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In: European Journal of Pharmaceutics and Biopharmaceutics, 150: 87-95

To refer to or to cite this work, please use the citation to the published version:

Peynshaert K., Devoldere J., Philips F., Vergauwe F., De Smedt S., Remaut K. (2020) Influence of pathogenic stimuli on Muller cell transfection by lipoplexes

European Journal of Pharmaceutics and Biopharmaceutics, 150: 87-95

DOI: [10.1016/j.ejpb.2020.03.009](https://doi.org/10.1016/j.ejpb.2020.03.009)

Influence of pathogenic stimuli on Müller cell transfection by lipoplexes

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Keywords: Müller cell, neuroprotection, nanoparticle, retinal drug delivery, mRNA, pDNA, hypoxia, hyperglycemia, oxidative stress, retina.

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ABSTRACT

Neuroprotection is a mutation-independent therapeutic strategy that seeks to enhance the survival of neuronal cell types through delivery of neuroprotective factors. The Müller cell, a retinal glial cell type appreciated for its unique morphology and neuroprotective functions, could be regarded as an ideal target for this strategy by functioning as a secretion platform within the retina following uptake of a transgene of our choice. In this *in vitro* study we aimed to investigate the capability of Müller cells to take up a standard liposomal vector (i.e. Lipofectamine 2000) and process its pDNA or mRNA cargo into the reporter GFP protein. By doing so, we found that mRNA outperformed pDNA in Müller cell transfection efficiency. Since neuroprotection is explored as a therapy for diabetic retinopathy and glaucoma, we furthermore examined the Müller cell's lipoplex-induced transfection efficiency and cytotoxicity in stressful conditions linked to these diseases – i.e. hypoxia, hyperglycemia and oxidative stress. Interestingly, Müller cells were able of maintaining high GFP expression regardless of these noxious stimuli. In terms of lipoplex-induced toxicity, hyperglycemia seemed to have a protective effect while hypoxia and oxidative stress led to a slightly higher toxicity. In conclusion, our study indicates that mRNA-lipoplexes have potential in transfecting Müller cells in healthy as well as diseased conditions.

1. INTRODUCTION

Müller cells are the dominant glial cell type in the retina and are responsible for the support of retinal neurons. Their unique anatomy matches this purpose since the Müller cell connects to each neuronal cell type by spanning the entire thickness of the retina, from the inner limiting membrane (ILM) to the outer nuclear layer (ONL). The Müller cell executes a wide variety of essential functions which nearly all assist in the functional, metabolic or structural support of retinal neurons. As an example, Müller cells assist in regulating synaptic activity by uptake of glutamate and serve as a soft but strong bedding for the neurons to grow on.¹ Furthermore, Müller cells handle the water and ion homeostasis within the retina and secrete neuroprotective and anti-oxidative factors in response to pathogenic stimuli.² On top of this natural neuroprotective behavior, Müller cells exhibit several beneficial properties which render them ideal targets for gene transfer. Indeed, their exceptional radial morphology allows them to interact with each neuronal cell type while their endfeet, which abut in the ILM, make the Müller cell a reachable target via intravitreal (IVT) injection (**Figure 1**).^{3,4} In contrast to neurons and photoreceptors, Müller cells are furthermore remarkably resistant to stress allowing them to survive in advanced stages of retinal disease.^{5,6} Based on these advantageous characteristics, we, and others, believe that the Müller cell could play a prominent role in ocular neuroprotection by functioning as a secretion platform within the retina.^{5,7-9}

Neuroprotection is a therapeutic strategy that focuses on the preservation of healthy neurons and the prevention of neuronal cell death by the delivery of neuroprotective agents like growth factors or anti-apoptotic proteins.¹⁰ It is currently explored for diseases involving retinal ganglion cell (RGC) and/or photoreceptor death of which the most commonly investigated diseases are glaucoma, retinitis pigmentosa and diabetic retinopathy.¹¹⁻¹⁸ In view of the short intravitreal half-life of proteins, an interesting tactic to ensure a prolonged neurotrophic effect is to deliver genes that encode for neuroprotective components.^{11,16} In this study we have looked into pDNA as well as mRNA as therapeutic genes for Müller cell transfection. Evidently, the use of pDNA is well-established in the field of retinal gene therapy due to its stability and long-term expression. However, the discovery that the incorporation of naturally occurring modified nucleosides into mRNA can greatly enhance and prolong its expression has recently also proven to be valuable for retinal therapies.¹⁹⁻²¹ Given that these genes could be successfully delivered to Müller cells, they could express the neurotrophic factors and secrete them in their surroundings to enhance neuron survival. To ensure efficient delivery of our mRNA and pDNA cargo *in vitro* we made use of the commercial carrier Lipofectamine 2000 throughout our study.

Since neuroprotection has been proposed as a treatment strategy for glaucoma and diabetic retinopathy,¹²⁻¹⁷ we wished to explore the influence of stress factors associated with these retinopathies on Müller cell transfection. We selected three pathological conditions that are easily simulated *in vitro*. As a first stress factor we have selected oxidative stress since this anomaly has been detected in several experimental (animal) models of glaucoma as well as diabetic retinopathy.²²⁻²⁴ We further selected hyperglycemia since this is the fundamental cause of diabetic retinopathy and elevated glucose levels are known to engender a variety of metabolic abnormalities and oxidative stress.²⁴ Finally, we chose hypoxia as a stress factor since diabetic retinopathy is correlated with decreased retinal blood flow²⁵ and hypoxic tissue has also been detected in glaucomatous eyes.^{26,27}

Taken together, in this study we explored the readiness of Müller cells to take up lipoplexes and process their gene cargo into proteins. We furthermore sought to compare the expression profiles of mRNA and pDNA in healthy Müller cells and assess the therapeutic potential of mRNA. Finally, we examined if pathogenic stimuli, as present in diseased retinal tissue, could influence the transfection efficiency and/or toxicity induced by lipoplexes *in vitro*.

2. METHODS

Cell culture of MIO-M1 cell line

The human Müller cell line Moorfields/Institute of Ophthalmology- Müller 1 (MIO-M1) was obtained from the UCL Institute of Ophthalmology, London, UK.²⁸ The MIO-M1 cells were cultured using DMEM GlutaMAX™ with low glucose (Gibco®, Paisly, UK) supplemented with 10% fetal bovine serum (Hyclone®, Cramilton, UK), 1% L-glutamine (Gibco®, Paisly, UK) and 2% penicillin – streptomycin solution (Gibco®, Paisly, UK). Cells were passaged at 80% confluency and incubated at 37°C with 5.0% CO₂.

Isolation and culture of bovine primary Müller cells

Bovine eyes were obtained from the local slaughterhouse and transported on ice. Excess extraocular tissue was removed and the eyes were rinsed with antibiotic water (10% Penicillin-streptomycin in PBS Gibco®, Paisly, UK)). The anterior segment of the eye was removed by cutting along the entire eye at 5 mm distance from the limbus. Next, the vitreous humor was squeezed out of the eye and an excess amount of CO₂ independent medium (ThermoFischer Scientific, 18045070) was poured into the eyecup to prevent it from drying. The eyecup was cut in 4 pieces after which the retina of 1 piece was transferred to a tissue grinder (VWR, 432-0203) containing 15 ml of separation medium (Advanced dMEM (Gibco®, Paisly, UK), 1% Glutamax, 1% Pen-Strep). After thorough grinding, the grinded retina

was poured into a 40 µm filter unit (Corning, CLS431750) mounted on a 50 ml conical tube and spun down at 300g for 5 minutes at room temperature. The supernatant was then discarded and re-suspended in 10 ml separation medium. After repeating the latter step 3 times, the cells were re-suspended in Müller growth medium (separation medium, 10% heat-inactivated FBS, 4 ng/ml epidermal growth factor (Sigma-Aldrich) and transferred to a CellBIND® T75 flask (Sigma, CLS3290). The flask was placed in a 5% CO₂ incubator, humidified atmosphere, 37°C and left undisturbed for a week. After 1 week all debris were washed away and 10 ml fresh medium was added. By now the Müller cells are growing into big clusters. During week 3 the cells were plated on CellBIND® multiwell plates (Corning, CLS3337) for experiments and passaged into other T75 flasks for further culture.

Plasmid purification, mRNA synthesis and labeling

gWIZ GFP (Promega, Leiden, The Netherlands) was amplified in transformed *E. Coli* bacteria and next isolated from this bacteria suspension using a Qiafilter Plasmid Giga Kit (Qiagen, Venlo, The Netherlands). pDNA concentration was determined on a NanoDrop 2000c (Thermo Fisher Scientific, Rockford, IL, USA) and adjusted to a final concentration of 1 µg/µl with HEPES buffer (20 mM, pH 7.2). GFP mRNA was produced by in vitro transcription of the pGEM4Z-GFP-A64 plasmid, of which the construct design can be found in reference 29.²⁹ The plasmids were purified using a QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands) and linearized using SpeI restriction enzymes (Promega, Leiden, The Netherlands). Linearized plasmids were used as templates for the in vitro transcription reaction using the T7 mMessage mMachine kit (Ambion, Life Technologies, Ghent, Belgium). The resulting capped mRNAs were purified using a RNeasy Mini kit (Qiagen, Venlo, The Netherlands). The mRNA concentration was determined on a NanoDrop 2000c ((Thermo Fisher Scientific, Rockford, IL, USA) and adjusted to a concentration of 1 µg/µl as done for pDNA. For fluorescent labeling of the mRNA and DNA with Cy5, we made use of the label IT® Nucleic Acid Labeling kit of Mirus Bio (Madison, WI), where Cy5 was added to the mRNA or DNA in a 1:1 ratio (v:w). This mixture was then incubated at 37°C for 2 hours followed by purification of the labeled nucleic acids using G50 microspin purification columns according to the manufacturer's instructions.

Lipoplex preparation

The lipoplexes were prepared according to the manufacturer's protocol applying a ratio of 1:3 (µg pDNA/mRNA to µL reagent). Briefly, the transfection agent Lipofectamine™2000 (Invitrogen, Belgium) was diluted in OptiMEM (Gibco®, Paisly, UK) and was left to incubate for 10 minutes at room temperature. The pDNA or mRNA (stock 1µg/µL) was prepared by diluting it in OptiMEM after which it was added in an equal volume to the diluted transfection reagent. After a 5 min incubation allowing for complexation of the nucleic acids with Lipofectamine, the lipoplexes were ready for use.

Lipoplex characterization

The hydrodynamic size and zeta potential of the lipoplexes were determined using a Malvern Zetasizer Nano (Malvern Instruments, Worcestershire, U.K.). For this purpose the lipoplexes were diluted in HEPES buffer prior to performing the measurements at 25 °C. Size measurements were done in triplicate with three runs per replicate and presented based on the number distribution. The zeta potentials were calculated from the electrophoretic mobility based on the Henry equation considering the Smoluchowski approximation. Zeta potential measurements were done in triplicate with two runs per replicate.

Nanoparticle incubation

Müller cells were seeded in a 24 well plate at a cell density of 10.000 cells per well applying 500 µl of medium per well. After 5 days of culture, 100 µl of the lipoplexes, prepared using the standard protocol in OptiMEM, was added to the cells and allowed to incubate for 24 hours at 37°C to evaluate gene expression and 4 hours to evaluate lipoplex uptake.

Stress exposure

Müller cells were exposed to stress factors for 48 hours in total: 24 hours prior to lipoplex incubation and during the 24 hour lipoplex incubation. To induce oxidative stress, cells were exposed to 75 µM Tert-butyl hydroperoxide (TBHP, 458139, Sigma-Aldrich, USA). To mimic hypoxia, the cell-containing well plates were placed in an incubator with 2% O₂ (instead of 21%) at 37°C and 5% CO₂. To generate hyperglycemia, glucose (G8644, Sigma-Aldrich, USA) was added to the cell culture medium to reach a final concentration of 25mM. Note that basic Müller cell culture medium (DMEM GlutaMAX™) already contains 5 mM of glucose.

Flow cytometry

All flow cytometry experiments were performed in 24 well plates. After stress and/or lipoplex treatment, cell culture medium was removed and cells were washed once with 500 µl PBS. Next, the cells were detached by applying 300 µl of 0.25% Trypsin-EDTA (Gibco®, Paisly, UK) after which the trypsin was neutralized by adding 500 µl of cell culture medium. This cell suspension was transferred to FACS tubes followed by a centrifugation step of 5 min at 300g. Then, the supernatant was removed and the cells were re-suspended in buffer (1% FBS, 0.1% sodium azide in PBS). After performing this wash cycle twice, the cells were re-suspended in 300 µl buffer and measured with a CytoFLEX™ (Beckman Coulter, Netherlands). Data analysis was done with Flowjo software (Tree Star Inc.).

MTT cell viability

Müller cells were seeded in a 24 well plate at a cell density of 10.000 cells per well and cultured for 5 days. After stress and/or nanoparticle treatment the medium was removed and the cells were washed once with PBS. Next, fresh cell culture medium containing 5 mg/ml of MTT reagent (Sigma-Aldrich, USA) was added to the cells and incubated for 3 hours at 37°C. After this incubation step, the medium was carefully removed and the formazan crystals were dissolved by incubation with 100% DMSO (Sigma-Aldrich, USA) on a shaker for 1 hour at room temperature. Finally, the absorbance was measured at 590 nm and 690 (background) with an Envision plate reader (Perkin Elmer, Zaventem, Belgium). The percentage of viability was then calculated by comparison with untreated cells representing 100% viability.

EVOS imaging System

Prior to executing the MTT assay protocol, all samples were imaged applying the EVOS FL Auto Cell Imaging System (Invitrogen, Paisley, UK) without any sample preparation.

Statistical analysis

All experiments were analyzed for statistical significance with a one or two-way ANOVA followed by the Bonferroni post hoc test to estimate significance between treated groups, or followed by the Dunnett post hoc test when compared to an untreated group. The results were considered as statistically significant if $p < 0.05$. The number of asterisks in the figures indicate the statistical significance as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All statistical analysis was performed with Graphpad Prism 5 software (San Diego, CA). Values are reported as the mean with standard deviation (SD).

3. RESULTS

Nanoparticle characterization

Figure 2 shows that pDNA and mRNA complexes had a similar size in HEPES buffer i.e. ~600 nm. Their zeta potential, a measure for their surface charge, were both negative though differed significantly: pDNA lipoplexes had a zeta potential of around -10 mV while their mRNA counterparts exhibited a zeta potential of -25 mV. This overall negative charge could be explained by the fact that the positively charged Lipofectamine is neutralized by its complexation with the negatively charged nucleic acids. It must be noted that our group has determined that Lipofectamine fully complexes the mRNA and pDNA at the Lipofectamine/nucleic acid ratio applied throughout this study.²¹

Transfection of healthy Müller cells by mRNA and pDNA-lipoplexes

To explore the potential difference in gene expression profile generated by pDNA and mRNA we exposed Müller cells to a dose range of gene-loaded lipoplexes from 0.2 to 1 μg for 24 hours. As shown in **Figure 3A**, the transfection efficiency with NPs containing mRNA was remarkably higher than for pDNA, with transfection maxima of 81 ($\pm 3\%$) and 21 ($\pm 1\%$), respectively. While a dose-dependent increase in transfection efficiency is apparent for pDNA between 0.2 μg to 0.4 μg , the transfection potential of mRNA did not augment significantly after 0.2 μg . Furthermore, while a seemingly downward trend is visually observed at highest dosages for pDNA, this effect is not significant.

Interestingly, despite the great contrast in transfection efficiency between the two gene types, the mean fluorescence intensity (MFI) of the live cell populations are situated in the same range for all dosages for both types (**Figure 3B**). Similar to the trend observed in transfection efficiency, pDNA does elicit a significant dose-dependent increase in MFI between 0.2 and 0.5 μg (white bars) while no significant changes in MFI are observed with mRNA for dosages higher than 0.2 μg (grey bars). Exposure of cells to nanoparticles more than often leads to cellular stress and/or toxicity.³⁰ To investigate the possible toxic effect of the lipoplexes on the Müller cells we have performed an MTT viability assay after 24 hour exposure to the nanoparticles. As shown in **Figure 3C**, both particles elicit a dose-dependent reduction in cell viability with significant toxicity initiating from a dose of 0.3 μg ($\pm 30\%$ cell death). However, no significant contrast between pDNA and mRNA was detected at any dose. To ensure qualitative confirmation of our flow cytometry data we looked into the morphology of the Müller cells after a 24 hour incubation with the lipoplexes. As can be derived from **Figure 3D**, Müller cell morphology and cell number correlates well with the toxicity trend observed by the MTT assay: increasing lipoplex dose results in a reduction in cell number as well as a shift from typical elongated Müller cell morphology to rounded cells.

A potential reason for the distinct transfection efficiency between the two types of lipoplexes could be a difference in uptake. To look into this hypothesis, we have determined the uptake efficiency and level per cell of lipoplexes containing fluorescently labeled nucleic acids after 4 hours of incubation. As illustrated in **Figure 4**, the uptake profile of mRNA and pDNA lipoplexes is very similar. Indeed, both particles are taken up by >90% of the cell population at all doses while exhibiting an obvious dose-dependent increase in fluorescence per cell.

Overall, we can conclude that pDNA lipoplexes are less efficient but equally toxic transfection agents compared to mRNA lipoplexes. Convinced of the merits of mRNA we pursued our study while focusing on the highly efficient mRNA lipoplexes.

To look into the impact of prolonged exposure to lipoplexes we have tested transfection efficiency and viability of Müller cells after a 48 hour incubation with mRNA lipoplexes. As can be derived from **Figure 5**, the transfection levels at 48 hours are comparable to those observed at 24 hours both in terms of transfection efficiency as well as MFI. Notably, Müller cell viability decreased by an average of 20% for all concentrations compared to the 24 hour time point, though this effect was only significant for the 0.5 µg dose. We can hence conclude that longer exposure to mRNA lipoplexes leads to more cellular toxicity without gaining in transfection efficiency. We therefore continued our study on stressed cells (see further) with a 24 hour exposure as timepoint.

While the human MIO-M1 cell line is well-characterized and applied worldwide, we wanted to have an indication if primary Müller cells were as eager to take up mRNA lipoplexes and express the mRNA cargo. To test this, we isolated primary Müller cells out of fresh bovine eyes and evaluated transfection levels after 24 hour exposure to mRNA lipoplexes in healthy conditions. As shown in **Figure 6**, the transfection efficiency observed in primary cells (grey bars) was remarkably similar to the level seen in the MIO-M1 cell type (white bars) and for the 0.3 µg dose even significantly higher ($\pm 15\%$). In case of MFI, fluorescence intensities were consistently higher in primary cells although this observation was not significant at any dosage. Although not significant, a slightly declining MFI for increasing mRNA concentrations can be perceived; a trend that is likely attributed to lipoplex-induced toxicity.

Transfection of stressed Müller cells by mRNA lipoplexes

To investigate the influence of retinal disease on the lipoplex-induced transfection efficiency and cytotoxicity of Müller cells we exposed MIO-M1 cells to noxious stimuli *in vitro*. Oxidative stress was represented by incubation with Tert-butylhydroperoxide (TBHP), an organic peroxide that is frequently applied in cell culture studies. It causes oxidative stress by its decomposition in unstable alkoxy and peroxy radicals which next react with cellular components.³¹ To simulate diabetic retinopathy we exposed the cells to 25 mM of glucose, a concentration established in literature.^{32–34} Finally, cells were exposed to 2% of O₂ instead of 21 % to imitate hypoxia. For all stress factors, cells were exposed to the stress for 24 hours prior to performing a 24 hour incubation with lipoplexes under stress conditions. This implies that the Müller cells were exposed to the noxious stimuli for 48 hours before the assay readout.

To look into the efficacy and cytotoxicity of lipoplexes while transfecting stressed Müller cells we selected three dosages (0.3; 0.5; 0.7 µg) to identify possible dose-dependent trends. 0.7 µg was chosen as the highest dose since this elicited a 50 % reduction in cell viability in healthy conditions. In **Figure 7** the viability of Müller cells in function of lipoplex dose and stress factors is displayed. To look into the potential cytotoxicity induced by the stress factors itself we have, on top of lipoplex-exposed

samples, also assessed cell viability with the MTT assay after 48 hour exposure for each stress factor as such.

As shown in **Figure 7A**, 48 hour exposure to hyperglycemia provoked a small but insignificant increase in cell viability. Interestingly, the viability of cells incubated with lipoplexes also increased when combined with hyperglycemia. At a dose of 0.7 μg cell viability even augmented from 49% (± 4) to 71% (± 10) without and with hyperglycemia, respectively. Compared to hyperglycemia, hypoxia had the opposite effect: while hypoxia as such did not induce any toxicity, the viability of nanoparticle-treated cells was significantly reduced upon exposure to a hypoxic environment, from 65% (± 5) to 40% (± 5) and from 49% (± 4) to 30% (± 4) for a dose of 0.5 and 0.7 μg , respectively (**Figure 7B**). As presented in **Figure 7C**, treatment with 75 μM TBHP, leading to a drop in cell viability to 67% (± 4) in untreated cells, did not significantly affect nanoparticle-mediated cytotoxicity. To confirm the toxicity trends observed with the MTT assay we looked for alterations in cell morphology and cell number with the EVOS imaging system. We found that, similar to the MTT results, lipoplex-treated cells in hyperglycemic conditions (**Figure 7D-3**) greatly resemble untreated cells (**Figure 7D-1**) regarding cell morphology as well as cell number. In great contrast, lipoplex-treated cells in hypoxic (**7D-4**) or oxidatively stressed conditions (**7D-5**) show a number of rounded dead cells and a visually distinct decrease in cell number.

Finally, **Figure 8** presents the percentage of living transfected Müller cells as well as their MFI in healthy and stressed conditions. Hypoxia seems to lead to a slight reduction in transfection efficiency although this effect is not significant. However, hypoxic conditions do result in a significant decline in MFI at 0.7 μg dosage. Furthermore, hyperglycemic conditions and oxidative stress do not significantly alter transfection efficiency nor the GFP expression per cell. Based on the fact that the transfection levels of living stressed cells is comparable to untreated ones for nearly all conditions, we can conclude that, the Müller cells that are able of surviving the induced stress are able of taking up the lipoplexes and expressing the mRNA.

4. DISCUSSION

In this study we aimed to get an impression on nanoparticle-induced gene expression in *in vitro* cultured Müller cells. To this end we applied the most straightforward set-up: a commercial lipid carrier (Lipofectamine) loaded with GFP-encoding nucleic acids in Müller cell line. This was a purposeful choice since, rather than looking for an ideal drug delivery carrier, we sought to examine general trends in Müller cell behavior toward nanoparticles and/or stress.

Comparison of Müller cell transfection and cytotoxicity induced by mRNA and pDNA lipoplexes

Characterization of the lipoplexes showed that mRNA and pDNA particles have a similar size in buffer which is rather large for a liposomal carrier, i.e. ~600 nm. Despite this similar size their zeta potential did differ, although both lipoplexes were negatively charged (**Figure 2**). Notably, we used the same N/P ratio for both nanoparticles (1:3 for μg pDNA/mRNA to μL reagent), since at this ratio the nucleic acids are fully complexed within the lipoplex ensuring no free nucleic acids can affect transfection.²¹ However, since mRNA strands are smaller in size, there is likely a larger number of mRNA strands per μg of nucleic acid as opposed to pDNA. This could signify that more negative charges are added to the lipofectamine which could next lead to a more negative overall surface charge for mRNA particles. Still, despite their difference in charge, both lipoplexes were taken up at similar levels in Müller cells (**Figure 4**). This comparable uptake did not result in similar levels of transfection efficiency. In contrast, mRNA-containing lipoplexes led to a 4 fold higher transfection efficiency in comparison to pDNA particles (**Figure 3A**). Since both mRNA and pDNA are fully complexed by the lipid particle, the presence of free nucleic acids does not influence this observation.²¹ On the other hand, while both particles are similar in size in buffer, it is well-established nanoparticles tend to aggregate in ion-rich cell culture medium, especially when exhibiting zeta potential below $\pm 30\text{mV}$.³⁵ Nevertheless, since the uptake profile of both lipoplexes is nearly identical it is unlikely that aggregation accounts for the substantial difference seen in GFP expression. Based on the latter observation, we hence suspect that the discrepancy in transfection profile is attributed to intracellular effects.

In fact, we are certain that the observed discrepancy is partly attributed to the basic difference in working mechanism and site of action between mRNA and pDNA: while pDNA requires transfer into the nucleus and transcription into mRNA, the GFP-encoding mRNA can be instantly translated into protein in the cytosol. Considering pDNA needs cell division to cross the nuclear envelope, it is likely that the rather slow division of MIO-M1 cells adds to the low transfection efficiency after 24 hours. Longer incubation times (≥ 48 hours) might therefore lead to a higher percentage of transfected cells. Nevertheless, it should be noted that the Müller cells in the adult retina are usually in a post-mitotic state which does not play in favor of pDNA. Once transfected the degree of expression per cell (MFI) was similar for mRNA and pDNA. Overall, mRNA therefore achieves a more beneficial expression profile since the MFI is as high as for pDNA though the number of GFP-expressing cells is substantially higher. Importantly, while the present study only compares pDNA and mRNA at one timepoint (24 hours), our group did look into the expression levels of mRNA in healthy Müller cells over a course of 3 weeks using another commercial carrier (Lipofectamine MessengerMAX). We found that, while for unmodified mRNA the GFP expression rapidly declined, specific chemical modifications (e.g. m¹ ψ U) could prolong high expression levels. Furthermore, pDNA expression levels never exceeded those obtained with mRNA, also at the longer time points.²¹ Nevertheless, it is probable that despite smart

mRNA modifications, pDNA will result in longer stable expression profiles than mRNA. The benefit of applying mRNA versus pDNA hence all comes down to which therapeutic you aim to deliver and which disease you are targeting.³⁶ It is evident that retinal degeneration originating from an identified genetic mutation will benefit the most from the intended permanent expression of pDNA. However, the transient expression of mRNA should – even for retinal therapies – not always be regarded as a downside. By contrast, our ability of fine-tuning the mRNA expression levels and/or time by differential mRNA modifications might be beneficial for therapies in which the long-term safety of enhanced protein expression is not yet entirely elucidated. In fact, the transient nature of mRNA might avoid the known detrimental effects caused by the long-term expression of some neurotrophic factors.^{21,37}

Next to efficacy, toxicity is an important parameter to consider when evaluating the potential of nanocarriers. To estimate particle-induced acute cytotoxicity we made use of the widely applied MTT assay and checked Müller cell morphology by microscopy. Both assays revealed a rise in cytotoxicity with increasing dose for both lipoplexes (**Figure 3C & D**). Based on the literature we expect this toxicity to be mainly attributed to the growing amount of lipid carrier rather than the nucleic acid fraction. Indeed, while liposomal carriers are often presented as relatively safe,^{38,39} several studies report on *in vitro* and *in vivo* toxicity induced by liposomes fabricated with cationic lipids.^{40,41} No significant difference in cell viability was detected between mRNA and pDNA-based lipoplexes. This observation is in line with our hypothesis that toxicity is caused by the lipid fraction, since the amount of lipid applied to the cells is exactly the same for both lipoplex formulations. When taking both protein expression and toxicity into account, we can conclude that mRNA is the preferred gene type for transfection of Müller cells since low dosages of mRNA lipoplexes produced high transfection efficiency with limited cytotoxicity.²¹ Moreover, these mRNA lipoplexes proved to induce comparable high levels of transfection efficiency as well as GFP expression per cell in primary Müller cells (**Figure 6**).

Influence of noxious stimuli on transfection efficiency and toxicity of mRNA-lipoplexes

As a next step we performed the very same experiments evaluating lipoplex-induced transfection efficacy and toxicity yet under influence of hyperglycemia, hypoxia and oxidative stress.

Hyperglycemia

Hyperglycemia was generated by culture of Müller cells in medium containing 25 mM of glucose. In cells exposed to hyperglycemia for 48 hours, a slight increase in cell viability was observed though this effect was not significant. Interestingly, cell viability was also higher for all lipoplex dosages in glucose-treated cells compared to untreated ones, yet the difference in viability was only significant for the highest lipoplex dose (**Figure 7A**). Also by microscopy we witnessed less toxicity in hyperglycemic conditions versus normoglycemic conditions (**Figure 7D**). It therefore seems that hyperglycemic

conditions boost the survival of Müller cells exposed to lipoplexes and thus have a protective effect. Enhanced Müller cell viability under influence of elevated glucose levels *in vitro* has been noticed before by Vellanki *et al.* They hypothesize that hyperglycemia provokes augmented entry of calcium in Müller cells which next stimulates cell proliferation.³⁴ In addition, studies in different cell types have shown that also other pathways can enhance cell proliferation as a response to hyperglycemia.⁴² It indeed seems logical that an increase in nutrient availability can stimulate the metabolism and simultaneously the proliferation of cells. Following this hypothesis an increase in the number of transfected cells could be expected, though this was not detected. Notably, it is well-established that the diabetic retina is characterized by Müller cell gliosis which usually involves Müller proliferation.^{43,44} It is important to recognize that while these *in vitro* results indicate that hyperglycemia is beneficial, Müller cell gliosis and enhanced proliferation is *in vivo* accompanied by many Müller cell alterations of which some can have a harmful effect on the retina.^{44,45} Hyperglycemia did not influence transgene expression since the number of GFP transfected cells and the MFI of the transfected cells was comparable to cells in normoglycemia (**Figure 8**). We can conclude that hyperglycemia influences Müller cell survival, yet does not affect the cell's ability to take up and process foreign mRNA.

Hypoxia

Exposure of Müller cells to hypoxia for 48 hours did not cause any cytotoxicity compared to cells cultured in normoxic conditions (**Figure 7B**). The same observation was made by Zhang *et al.* and Winkler *et al.* who did not detect significant cell death in hypoxic rat primary Müller cells and human primary Müller cells, respectively.^{46,47} These observations are a logical consequence of the Müller cell's unique energy metabolism which is largely based on glycolysis rather than the highly oxygen-dependent process of respiration. This implies that, even under normoxic conditions, Müller glia have a low rate of oxygen consumption.⁴⁷ The resistance of Müller cells to hypoxia could be a general trait attributed to glial cells. Indeed, also astrocytes – the dominant glial cell type of the central nervous system – were found to be resistant to hypoxia thanks to their high glycolytic capacity. It seems that astrocytes, and likely Müller glia as well, are able of maintaining their energy reserves despite oxygen deprivation as long as there is glucose available to fuel their anaerobic metabolism. Interestingly, the hypoxia resistance of glial cells is in great contrast to the vulnerability of the neurons they support; many studies demonstrate that neurons are highly sensitive to ischemia or lack of oxygen.^{45,48,49}

While no change in cytotoxicity was observed in response to hypoxia alone, the combined treatment of hypoxia and higher dosages of lipoplexes ($\geq 0.5 \mu\text{g}$) did lead to significant toxicity as observed by the MTT assay and microscopy (**Figure 7B & D**). More importantly, the drop in cell viability was more substantial compared to lipoplex treatment alone. This observation was rather unanticipated

considering the lack of cytotoxicity observed in hypoxic Müller cells. Yet, while the Müller cell can compensate for the hypoxia-induced stress, the addition of nanoparticle-elicited stress clearly exceeds the Müller cell's ability to adapt, ultimately resulting in cell death. We therefore assume that the basic metabolism of the Müller cell is not highly oxygen-dependent, yet the coping mechanisms it upregulates to endure the lipoplexes, likely is. When examining the data on transfection efficiency and level of gene expression (MFI) we notice that for each dose tested, the transfection efficiency and MFI is slightly lower for hypoxia-treated cells than for cells kept in normoxia, yet this effect is only significant for the highest dose (**Figure 8**). Since liposomes are known to enter the cell via endocytosis, an active uptake process, this trend could be due to decreased uptake of the lipoplexes in hypoxic conditions.^{50,51} On the other hand, lipoplex uptake could be similar in both conditions but the translation of mRNA into the GFP protein might be affected by oxygen deprivation as stated by Andreev *et al.*⁵² Overall, we can summarize that hypoxia intensifies lipoplex-induced cytotoxicity but does not greatly affect the efficacy of the lipoplexes in the remaining live cells.

Tert-butylhydroperoxide

A 48 hour incubation with 75 μM of TBHP evoked significant Müller cell death (**Figure 7C**). Ostensibly this does not seem to correlate well with other reports in the field, since exposure to the peroxide H_2O_2 did not affect MIO-M1 cells,⁵³ and only elicited very limited apoptosis in rat primary Müller cells.⁴⁶ In fact, we also applied H_2O_2 as an inducer of oxidative stress during our initial experiments and did not observe any cytotoxicity even at concentrations above 1500 μM (data not shown). We therefore decided to continue our studies with TBHP based on the following facts: 1) H_2O_2 is rapidly degraded and is eliminated from cell culture medium within the hour at concentrations around 100 μM ,⁵⁴ and 2) in contrast to H_2O_2 , TBHP was found to evoke consistent cellular stress and was thus proposed as a more suited compound for studies investigating oxidative stress.³¹ It is well-established that generation of ROS and the associated oxidative stress can cause cellular damage on multiple levels including e.g. lipid peroxidation and DNA damage.³¹ Consequently, the TBHP-induced cytotoxicity in Müller cells observed in our experiment is in line with these findings. Co-treatment of TBHP and lipoplexes did provoke more cytotoxicity than lipoplex treatment alone for all dosages tested, although the effect was never significant (**Figure 7C**). Seeing the separate treatments each evoked substantial cell death we did anticipate the combined treatment to be even more harmful. In spite of the extensive stress and accompanying cytotoxicity elicited by co-treatment of lipoplexes and TBHP, the transfection efficiency and MFI in TBHP-treated cells was similar compared to untreated cells (**Figure 8**). This is a hopeful outcome for our neuroprotective strategy since it seems that regardless of cellular toxicity, the surviving cells are able of maintaining a high rate of transgene expression. The latter conclusion is substantiated by Bhatia *et al.* who witnessed that the herbicide paraquat could induce severe oxidative

stress in astrocytes leading to reduced cell numbers. More importantly, they also noticed that the astrocytes that were able of surviving the oxidative insult continued to perform their neuroprotective functions.⁵⁵

5. CONCLUSION

The principal goal of this study was to explore the potential of mRNA and pDNA as a therapeutic for neuroprotection in healthy and diseased Müller cells. Here, we found that mRNA lipoplexes outperformed DNA lipoplexes in Müller cell transfection the number of transfected cells was strikingly higher. To further examine the potential of mRNA in this context, future experiments should determine the transience of the mRNA-induced GFP expression since this is an important requirement for the neuroprotective strategy. Remarkably, none of the stress factors applied, greatly influenced the transfection efficiency or the MFI induced by mRNA lipoplexes. We did observe that hypoxia and oxidative stress sensitized Müller cells to lipoplex toxicity while hyperglycemic conditions had the opposite effect. Naturally, the experimental set-up applied in this study is elementary since diseases usually lead to multifactorial changes in the cellular environment and the influence of surrounding cell types is absent in the Müller monoculture. Future experiments could therefore focus on confirming these trends in more complex systems such as retinal explants and/or investigate the effect of a combination of stress triggers. Since both diabetic retinopathy and glaucoma are chronic diseases, the influence of longer exposures to stress could also be evaluated. Nevertheless, our observations support the strategy to apply Müller cells as secretion platforms in the diseased retina since this suggests that, despite a stressful environment, Müller cells would be able of processing nanoparticles and expressing the transgene of our choice.

6. ACKNOWLEDGEMENTS

Karen Peynshaert is a postdoctoral fellow of the Research Foundation-Flanders, Belgium (FWO-Vlaanderen, grant 12Y2719N). We would like to thank the Laboratory of Pharmaceutical Microbiology (Ghent University, Belgium) for the use of their EVOS imaging system.

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FIGURE CAPTIONS

Figure 1. The Müller cell as a secretion platform for neurotrophic factors following intravitreal injection of transgene-carrying nanoparticles. M: Müller cell; ILM: inner limiting membrane; NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; PRS: photoreceptors.

Figure 2: Characterization of lipofectamine 2000 – NA complexes by dynamic light scattering. A) size B) zeta-potential (n ≥ 3).

Figure 3: pDNA lipoplexes are less efficient but equally toxic transfection agents compared to mRNA lipoplexes. A) Transfection efficiency of healthy Müller cells by mRNA and pDNA lipoplexes determined by flow cytometry (n≥5). B) mean fluorescent intensity (MFI) of living transfected cells as measured by flow cytometry (n≥5). C) Müller cell viability following transfection with DNA or mRNA lipoplexes as measured by the MTT assay. Both NPs induce significant cytotoxicity starting from a dose of 0.3 µg (n≥3). D) Cell morphology after lipoplex incubation as imaged with the EVOS Cell Imaging System; scale bar = 50 µm.

Figure 4: The uptake profile of mRNA and pDNA lipoplexes in MIO-M1 cells is highly similar. A) Uptake efficiency of healthy Müller cells after 4 hours of exposure to mRNA or DNA lipoplexes, determined by flow cytometry (n=3). B) mean fluorescent intensity (MFI) of the entire cell population as measured by flow cytometry (n=3).

Figure 5: 48 hour exposure to mRNA lipoplexes results in comparable transfection levels, yet higher toxicity. A) Transfection efficiency of healthy Müller cells by mRNA lipoplexes determined by flow cytometry (n=3). B) mean fluorescent intensity (MFI) of living transfected cells as measured by flow cytometry (n=3). C) Müller cell viability following transfection with mRNA lipoplexes as measured by the MTT assay (n=3).

Figure 6: Similar trends in transfection efficiency and MFI are observed with mRNA lipoplexes in primary Müller cells. Transfection efficiency (A) and MFI (B) of living primary Müller cells transfected with mRNA lipoplexes, measured by flow cytometry (n=3).

Figure 7: Müller cell viability and morphology after exposure to stress factors and/or mRNA lipoplexes as measured by the MTT assay and EVOS, respectively. A) Hyperglycemia, 25mM glucose (n=3). B) hypoxia (2% O₂) (n=6) C) oxidative stress by exposure to 75µM TBHP (n=3). D) 1: untreated; 2) 0.5 µg mRNA lipoplexes; 3) 0.5µg mRNA lipoplexes + hyperglycemia; 4) 0.5 µg mRNA lipoplexes + hypoxia; 5) 0.5 µg mRNA lipoplexes + 75 µM TBHP. Scale bar = 50 µm.

Figure 8. Transfection by mRNA lipoplexes in Müller cells exposed to pathogenic stimuli. A) transfection efficiency B) MFI (n≥3).