye as independent variable was fitted to the data. Since measurements for each dye were taken from different plates, the data can be seen as clustered; therefore, stochastic independence of the observations cannot be assumed. This is accounted for by means of a cluster-robust variance-covariance matrix. For each type of exposure, the mean viability of HCEC cells exposed to TB06 was compared to the mean viability of all other dyes, respectively, resulting in a Dunnett-test to adjust for the multiplicity that arises from simultaneously testing more than one hypothesis. Moreover, the Bonferroni-procedure was applied to account for multiplicity with regard to the two time points (immediate and delayed).

Using a significance level of $\alpha = 5\%$ the multiplicity-adjusted p-values are reported for each type of exposure. For descriptive purposes, similar comparisons were done with the other two cell lines.

The data analysis was conducted in R using the multcomp-package for multiple comparison procedures and the multiwayvcov-package for the calculation of the robust covariance matrix.

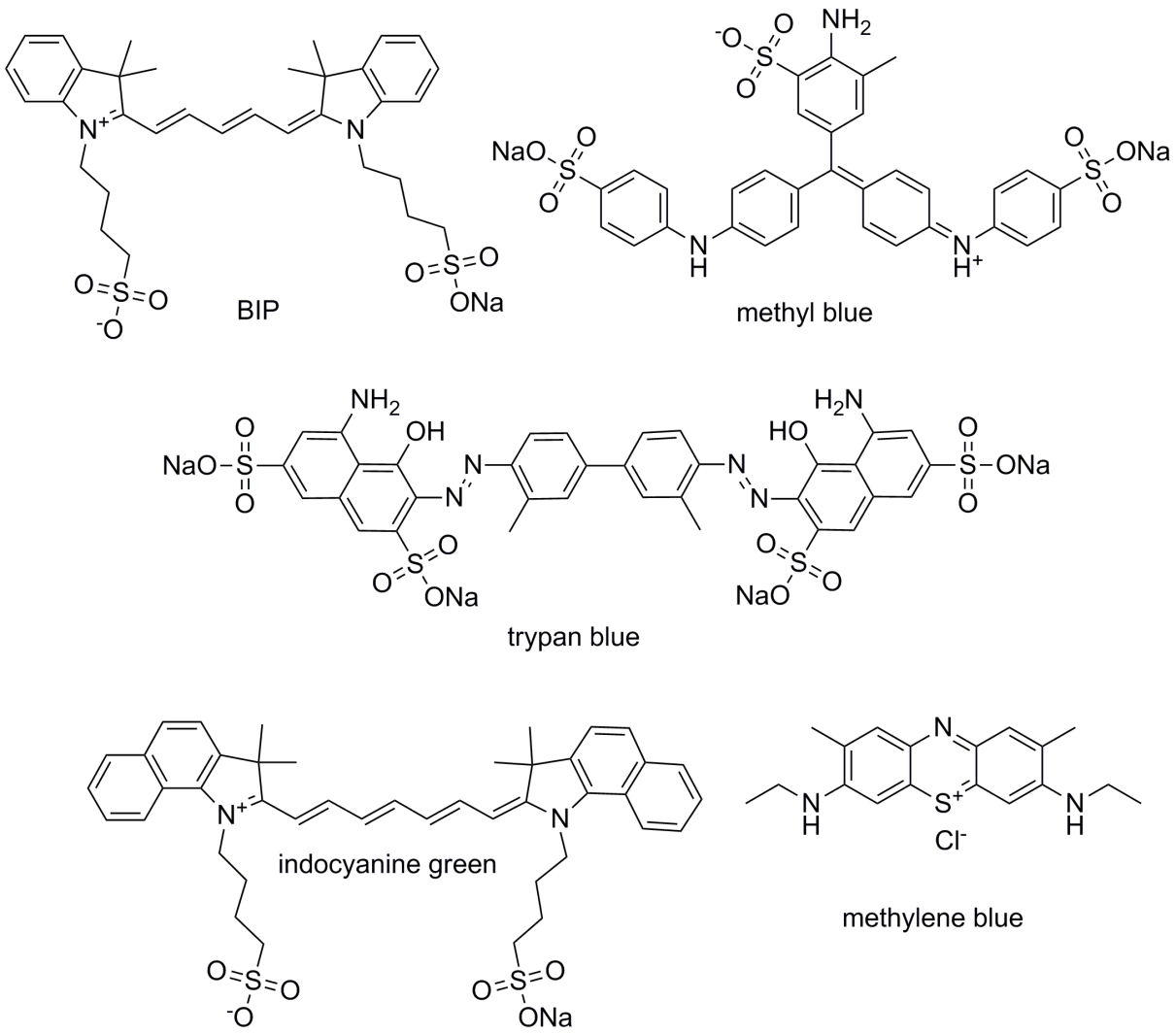

Figure 1. Structures of the dyes in this study

Results

Cataract induction

The protocol developed by van Vreeswijk and Pameyer [8] was used, consisting of heating eyes in a microwave oven. A power of 800 W and an exposure time of between 7 and 8 seconds was found to induce cataract efficiently. The time required depended slightly on the weight of the eye (between 6.5 and 7.9 g). Longer exposure resulted in explosion of the eye.

Staining

Table 1 lists the dyes and dye combinations which were used for staining the lens capsule. BIP stains the capsule in green, as do dye combinations with BIP as major component and TB as minor component. More TB than BIP leads to blue staining. MB in combination with TB results in reddish staining, whereas TB with MeB results in blue staining.

While the combinations of MB and MeB with diluted TB were sufficient for staining, MB and MeB alone did not stain the capsule. The weakest staining was observed for ICG.

Table 1. Overview of all dyes and dye combinations which were tested.

Dye	abbreviation	Staining intensity	Color of stain	Toxicity compared to TB 0.06%
TB 0.06%	TB06	(baseline)	blue	(baseline)
BIP 0.06%	BIP06	about as baseline	green	less toxic
BIP 0.03%	BIP03	much less than baseline	green	less toxic
TB 0.015% BIP 0.015%	TB015BIP015	about as baseline	blue-green	about as baseline
TB 0.015% BIP 0.03%	TB015BIP03	stronger than baseline	green	slightly less toxic
TB 0.03% BIP 0.015%	TB03BIP015	about as baseline	blue	about as baseline
MB 0.025%	MB025	no staining	n.a.	strongly phototoxic
TB 0.06% MB 0.025%	TB06MB025	stronger than baseline	reddish blue	about as baseline
TB 0.03% MB 0.025%	TB03MB025	much less than baseline	pink	about as baseline
MeB 0.025%	MeB025	no staining	n.a.	about as baseline
TB 0.06% MeB 0.025%	TB06MeB025	much stronger than baseline	blue	slightly more toxic
TB 0.03% MeB 0.025%	TB03MeB025	about as baseline	blue	more toxic
ICG 0.5%	ICG5	much less than baseline	green	slightly less toxic

Stability of BIP solutions

BIP was soluble in PBS, and the solution was stable for more than one year. This is in clear contrast to ICG and DSS (first described by Haritoglou et al. [11]) a dye similar in structure to ICG. ICG is available only as powder, and solutions must be made up fresh every day.

Toxicity

The HCEC cell line was chosen as the most relevant permanent cell line for the staining of the anterior capsule. ARPE cells have been used extensively by us and others to record toxicity of dyes used in vitreoretinal surgery, and we used it to cross-check the toxicity of the new dyes. We included also the HeLa cell line, as we found in orienting experiments that this cell line is much more sensitive to dyes and other agents immediately after exposure than both of the former cell lines.

TB at 0.06% did show some toxicity, which was also observed before for ARPE cells [10]. Toxicity was most pronounced in HeLa cells when assayed immediately after exposure, but less so in HCEC cells, and even less in ARPE cells. BIP was found not to be toxic (when used alone, it was less toxic than TB), and also its combinations with TB were not more toxic than TB.

Toxicity of MB was considerable, already in the dark. This is not surprising, as it is known to have photodynamic properties, for which the ambient light might already be sufficient [12]. It was therefore a surprise that its combination with TB 0.06% was much less toxic.

In general, HeLa cells showed a considerable depression of cell activity, independent of the dye used. This was most pronounced immediately after exposure. The sensitivity of HeLa cells was much higher than that of ARPE cells (tested before) and that of the HCEC cells.

Table 2. Mean viability (in % of PBS) ± SEM (standard error of the mean) for the HCEC cell line.

HCEC	Mean ± SEM			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
TB06	73±4.4	74±4.1	68±2.9	64±3.3
BIP06	96±3.4	98±2.5	85±2.5	85±1.5
BIP03	95±2.9	100±3.1	87±2.7	90±2.2
TB015BIP015	99±3.6	98±2.0	81±3.9	61±3.4
TB015BIP03	99±3.2	101±2.5	83±2.3	64±4.0
TB03BIP015	92±3.1	91±2.7	84±2.7	59±3.4
TB03BIP03	88±3.0	91±4.4	75±1.9	67±2.6
TB06BIP06	81±3.7	91±4.2	68±2.1	60±3.1
MB025	21±1.1	6±1.0	24±2.1	4±0.7
TB06MB025	99±2.3	106±3.1	83±2.1	90±3.6
TB03MB025	85±3.1	52±2.5	64±1.4	7±1.2
MeB025	62±3.8	93±1.9	62±2.2	44±1.8
TB06MeB025	66±2.4	89±2.6	46±2.4	44±2.6
TB03MeB025	71±2.6	74±3.0	63±2.4	55±3.9
ICG5	90±2.6	111±1.9	88±2.1	79±2.1

Table 3: Estimated difference of survival in HCEC cells (in % relative to TB06) ± SE as well as Bonferroni-adjusted Dunnett-test p-values for comparisons between TB06 and the other dye combinations. A negative sign indicates that the mean survival after exposure to TB06 is lower than survival after exposure to the other dye.

	Linear model estimate ± robust SE (Dunnett-test p-values)			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
BIP06	-23±2.6 (8.4e-15)	-24±11.2 (0.23)	-17±4.0(2.5e-4)	-21±4.6 (2.1e-4)
BIP03	-22±6.3 (9.6e-3)	-26±11.0 (0.14)	-19±5.7(8.9e-3)	-26±5.5 (2.6e-5)
TB015BIP015	-26±6.4(1.1e-3)	-24±9.6 (0.10)	-13±5.7(0.17)	3±7.7 (1)
TB015BIP03	-26±6.2 (5.2e-4)	-27±10.0 (0.06)	-15±4.2(4e-3)	-1±10.1 (1)
TB03BIP015	-20±8.4 (0.22)	-17±10.7 (0.63)	-16±5.6(0.04)	5±9.0 (1)
TB03BIP03	-15±2.6 (4e-8)	-17±3.3 (2.1e-5)	-7±5.3(1)	-3±3.7 (1)
TB06BIP06	-8±3.1 (0.16)	-17±4.3 (7.5e-4)	0±4.1(1)	4±1.0 (7.6e-4)
MB025	52± 6.6(3.5e-13)	68± 9.8(1.9e-10)	43±6.2(8.5e-11)	60±6.4 (2e-16)
TB06MB025	-26±5.9 (2.5e-4)	-32±10.2 (0.02)	-15±2.1(5.8e-11)	-26±8.3 (0.02)
TB03MB025	-12±9.7 (1)	22± 5.0(9.2e-5)	4± 4.3(1)	57± 5.4(2e-16)
MeB025	11±9.7 (1)	-19±9.5 (0.30)	6± 4.1(0.96)	20±6.9 (4.7e-2)
TB06MeB025	2±5.7 (1)	0± 9.5(1)	5± 6.2(1)	9±4.8 (0.54)
TB03MeB025	6±7.1 (1)	-15± 10.0(0.69)	22±4.9(1e-4)	20±7.2 (0.06)
ICG5	-17±7.0 (0.17)	-37±10.0(2.3e-3)	-20±4.5(9.5e-5)	-15±6.8 (0.26)

Table 3 lists whether any of the new dyes or dye combinations is more toxic (positive values) or less toxic (negative values) that TB at 0.06%. It is remarkable that TB 0.06% is not the least toxic of the dyes.

Higher toxicity was found for MB alone, and the combination of TB 0.03% with MB, in all three cell lines tested, and for both immediate toxicity and delayed toxicity. For other combinations of TB and MB, and for TB with MeB, delayed toxicity differed between the cell lines.

BIP at 0.06% was found to be much less toxic than TB at 0.06%. Its staining ability is comparable to TB. Taking into account the reduced toxicity of BIP, this new dye appears better suited for capsulorhexis than TB.

Combinations of BIP with TB were found to be significantly less toxic than TB alone. Depending on the desired color, the combinations of TB and BIP can be adjusted: At equal concentrations, the blue color from TB is prevalent, whereas mixtures with less TB than BIP give a green color.

Results of staining

The staining for dyes with low toxicity is shown in Fig. 1. The strongest staining was observed for TB 0.03% BIP 0.015%, and the color of the stain was blue. The strongest green stain was observed for BIP 0.06% and for the combination TB 0.015% BIP 0.03%.

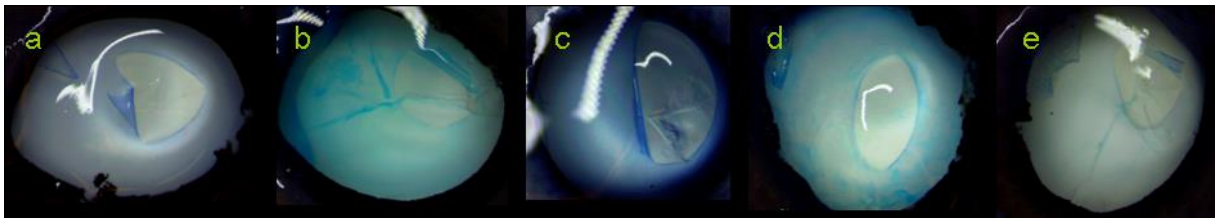

Figure 2. Staining of lens capsule with dyes. a: TB 0.06%; b: BIP 0.06%; c: TB 0.03% BIP 0.015%; d: TB 0.015% BIP 0.03%; e: TB 0.015% BIP 0.015%.

The staining results for other dyes and dye combinations, and toxicity results for ARPE and HeLa cell lines can be found in the Supplementary Material.

For obtaining the same level of blue staining as TB 0.06%, TB 0.03% + BIP 0.015% is sufficient. Reducing the concentration of TB still results in visible staining. A higher amount of BIP over TB, TB 0.015% + BIP 0.03%, gives a more greenish stain.

Discussion

For staining of the capsule, TB 0.1%, ICG 0.25%, and MB 0.1% have been recommended [13] (also reviewed in [14]). Toxicity was not investigated there, but by others, after inadvertent administration of MB 0.1% during capsulorhexis [15]. On the basis of our results, the use of MB alone must be strongly advised against. MB has a very strong photodynamic effect [16], and any light exposure will lead to potential damage of cells which have come in contact with MB.

Toxicity was measured with three different permanent cell lines, preferred over primary cell lines according to the ISO 10993-5:2000(E) norm [9]. The use of permanent cell lines avoids possible variations from corneal endothelial cells derived from animal eyes, nevertheless, one should be aware of the possible limitations of using permanent cell lines.

MeB and TB, as single dyes, have also been tested in a similar setup before [17], with similar results in staining and toxicity.

In view of the toxicity of MB alone, it was very surprising to see that MB in combination with TB is much less phototoxic. Interference of other dyes (or other agents) with photodynamic effects of MB appear not to have been reported before. MB is thought to be phototoxic due to the production of singlet oxygen [18], and TB reacts rapidly with singlet oxygen [19]. Thus because of very close distance between MB and TB in the stained tissue (and cells) TB might act as singlet oxygen scavenger.

BIP is a dye with a low toxicity profile and good staining ability for the anterior capsule. As a solution of 0.06%, it is still transparent, whereas TB at the same concentration is not transparent. It might therefore be worthwhile to investigate the use of BIP also for vitreoretinal surgery.

What was known before

No safe dye was known which was able to stain the lens capsule in green.

What this paper adds

- A dye new to ocular surgery, BIP, alone or in combination with TB, is a safe and effective dye for staining the anterior capsule in green.
- Combinations of TB with BIP give strong staining
- MB alone is a very unsafe dye for staining, due to its severe phototoxicity.
- In combination with TB, the toxicity of MB is greatly reduced.

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New stains for anterior capsule surgery

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Supporting Information

Supplementary Table S1.
Mean viability (in % of PBS) ± SEM (standard error of the mean) given for ARPE and HeLa cell lines for each type of exposure

Supplementary Table S2
Estimated difference of survival (in % relative to TB06) ± SE as well as Bonferroni-adjusted Dunnett-test p-values for comparisions between TB06 and the other dye combinations

Supplementary Figure S1.
Cataract induction

Supplementary Figure F2.
Staining results

Table S1. Mean viability (in % of PBS) ± SEM (standard error of the mean) given for ARPE and HeLa cell lines for each type of exposure

ARPE	Mean ± SEM			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
TB06	89 ± 2.7	74 ± 2.6	79 ± 4.4	78 ± 3.2
BIP06	104 ± 2.8	106 ± 3.5	107 ± 2.7	104 ± 2.4
BIP03	100 ± 4.3	94 ± 2.3	94 ± 1.9	91 ± 1.4
TB015BIP015	76 ± 4.0	71 ± 2.3	72 ± 2.6	65 ± 2.3
TB015BIP03	82 ± 4.5	78 ± 2.3	80 ± 2.8	70 ± 2.5
TB03BIP015	79 ± 4.0	75 ± 2.5	77 ± 3.0	66 ± 3.5
TB03BIP03	101 ± 3.5	85 ± 2.3	88 ± 2.9	89 ± 2.7
TB06BIP06	97 ± 3.9	86 ± 2.0	86 ± 2.3	86 ± 2.7
MB025	75 ± 5.7	19 ± 1.3	74 ± 3.6	8 ± 2.0
TB06MB025	94 ± 3.9	80 ± 3.7	111 ± 3.3	81 ± 5.2
TB03MB025	85 ± 3.5	77 ± 3.1	108 ± 3.2	18 ± 4.7
MeB025	79 ± 3.0	71 ± 2.3	88 ± 3.1	72 ± 2.4
TB06MeB025	71 ± 2.5	65 ± 4.4	92 ± 3.3	93 ± 5.1
TB03MeB025	74 ± 3.2	77 ± 3.4	91 ± 3.3	82 ± 3.8
ICG5	116 ± 3.5	117 ± 7.6	120 ± 3.6	93 ± 3.1

HeLa	Mean ± SEM			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
TB06	48±1.4	41±1.5	93±3.0	80±5.1
BIP06	55±4.3	59±2.2	112±3.5	108±4.1
BIP03	57±2.7	53±3.7	94±5.0	99±4.3
TB015BIP015	61±4.0	35±1.3	78±5.1	82±2.5
TB015BIP03	62±3.9	38±1.2	91±5.0	87±4.0
TB03BIP015	59±1.9	38±1.8	75±3.0	83±2.5
TB03BIP03	45±2.0	39±3.3	101±8.1	87±6.0
TB06BIP06	30±2.5	45±3.2	101±3.1	76±5.5
MB025	48±3.3	41±1.5	15±3.2	3±0.3
TB06MB025	68±4.0	12±1.4	72±2.3	49±2.0
TB03MB025	24±2.1	14±1.1	65±3.8	6±0.6
MeB025	65±3.3	27±1.6	70±3.8	76±3.6
TB06MeB025	88±2.9	36±1.3	71±4.2	73±2.8
TB03MeB025	18±1.7	24±1.1	62±2.0	51±2.9
ICG5	43±4.6	36±2.1	110±4.7	91±3.6

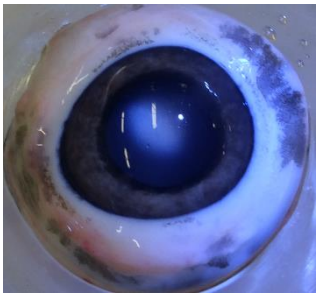
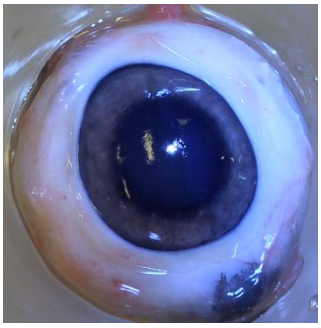
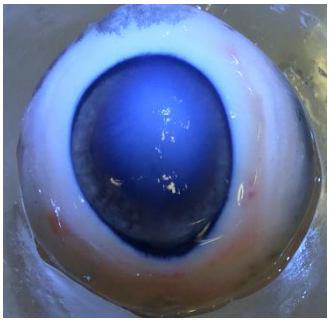
Table S2. Estimated difference of survival (in % relative to TB06) ± SE as well as Bonferroni-adjusted Dunnett-test p-values for comparisions between TB06 and the other dye combinations. A negative sign indicates that the mean survival after exposure to TB06 is lower than survival after exposure to the other dye

ARPE	Linear model estimate ± robust SE (Dunnett-test p-values)			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
BIP06	-15 ±6.1 (0.22)	-33±8.6(2.2e-3)	-28±10.2(0.06)	-26±6.7(2.7e-3)
BIP03	-11 ±3.4(0.02)	-20±6.8(0.04)	-15±9.9(0.77)	-13±5.7(0.25)
TB015BIP015	13 ±6.0(0.45)	2±3.5(1)	7±6.5(1)	13±5.0(0.09)
TB015BIP03	7 ±4.5(1)	-4±4.2(1)	-1±4.4(1)	8±2.4(0.02)
TB03BIP015	10 ±4.9(0.51)	-2±3.9(1)	2±5.7(1)	12±2.6(2.3e-4)
TB03BIP03	-12 ±2.1(8.6e-8)	-12±5.6(0.36)	-8±6.0(0.85)	-11±4.2(0.14)
TB06BIP06	-8 ±4.5(0.69)	-12±5.0(0.14)	-7±6.1(1)	-8±2.9(0.07)
MB025	14 ±15.2(1)	55±5.3(2e-16)	5±13.4(1)	70±8.2(9.5e-15)
TB06MB025	-5 ±5.4(1)	-6±5.7(1)	-32±10.8(0.03)	-3±12.8(1)
TB03MB025	4 ±6.2(1)	-4±6.0(1)	-29±10.5(4.5e-2)	59±8.9(1.2e-9)
MeB025	10 ±7.2(1)	3±4.8(1)	-9±11.7(1)	6±7.0(1)
TB06MeB025	18 ±4.3(1.3e-3)	9±11.3(1)	-13±12.6(1)	-15±13.2(1)
TB03MeB025	15 ±5.9(0.15)	-3±6.9(1)	-12±12.6(1)	-3.8±11.0(1)
ICG5	-27 ±5.8(7.1e-5)	-43±16.8(0.11)	-41±11.2(2.7e-3)	-15±9.3(0.81)

HeLa	Linear model estimate ± robust SE (Dunnett-test p-values)			
Dye	Immediate dark	Immediate light	Delayed dark	Delayed light
BIP06	-7±9.5 (1)	-18±4.1 (3.9e-4)	-18±8.3(0.26)	-29±7 (7.6e-4)
BIP03	-9±6.0 (1)	-12±7.5 (0.97)	0±5.4(1)	-19±10 (0.38)
TB015BIP015	-13±1.9 (2e-10)	7±4.4(1)	16±12.2 (1)	-2±12.1 (1)
TB015BIP03	-15±3.7 (2.1e-3)	3±3.6(1)	2±11.3 (1)	-8±12.9 (1)
TB03BIP015	-12±3.3 (7e-3)	4±4.2(1)	19±5.6 (0.01)	-3±13.1 (1)
TB03BIP03	3± 3.1(1)	3±4.8(1)	-7±24.6 (1)	-7±8.9 (1)
TB06BIP06	18±5.4 (0.02)	-4±2.3 (0.80)	-8±8.7 (1)	4±5.0 (1)
MB025	0±2.2(1)	0±3.3(1)	78±10.1(1.1e-12)	77±12.5 (2e-8)
TB06MB025	-21±9.5 (0.39)	30±2.7 (2e-16)	22±7.3 (0.04)	31±10.5 (0.04)
TB03MB025	24± 4.1(1.5e-7)	27±4.5 (5.8e-8)	29±10.6(0.08)	73±11.6 (5.9e-9)
MeB025	-18±2.6(8.8e-15)	14±3.7 (2e-3)	24±10.0(0.19)	4±13.2 (1)
TB06MeB025	30±4.4(7.9e-10)	18±3.8 (6.1e-5)	31±4.8(2.9e-9)	29±7.2 (9.3e-4)
TB03MeB025	-41±2.2 (2e-16)	6±3.6(0.93)	23±11.2(0.38)	7±13.1 (1)
ICG5	5± 10.6 (1)	5±5.1 (1)	-16±7.2 (0.24)	-12±9.4 (1)

Figure S1. Cataract induction

weight in grams, duration of irradiation in seconds

6.8 g 7 s	7.5 g 7.5 s	6.4 g 8 s
		
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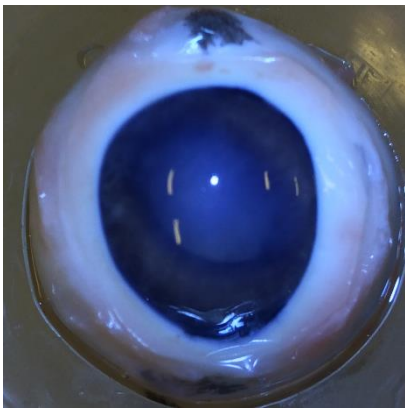
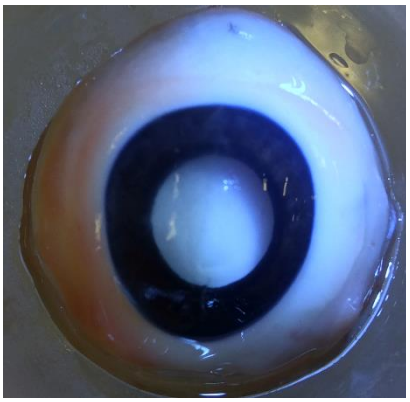
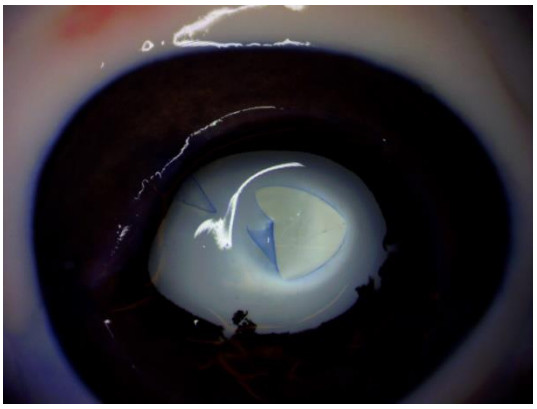
		

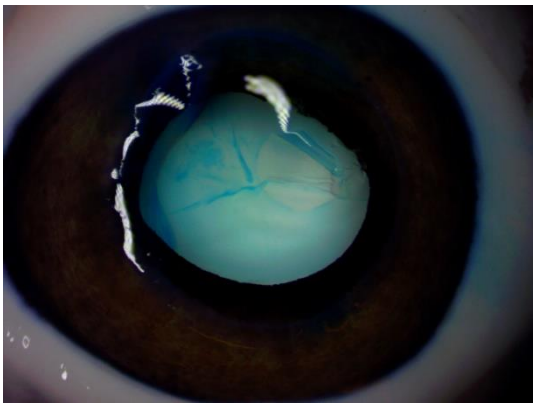
Figure S2. Staining results.

Dyes

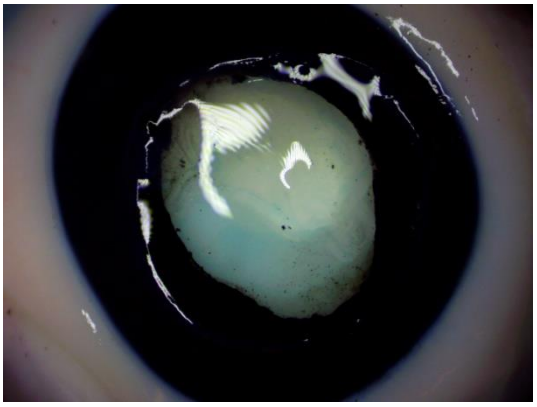
TB 0.06%



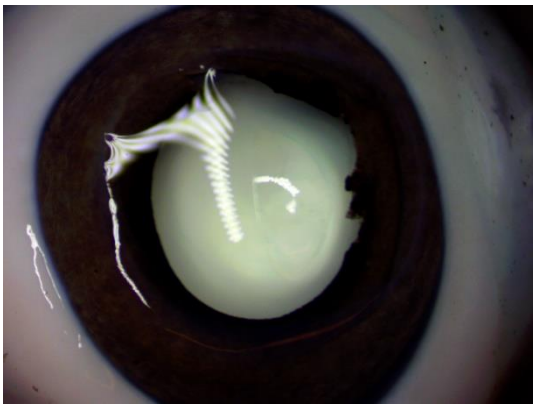
BIP 0.06%



BIP 0.04%

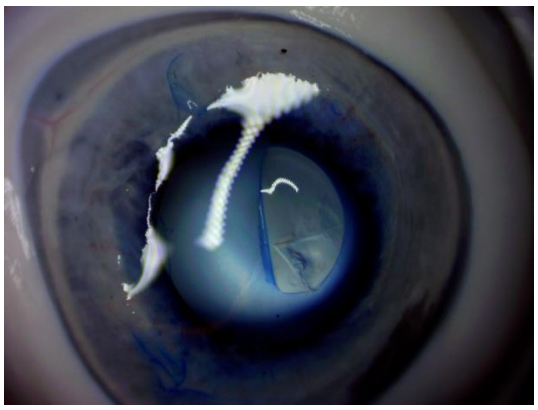


BIP 0.03%

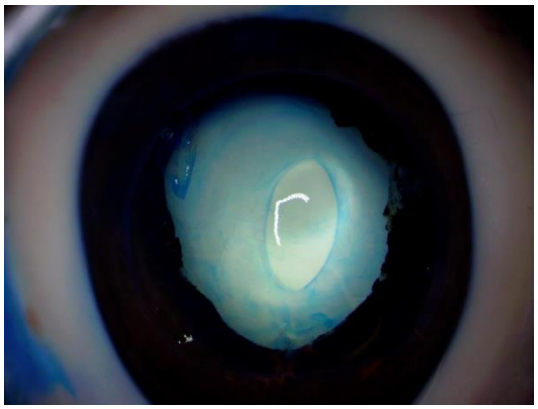


Dyes

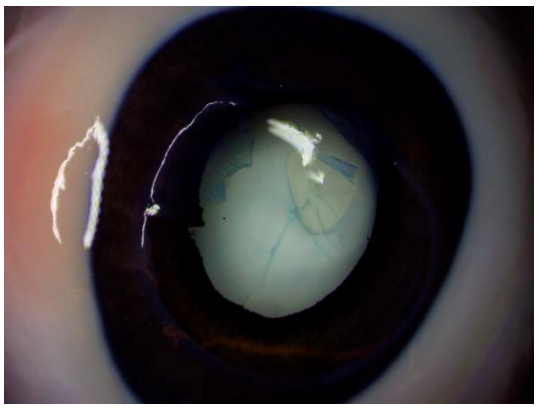
TB 0.03% BIP 0.015%



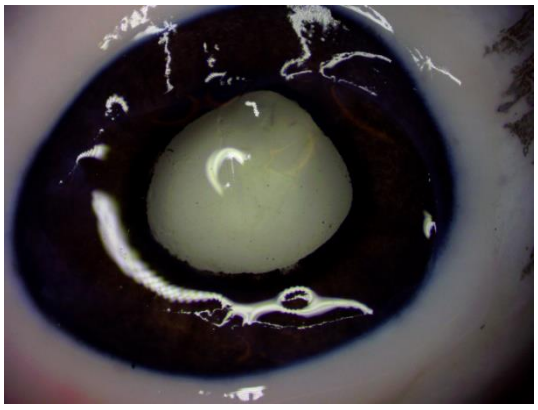
TB 0.015% BIP 0.03%



TB 0.015% BIP 0.015%

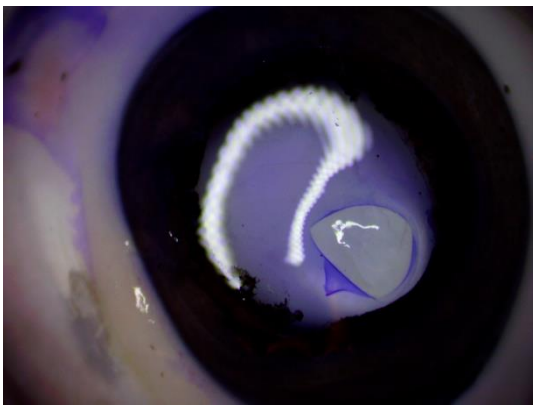


MB 0.025%

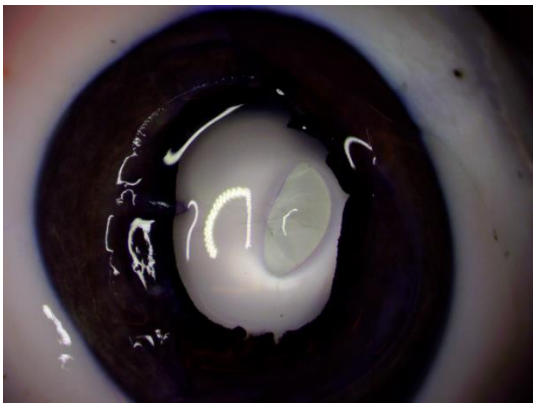


Dyes

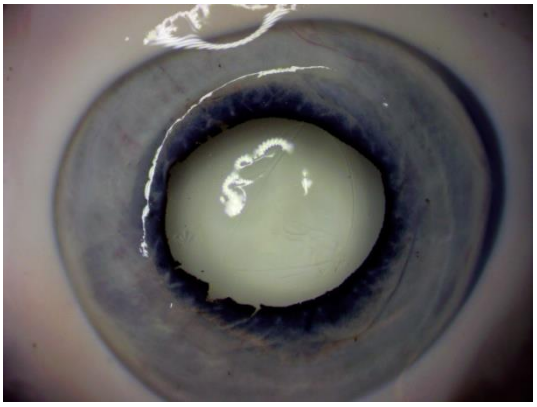
TB 0.06% MB 0.025%



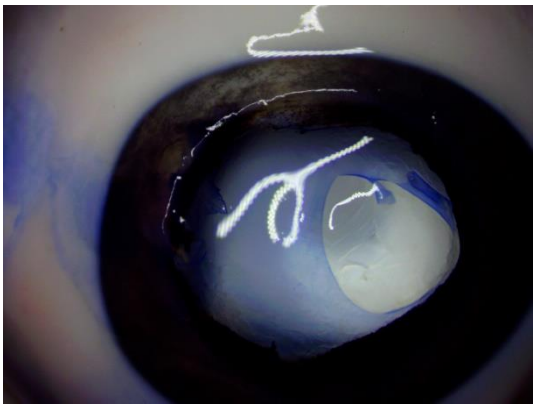
TB 0.03% MB 0.025%



MeB 0.025%

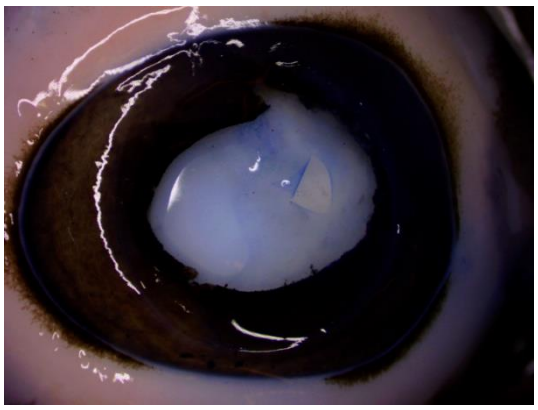


TB 0.06% MeB 0.025%

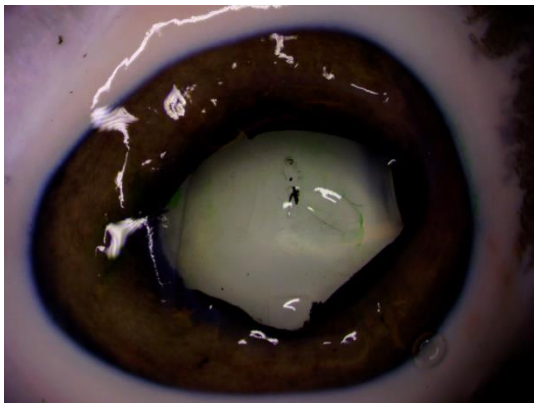


Dyes

TB 0.03% MeB 0.025%



ICG 0.5%



MB: Methylene Blue

MeB: Methyl Blue

Paper V

New cyanine dyes as vital stains in vitreoretinal surgery

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Abstract

Purpose: To design, synthesize, and investigate new cyanine dyes for potential use in vitreoretinal surgery.

Methods: Dyes were synthesized and purified by column chromatography. Cytotoxicity was evaluated on ARPE-19 cell line, immediately after exposure to dyes and after further 24 hours, including phototoxicity assessment. Staining ability was tested on polyvinylidene difluoride filter (model for Inner Limiting Membrane).

Results: Two new cyanine dyes, DG-99 and MA-28, were found to stain model for ILM in green, showing no toxicity on ARPE cells. Both dyes are stable in aqueous solution with adequate additives.

Conclusion: DG-99 and MA-28 enable staining of the ILM in green without damage to pigment epithelium cells.

Introduction

Vital dyes used in ophthalmology facilitate the removal of inner limiting membrane (ILM) and epiretinal membrane (ERM) by staining the tissue of interest. Staining the membrane makes it easier to be located, ensures its complete removal, and decreases the risk of damaging the retina.

A few cyanine dyes were suggested for staining of the ILM during vitreoretinal surgery i.e. indocyanine green (ICG), and infracyanine green.[1] ICG, a dye that has an affinity to ILM and stains well the membrane in patients,[2] is controversial when it comes to its toxicity experiment results.[3] It is also not stable in aqueous solution and needs to be prepared freshly each time in a clinic.[4] Another cyanine dye, (2-[5-[3,3-dimethyl-1-(4-sulfobutyl)-1,3-dihydro-indol-2-ylidene]-penta-1,3-dienyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium sodium (BIP) was investigated by us for potential use in vitreoretinal surgery. It is safe for ARPE-19 cells even at high concentration, soluble in PBS, and stable in water solution, but it did not stain the human ILM. This dye was found to be suitable to stain lens capsule during cataract surgery.[5]

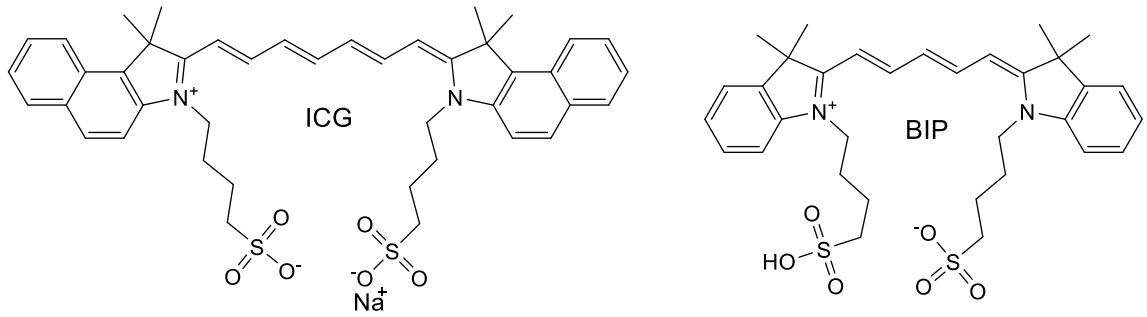


Figure 1. Structures of ICG and BIP, that were the starting point for design of new cyanine dyes.

We wanted to examine if it is possible to combine desired characteristics of ICG and BIP by designing new cyanine dyes, and how different structure modifications would influence its properties.

A dye used in ocular surgery must meet a few requirements. It needs to be safe for retinal epithelium and other cells exposed to it, therefore we investigated toxicity and phototoxicity on ARPE-19 cells immediately after exposure to dye and with a delay of 24 hours. Staining ability was tested on MultiScreen BV Filter Plate and Collagen I membrane as described by Wilińska et al.[6]

Fourteen dyes were designed and synthesized in the laboratory, that were new members to cyanine family and were further subjected to staining ability, toxicity and stability investigation.

Materials and Methods

Chemistry

The structures, synthesis procedures, MALDI spectra, and absorbance spectra for the dyes presented in the paper are provided in the Supporting Information.

Staining models

Collagen membranes (CCC) were from Viscofan (Weinheim, Germany). MultiScreenHTS BV (PVDF) filter plates were from Merck (Darmstadt, Germany).

For staining investigation the PVDF filter was used as model for ILM and CCC collagen membrane was used for ERM model. Punches (diameter 5 mm) of the CCC membrane were placed on a filter plate with a hypodermic needle. Membranes and filters were washed with 100 µl PBS and incubated with 100 µl PBS for 30 minutes. Subsequently, PBS was removed by dynamic vacuum and 50 µl of dye per well was loaded. After 30 s incubation, the dye solution was removed by vacuum and membranes and filters were washed 3 times with PBS. Membranes were transferred to 96-well clear flat-bottom plate and 20 µl of PBS were added to keep the membranes moist. Pictures of filters and membranes were taken.

Toxicity

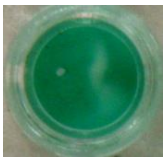
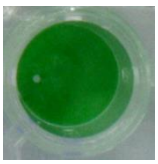
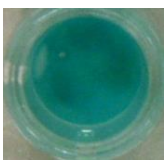
The human retinal pigment cell line (ARPE-19) was from ATCC (cell line CRL-2302; ATCC, LGC Standards GmbH, Wesel, Germany).

As previously described by Awad at al. [7] ARPE cells were seeded in 96-well plates and grown to confluency. After washing with PBS, cells were incubated for 30 minutes with 50 µl of dye solutions (concentration 0.1%) per well. Dyes were removed, and cells were washed 3 times with 200 µl of PBS. Cells that were tested for phototoxicity were further exposed to 15 min of strong light in 100 µl PBS. The WST-1 reagent was used to determine survival of cells. Toxicity and phototoxicity was assayed immediately after exposure to dye/dye and light, or after further 24 hours incubation with fresh medium.

Results

Staining

AG-04 is not soluble in water, PBS, or BSS and was not tested for staining ability.

Dye	Filter	Membrane	Dye	Filter	Membrane
DG-99			MA-14		
AG-03			MA-15		
AG-05			MA-16		

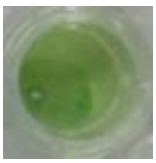
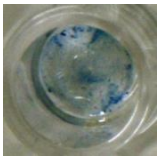
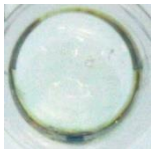
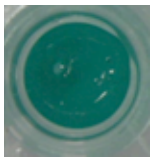
Dye	Filter	Membrane	Dye	Filter	Membrane
AG-06			MA-17		
MA-09			MA-18		
MA-10			MA-19		
MA-12			MA-28		

Table 1. Results of staining of PVDF filter and CCC membrane models with new cyanine dyes. All solutions used were 0.1% dye concentration

DG-99, AG-03, AG-05, AG-06, MA-14, MA-15, and MA-28 stained well the model for ILM in green or greenish blue, and were further subjected to toxicity investigation. MA-09, MA-12, MA-16, and MA-19 did not stain the filter at all.

MA-09, MA-10 and MA-14 were not stable in solution, so they did not qualify for toxicity tests.

Toxicity

Toxicity and phototoxicity of dyes DG-99, MA-28, AG-03, AG-05, AG-06, and MA-15 was tested according to the method described by Awad et al.[7] Substances were tested in a concentration range from 0.01% to 0.2% or 0.5%, with planned potential use at 0.05% or 0.1%.

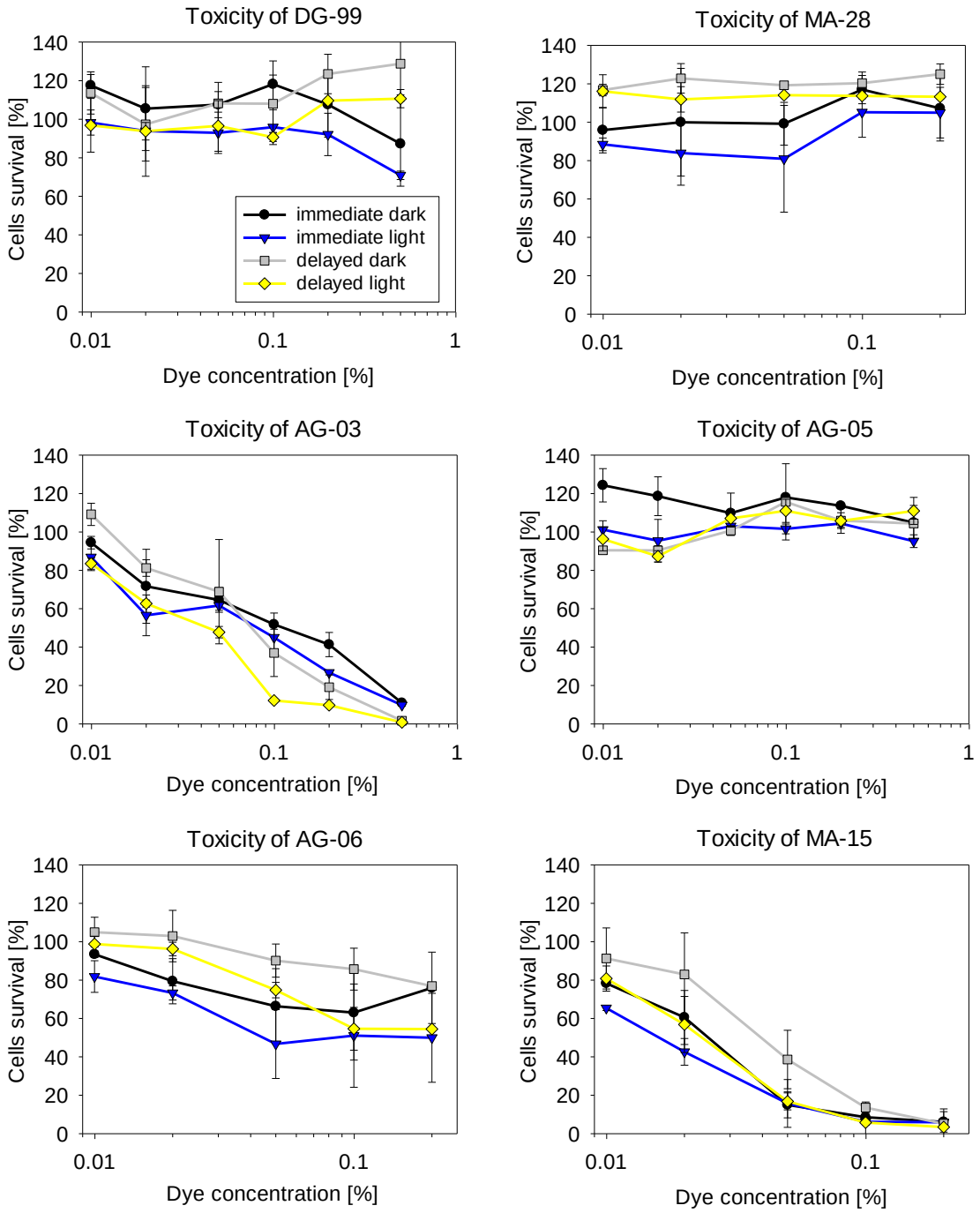


Figure 2. Toxicity and phototoxicity of DG-99, MA-28, AG-03, AG-05, AG-06, MA-15.

AG-03 and MA-14 showed toxicity to ARPE cells already at 0.02% concentration. Cells incubated with AG-06 showed decrease in viability starting at 0.05% immediately after exposure to dye/dye and light. DG-99 and MA-28 were considered safe up to 0.2%

concentration. AG-05 did not cause decrease in viability up to highest tested concentration of 0.5%.

Dyes intended for use as a medical device need to be subjected to two step sterilization including filtering and autoclaving. AG-05 is temperature sensitive and degrades during autoclaving process resulting in color change of the solution to brownish. It could not be further considered as a potential dye for vitreoretinal surgery.

Stability

Stability in aqueous solution of DG99 and MA28 was investigated.

DG-99 was stable in water, but not in PBS or BSS. For it to be stable in BSS, addition of 0.4% Kolliphor RH-40 was needed to 0.1% dye solution. DG-99 was also found to be stable in following solutions: 4% D-isosorbide in water, 4% dimethyl ether isosorbide in water, 5% mannitol in water.

MA-28 was not stable in PBS or BSS. Solutions of MA-28 in water were stable. To stabilize MA-28 in PBS or BSS solution, addition of 5% diglycerol was required. Dye at 0.1% was also found to be stable in 4% D-isosorbide in water, 4% dimethyl ether isosorbide in water, 5% mannitol in water. Kolliphor RH-40 did not stabilize the dye solution in PBS or BSS.

Discussion

For ILM visualization in green few dyes were suggested including ICG, that is not soluble in PBS and not stable in aqueous solution.[4] Therefore, the ICG powder needs to be reconstituted in water and subsequently diluted with balanced salt solution each time in the clinic. Moreover, opposing results were reported regarding its safety in intraocular use. (See SI by Awad et al.[7])

A new green dye is desired that would have the same ability as ICG to stain ILM in green but would overcome its shortcomings. It should be stable in aqueous solution to be delivered to clinics as ready-to-use product, and its safety to RPE cells should be ensured.

Fourteen new compounds were synthesized and tested. Their structure was inspired by currently used or previously suggested substances.

Our study confirms that even small alteration in the structure of molecules can lead to substantial changes in substance characteristics. We have also developed an organized and systematic way to investigate new potential dyes for vitreoretinal surgery, to ensure quality and safety of proposed substances before clinical use.

In summary, two of the investigated dyes met all the requirements for a new potential dye to stain ILM. They combine ICG staining ability, but are safe to ARPE cells, even when exposed to light. Both of those dyes can be stabilized in the solution with addition of diglycerol (for MA-28), which serves at the same time as sinking agent [6], and Kolliphor RH-40 (for DG-99).

References

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4. Penha FM, Rodrigues EB, Maia M, et al. Biochemical analysis and decomposition products of indocyanine green in relation to solvents, dye concentrations and laser exposure. *Ophthalmologica* 2013; 230(Suppl. 2): 59-67.

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