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Evaluation of transfection of A7 astrocytes with gemini nanoparticles for retinal neuroprotection in a dynamic microfluidic cell culture system

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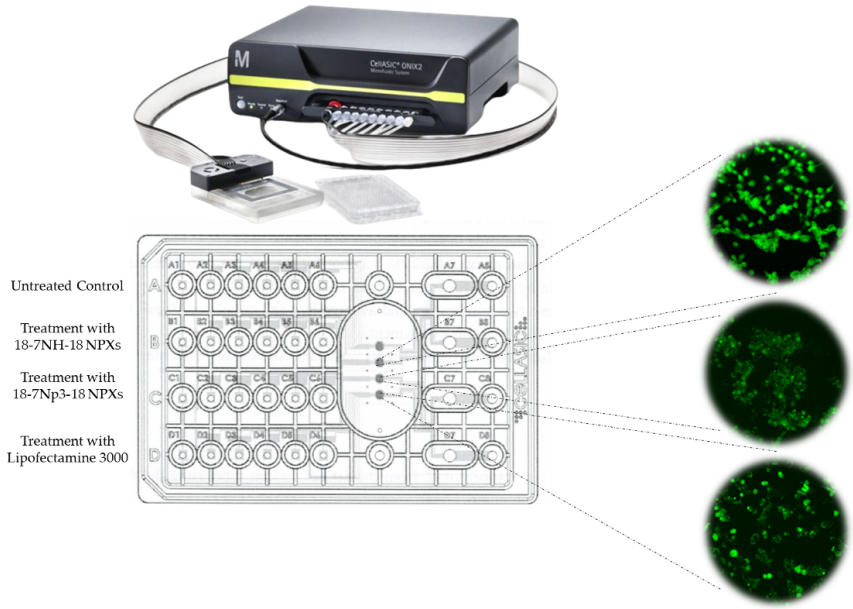
Graphical Abstract



Abstract:

Glaucoma is a neurodegenerative disease of the retina and optic nerve, with elevated intraocular pressure as one of the main contributing factors. The traditional *in vitro* methods to evaluate new gene delivery systems for neuroprotective treatments of glaucoma require testing in cell monolayers under static conditions. Therefore, a microfluidic technology that provides a dynamic simulation of cell exposure to nanoparticles (NPs) could be a useful model for rapid screening. In this study, CellASIC ONIX2 microfluidic platform was used to evaluate and optimize culture and transfection conditions to assess cell viability and transfection efficiency (TE) of gemini NPs carrying GFP-encoding plasmid in A7 astrocytes, one of the targeted retinal cells for glaucoma treatment. The average TE (inlet-middle-outlet of culture chambers) by gemini nanoplexes, 18-7NH-18 NPXs, and 18-7Np$_3$-18 NPXs was >85% and > 25%, respectively, after 5 h of perfusion with combined average fluorescence intensity (FI) (caFI) of about 600 and < 100, respectively, whereas TE after Lipofectamine 3000 was > 70% and caFI of < 100. The results have shown that compared to 2D monolayers, the microfluidics technique provides better exposure of cells to NPs, is suitable for kinetic mapping of gene expression, and is a useful tool for cell growth and live evaluation of gene delivery system interaction with cells.

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Keywords: glaucoma; gene delivery, non-viral, microfluidic; CellASIC ONIX2; M40S-03 microfluidic plate; astrocytes; gemini nanoparticles

Rationale, Purpose, and Limitations

This study evaluated a microfluidic cell culture platform designed to grow cells under dynamic conditions for suitability as an *in vitro* screening tool for nanoparticle (NP)-based gene delivery systems. The main purpose was to generate transfection efficiency (TE) data in A7 astrocytes grown in the microfluidic environment and compare some of the pharmacokinetic parameters of TE (onset, duration, and extent of gene expression) to data obtained from our previous studies with A7 cells grown in traditional 2D monolayers, after treatment with gemini NPs carrying green fluorescent protein (GFP) encoding plasmid. The advantages of screening NPs in microfluidic environments include flexibility of dosing and duration of exposure of cells to NPs, precise control over growth, treatment, and cell staining, as well as long-term monitoring of cells by confocal microscopy. However, the culture conditions, such as chamber coating, cell seeding density, and NP concentration, have to be carefully optimized for each type of cell to establish a suitable protocol.

Introduction

Glaucoma is a neurodegenerative disease resulting from prolonged elevated intraocular pressure (IOP) in the eye as one of the main factors.[1] While the management of the IOP is an important part of the treatment regime in glaucoma, additional neuroprotective approaches are needed for the retina and optic nerve. Research in the past decade showed that neuroprotective approaches could help prevent retinal ganglion cell (RGC) damage and could be essential to preserve vision in patients over time. Neurotrophic factors (NTFs) among neuroprotective compounds were shown to be especially useful.[2-4] However, efficient and reliable delivery of these agents to the retina is still a major obstacle. Retina-targeted gene therapy with NTFs could provide a suitable concentration of NTFs necessary to protect RGCs due to NTF deficiency.[5] Non-viral gene delivery systems (nanoparticles, NPs) may provide many advantages compared to viral vectors in terms of safety; however, their efficacy still requires improvements.[1] In the retina, there are several cell types that could be targeted with NPs encoding NTFs. These include RGCs, astrocytes, Muller cells, and retinal pigment epithelial cells. However, to learn more about the transfectability of these cells, the kinetics of gene expression, and the quantitation of expressed NTFs, a suitable model system is needed.

The development of non-viral delivery systems typically includes the TE screening of a large number of compositions *in vitro* prior to *in vivo* studies.[6] The usual *in vitro* methods involve the incubation of various NP compositions with cell monolayers under static conditions, and the TE is evaluated by flow cytometry, confocal microscopic imaging, or protein assays. A microfluidic technology that provides for the dynamic simulation of cell exposure to NPs could be a useful model for the rapid screening of TE.[7] In this study, we have evaluated the TE of gemini NPs in one of the potential target cells, astrocytes, located mostly in the innermost retinal layers. Astrocytes are located close to RGCs within the retina nerve fiber layer and optic nerve head, and the expressed NTFs could more directly affect RGCs.

Our group is developing gemini surfactant NPs (gemini NPs) for ocular gene delivery. Gemini surfactants are a chemically versatile group of compounds with varying spacer and tail modifications. The first-generation gemini surfactants are a group of dicationic amphiphilic molecules that contain two quaternary ammonium groups separated by a carbon chain spacer (s) and two hydrophobic carbon tails (m) of various chain lengths, abbreviated as m-s-m. The second-generation compounds contain modifications in the spacer, such as an NH functional group, referred to as m-7NH-m, which, due to the pH sensitivity, facilitates the endosomal escape of NPs. Previously we have shown the potential of some gemini NPs as non-viral gene delivery systems; however, as the library of the gemini surfactant molecules increases, the efficient screening of novel NP compositions is even more imperative for identifying lead NPs.

Studies have shown the usefulness of various microfluidic platforms for *in vitro* culturing and evaluation of cellular functions, drug screening, and production of NPs and specialized cells for cell therapies,[8-10,] but there are limited studies for gene delivery testing in this type of system. The CellASIC microfluidic system uses a novel technology for culturing and testing treatments

of cell layers or spheroids on microfluidic plates. It could be used for the screening of non-viral gene delivery formulations. It enables live cell imaging and multiparametric environmental control over temperature, culture conditions, and gas composition. The system is used in conjunction with customized microfluidic plates, a variety of which exist for use with different cell cultures. Plates contain four culture chambers and eight well groups per chamber (one dedicated gravity perfusion well, four solution wells, one cell-loading well, and two waste collection wells), allowing the evaluation of up to four different experimental conditions simultaneously. Cells are manually loaded into the plate, either by gravity (capillary action) or pressure-driven loading. A manifold covering the plate creates a vacuum seal, ensuring that temperature and gas control and solution switching occur independently and hands-free. Experimental steps and parameters are then programmed into the CellASIC software, creating a protocol that, when executed, guides the system through the experiment. All required solutions, such as cell suspensions, growth media, experimental samples, or staining dyes, are loaded into the appropriate solution wells before sealing the plate to the manifold. Thus, once the protocol is initiated, the environment remains sealed for the duration of the experiment, reducing the potential for contamination during cell imaging. Furthermore, the CellASIC system uses pressure-driven media perfusion, creating a dynamic cell culture, and environmental controls and solution switching can be adjusted in the software's manual mode. The microfluidic plates are equipped with four cell culture chambers with a volume of 0.9 µL each within a larger viewing chamber designed for use with inverted microscopes. The small culture chamber size allows for easy identification of single cells and evaluation of gene expression over time.

In this study, we used the CellASIC microfluidic platform to culture A7 astrocytes and evaluated the transfection properties of two gemini nanoplexes (NPXs) we have developed and tested previously in a 2D 96-well-plate monolayer culture environment along with a reference transfection agent, Lipofectamine 3000.[11] We selected the parent unmodified 18-7NH-18 NPXs and its peptide-conjugated version, the immunoglobulin superfamily (IgSF)-derived FASNKL peptide-conjugated 18-7N(p_3)-18 5:1 pNPXs which were evaluated in our previous study both *in vitro* in A7 astrocytes and in CD1 mice *in vivo*.[12]

Materials and Methods

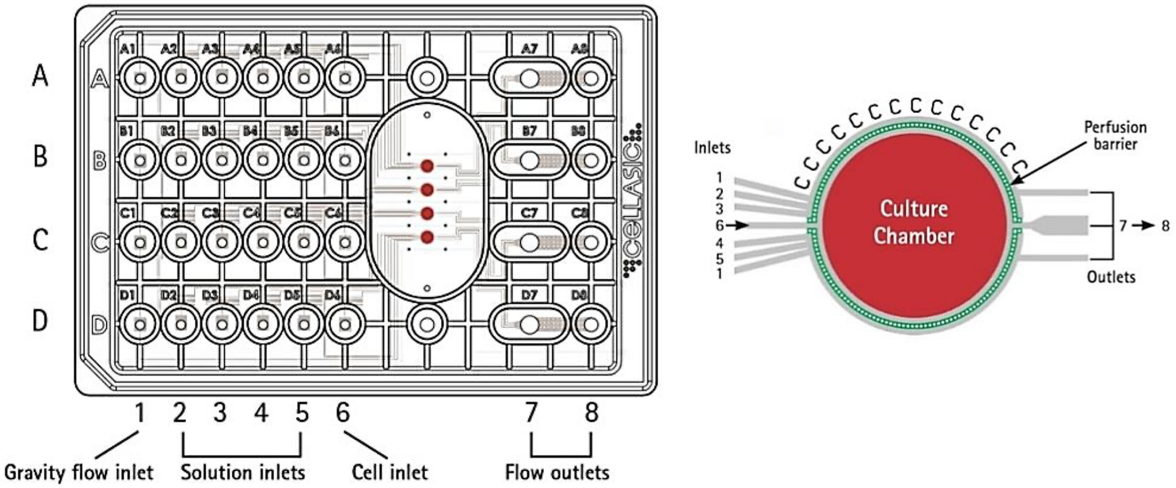
Culture and transfection of A7 Astrocytes using CellASIC Microfluidic System

Rat A7 astrocytes (received from Dr Jeremy Sivak, University of Toronto) were cultured in DMEM/high glucose media (Hyclone, GE Healthcare Life Sciences, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% Pen/Strep (Hyclone) at 37°C in a 95% air/5% carbon dioxide. At about 80% confluency, cells were detached using Tryple express solution (Thermo Fisher Scientific, Waltham, MA, USA), collected, centrifuged, and the cell pellet was resuspended in media without serum at 1×10^6 cells/mL. A 10 µL aliquot of the cell suspension was loaded into each of the four chambers of the M04S-03 CellASIC ONIX microfluidic plate (Millipore-Sigma, Oakville, ON) (Figure 1).

The microfluidic plate is a single-use plate primed with a phosphate-buffered saline (PBS) solution. The plate has four separate culture units (A-D) corresponding to each chamber. Each culture unit has its solution inlets (1-5), cell inlet (6), and shared flow outlets (7-8). In this study, 10 µL of A7 astrocyte cell suspension 1×10^6 /mL was pipetted into the inlet well 6 of each of the four culture units A-D to be loaded by capillary action into the culture chambers. Inlet wells 2-5 of each of the four culture units A-D were filled with media and staining solution. The plate was sealed with the ONIX2 manifold, which allows for a vacuum line and gas line to control the flow rate in each well using air pressure, and was mounted on the Zeiss LSM710 confocal laser scanning microscope (CLSM; Carl Zeiss, Germany) holder. In this study, well 4 of each of the four culture units A-D were filled with 350 µL of complete media (DMEM/high glucose media supplemented with 10% FBS and 1% Pen/Strep; well 3 of each of the four culture units A-D were filled with 10 µL NPXs (0.5 µg gWIZ plasmid dose) in 100 µL basic media (DMEM/high glucose media without FBS) or only basic media if used as a control; well 2 of each of the four culture units A-D were filled with MitoTracker

Deep Red FM viability dye; well 1 of each of the four culture units A-D was left empty; well 6 of each of the four culture units A-D was filled with 10 µL of A7 astrocyte cell suspension 1×10^6 /mL; and unit A7 and A8 were filled with 10 µL of PBS.

A:



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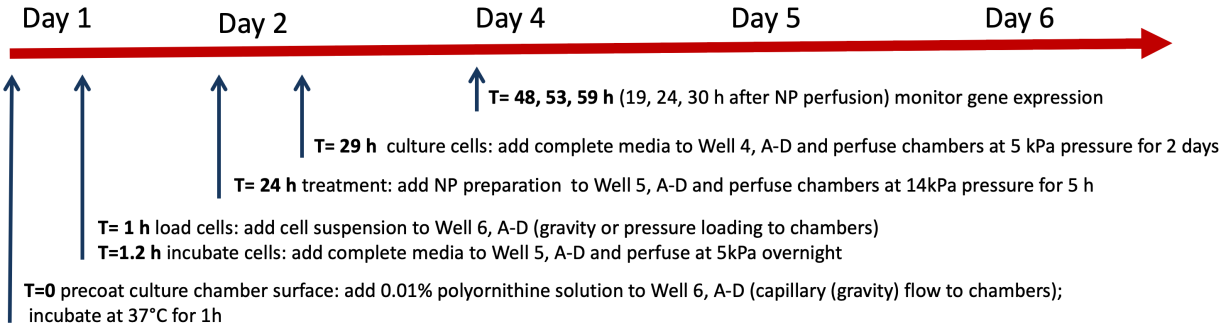

Figure 1A. Plate configuration of the CellASIC ONIX M04S-03 microfluidic plate for monolayer cell culture. Each of the four culture units labeled A-D leads to a culture chamber seen to the right of the cell inlet (6). Each unit has an independent gravity flow inlet (1), solution inlets (2-5), cell inlet (6), and shared flow outlets (7-8). Beside the plate, a more detailed view of one of the culture chambers of the microfluidic plate is shown. The culture units' air channels separate A-D. The cell inlets (6) feed directly into their respective culture chamber, and the perfusion barrier keeps outflow from other inlets. (CellASIC ONIX2 User Guide, Millipore-Sigma).

B:Experimental design for a typical protocol scheme for a transfection experiment.

The CellASIC ONIX2 Software was used to select the desired protocol and cell culture conditions. As recommended, wells 2-5 were under 5 kPa to provide an adequate flow of growth media to nourish the cells. The operating parameters, including pressure, temperature, and gas flow, were set in the protocol at a pressure of 0.5 psi (optimal 0.5-1 psi or 3.4-6.9 kPa) and a temperature of 37°C.

In the plates optimizing culturing conditions, after the A7 astrocyte cells were loaded into the plate, images were taken in each chamber using CLSM to observe cell loading and morphology at 0, 18, and 22 h. At 22 h, live images were taken of cells stained with MitoTracker Deep Red (MTDR), a red-fluorescent viability dye for mitochondria in live cells. The experimental plates initiated treatment at 24 h by perfusing cells with NPXs for 5h at 14kPa pressure. Subsequently, images of green fluorescent protein (GFP) expression using the 488 nm laser were taken at 48, 53, and 59 h, corresponding to 19, 24, and 30 h after NPX perfusion.

TE and FI were estimated from the images of the culture chambers using ZEN 3.2 software

(Zeiss). The number of transfected cells per total number of cells in the culture chamber was determined (TE) by counting fluorescent cells above the upper limit of autofluorescence (ULA).

$$TE = \left(\frac{number\ of\ cells\ with\ fluorescence > ULA}{total\ number\ of\ cells}\right) * 100$$

The average FI (aFI) of the transfected cell population was measured on images from three selected 0.5×10^{-4} cm^2 areas with the highest cell density on each of the inlet, middle, and outlet images using the Measurement function of the ZEN 3.2 software and reported as mean ± s.d. (n=3) aFI values for each treatment. Combined average FI (caFI) values represent the overall average of the aFI values from the inlet, middle, and outlet areas.

TE studies in monolayer cultures

For comparative purposes, A7 astrocyte cells were cultured and treated with NPXs as a static monolayer culture in 96-well plates, as reported and published before.[12] The brief procedure in the 2D experiments, described here for comparative purposes, is as follows: 15,000 cells/well were seeded in 96-well flat bottom tissue culture plates (Grenier Bio-one, Monroe, NC, USA). The next day, at about 80–90% confluency, the complete media was replaced with fresh basic media without serum. Cell monolayers were dosed with 10 µL NPX formulations containing 0.5 µg of pDNA per well and incubated for 5 h at 37°C, followed by replacing the basic media with complete media and incubation for 48 hrs. After the incubation, cells were detached using Accutase™ (Innovative Cell Technologies, San Diego, CA, USA), stained with 500 nM MitoTracker Deep Red FM (Thermo Fisher Scientific), and incubated for 30 minutes. After incubation, TE (%) and viability were analyzed by an Attune flow cytometer (Life Technologies, Carlsbad, CA, USA).

All statistical analyses were carried out on a GraphPad Prism (GraphPad software, La Jolla, CA, USA), data presented as mean ± s.d. One-way ANOVA with Sidak's multiple comparison tests were carried out to analyze the data sets and $p < 0.05$ was considered as significant.

Nanoplex formulations

Gemini NPXs, 18-7NH-18 NPXs, made from gemini surfactant 18-7NH-18 (N, N'-(azanediylbis(propane-3,1-diyl))bis(N,N-dimethyldodecan-1-aminium), or 18-7Np_3-18 NPXs made from its peptide modified derivative (peptide$_3$: FASNKL) synthesized in-house[12]) and 0.5 µg/dose GFP-encoding reporter plasmid (gWIZ ™ GFP plasmid, Aldevron, Fargo, ND, USA) (G:P) at 5:1 charge ratios (ρ±) were made by complexing the required volume of plasmid solution with a calculated volume of gemini surfactant stock solution to obtain the required ρ± at room temperature for 15 minutes.[12] Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) complexed with gWIZ-GFP was used as a reference standard.

Results and Discussion

3.1. Evaluation and optimization of A7 astrocytes culturing conditions in microfluidic plates

Perfusion culture in the M04S-03 microfluidic plate allowed for continuous real-time live cell imaging in the culture chambers for morphological, viability, and TE analysis of the A7 astrocytes. The perfusion culture conditions must be optimized for each cell type and specific experimental protocols. First, the loading and culturing of A7 astrocytes on uncoated culture chamber surfaces were evaluated (Figure 2). At time 0, the loading of 10µL of cells (0.5×10^6 cells/mL in complete media) by gravity into chambers 1-4 produced uniform distribution but with a relatively low confluency. The cells appeared round and viable (Figure 2A). At 18 h, the cells were still round but smaller and with darker density and still did not show signs of attachment to the chamber surface. Despite the morphological change, the cells in the culture chamber were viable as shown in Figure 2B, after MitoTracker Deep Red staining.

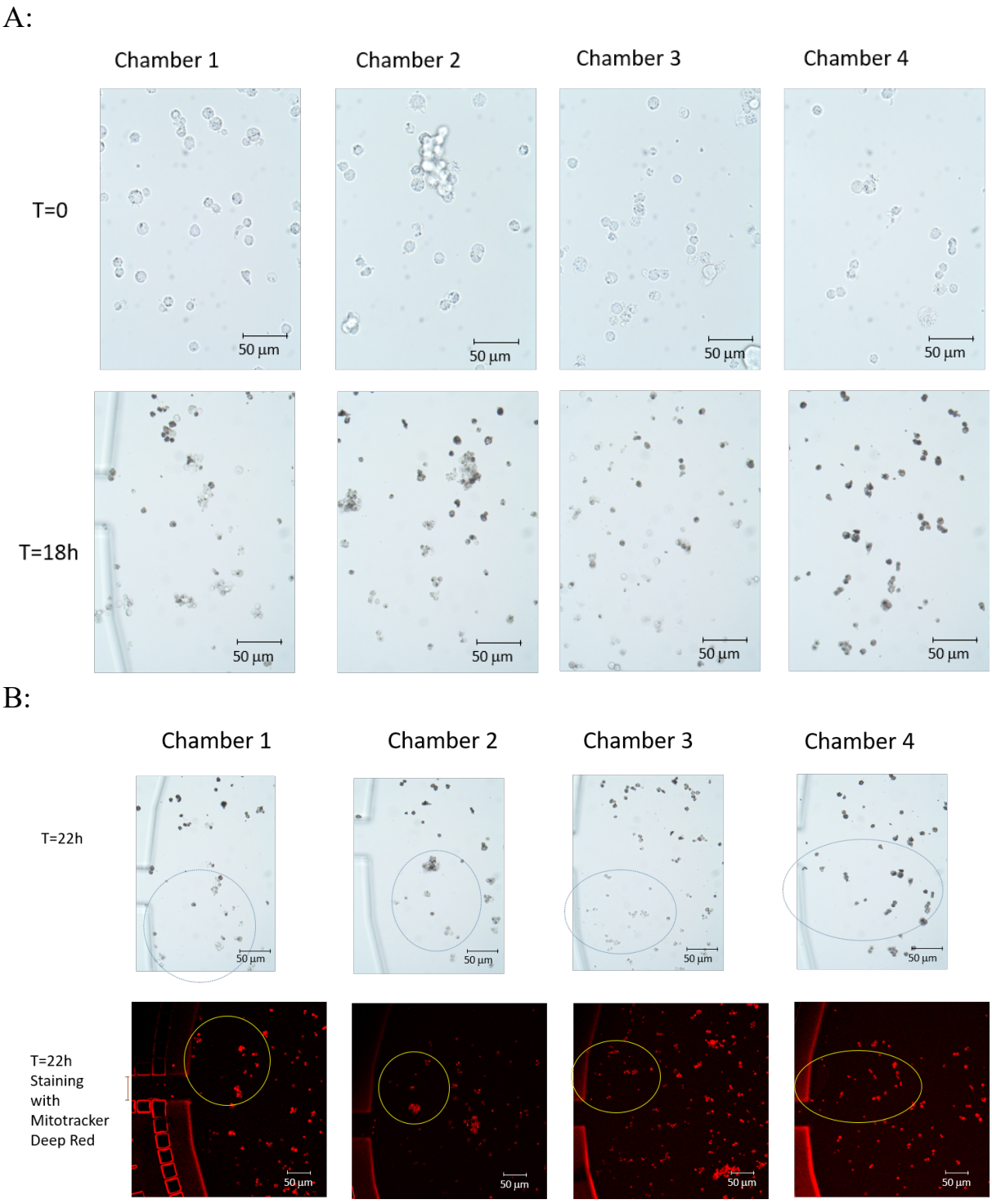

Figure 2. Live images of A7 astrocyte cells in the CellASIC ONIX M04S-03 uncoated microfluidic plate captured with CLSM. All images: Bar 50 µm.

A. Cell loading and morphology of A7 astrocyte cells in uncoated culture chambers 1-4 at time 0 and 18 h.

B. Cell loading and viability observed after 22 h of perfusion. A7 astrocytes were stained with MitoTracker Deep Red (second row) showing fluorescence in the viable cell population. Note: In the second row, images are inverted relative to the top row. Due to the two camera setup on the confocal microscope, the light microscopic vs confocal images appear inverted, i.e. upside-down, please see the encircled areas as guide in each corresponding image accordingly.

To achieve attachment, the cells were loaded into the chambers after pre-coating the chamber surfaces with 50 µL 0.01% polyornithine. The cells (10 µL 1×10^6 cells/mL in complete media) were loaded by gravity across the entire surface (Figure 3, T=0). Images taken at the inlet and outlet locations within the chamber show that the incoming cells at the inlet area first accumulate and subsequently migrate toward the outlet and attach to the entire surface area. Similar cell distribution was observed when the cells were loaded by pressure at 14 kPa. Cell confluency continued to be typically higher around the inlet area compared to the middle or outlet areas of the chamber at T=18 h and onward. This occurred at lower or higher flow rates between 10-18 kPa pressures corresponding to about 10-30 µL/h.

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Table 1. Microfluidic Culture Protocol Development Parameters for A7 Astrocytes

a) Method development process parameters

Parameter set	Surface coating	Cell-loading conditions	Perfusion of culture media (flow rate)	Perfusion of NP formulations
1	none	capillary method	14 kPa	-
2	0.01% polyornithine	capillary method	10-20 kPa	-
3	0.01% polyornithine	pressure method; 1.7 kPa	5-20 kPa	-
4	0.01% polyornithine	pressure method; 1.7 kPa	5-20 kPa	-
5	0.01% polyornithine	capillary method	10-20 kPa	5-14kPa

b) Optimized protocol for CellASIC® ONIX2 M04S-03 microfluidic plate well-set

Well No.	Reagents	Plate Action
6, A-D	50 µL 0.01% polyornithine coating (loading by gravity)	Incubate at 5% CO_2, 37°C, 1 hour, remove fluid from wells 7 and 8 A-D, and incubate for 1 hour before loading cells
6, A-D	10 µL PBS (loading by gravity)	Incubate at 5% CO_2, 37°C, 10 min to rinse wells
6, A-D	10 µL A7 astrocyte cell suspension (loading by gravity)	Incubate plate for 30 minutes after cell loading
4, A-D	350 µL Complete media	Perfuse cells at 5% CO_2, 37°C, 5 kPa, overnight
3, A-D (control or treatment)	10 µL NPXs in 100 µL incomplete media or 100 µL incomplete media (control)	Perfuse cells at 5% CO_2, 37°C, 14 kPa, 5 hours
4, A-D	350 µL complete media	Perfuse cells at 5% CO_2, 37°C, 5 kPa, 48 hours
2, A-D	2 µL 1 mM MitoTracker DR viability stain in 200 µL complete media	Perfuse cells at 5% CO_2, 37°C, 7 kPa, 2.5 hours
1, A-D	Empty	
7 and 8, A-D		Outlet flow collection

The cells attached well to the chamber surfaces and spread and showed morphologies similar to 2D cultures in plates. The various culture parameters and optimum settings are summarized in Table 1.

2. Transfection of A7 astrocytes with gemini NPXs

Physicochemical characterization of the NPXs was previously carried out and indicated 18-7NH-18 NPXs had particle size of 199.80 ± 1.83 nm with a PDI of 0.27 ± 0.01 and zeta potential of 30.18 ± 0.36 mV, whereas 18-7N(p_3)-18 pNPXs had particle size of 257.40 ± 7.70 nm, PDI 0.2 ± 0.01 and zeta potential of 37.13 ± 0.85 mV [12].

The perfusion of the NPXs was initiated at T=24 h, for 5 h continuously at 14 kPa optimum pressure. This exposed the cells to NPXs at approximately 20 µL/h flow rate. The cells appeared healthy and remained attached after the transfection (Figure 3, T=24 h + 5 h) when perfused with complete media.

Figure 4 shows the light and confocal microscopic images and the corresponding calculated average FI (aFI) of GFP-expressing cells in the inlet, middle, and outlet areas of the culture chambers for the three different NP treatments. The cells transfected with 18-7NH-18 NPXs, 18-7Np_3-18 NPXs, and Lipofectamine 3000 started to show GFP expression at 19 h after NPX perfusion (Figure 4A, Chambers 2, 3, and 4). The highest intensity GFP expression was observed with 18-7NH-18 NPXs (Figure 4 A Chamber 2 and B).

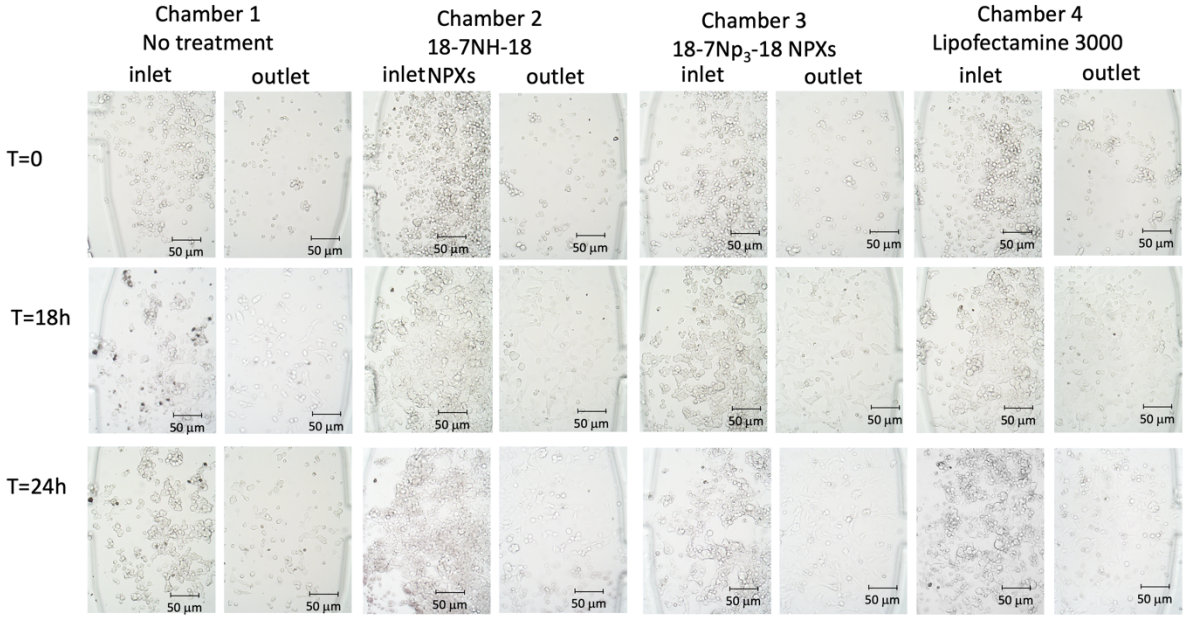
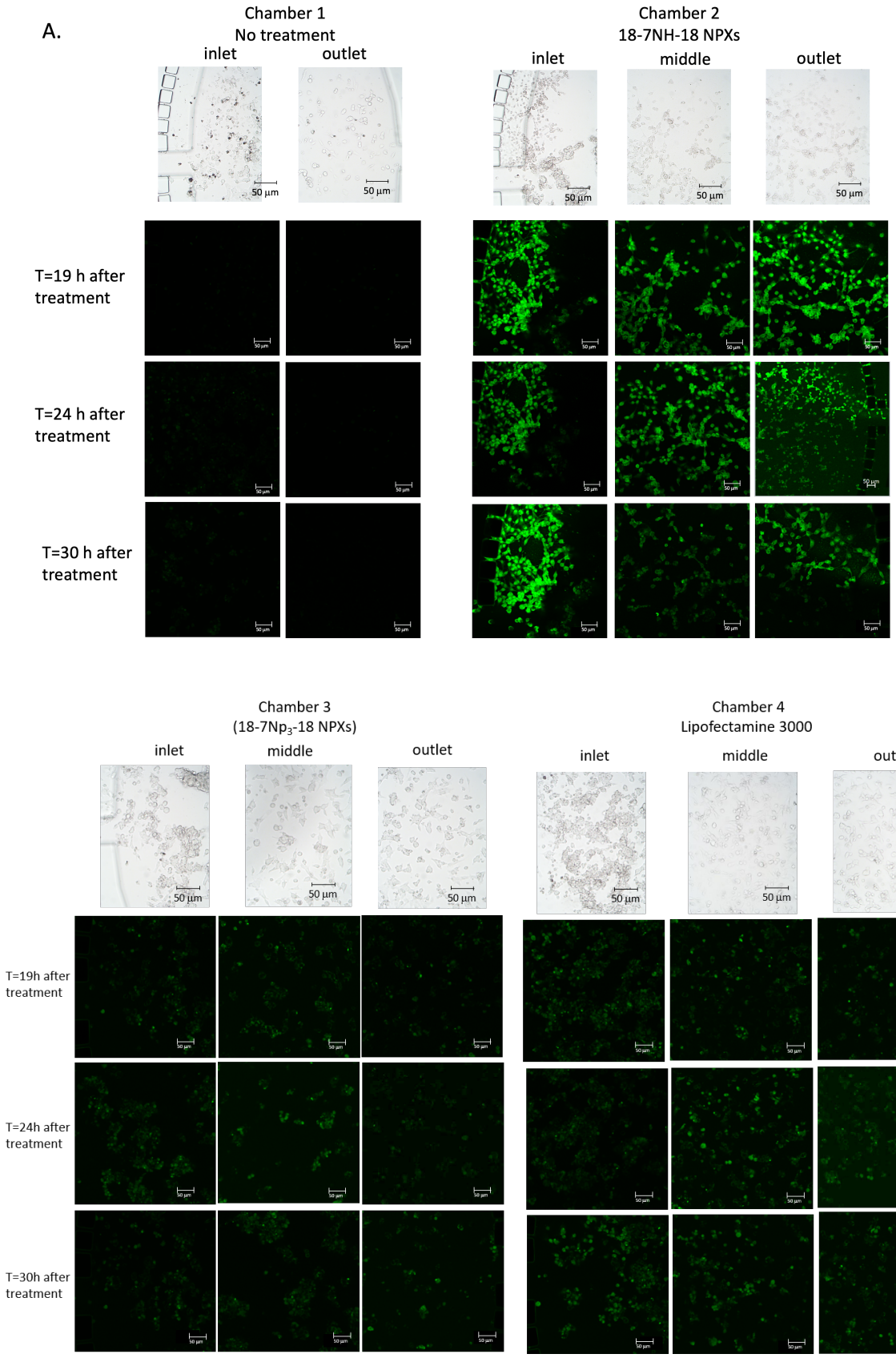


Figure 3. Cell loading and morphology of A7 astrocyte cells in the CellASIC ONIX M04S-03 microfluidic plate coated culture chambers 1-4 at time 0, at 18 h, and at 29 h (treatment started after 24 h preincubation and perfusion with NPXs was maintained for 5 h). Prior to cell loading, the culture chambers were pre-coated with 50 µL 0.01% polyornithine. Chamber 1: untreated control; Chamber 2: treatment with 18-7NH-18 gemini NPXs; Chamber 3: treatment with 18-7Np$_3$-18 gemini NPXs; Chamber 4: treatment with Lipofectamine 3000. Images were captured both at the inlet and outlet of the culture chambers. All images: Bar 50 µm.

The cells transfected with 18-7NH-18 NPXs in the culture chambers showed high TE at 85-90 % of cells expressing GFP up to 30 h (Figure 4 B Table). The aFI values at 19 h were similar (no significant difference) in the inlet, middle, and outlet areas of the culture chambers (Figure 4 B). Comparatively, the number of transfected cells and the aFI with 18-7Np_3-18 NPXs and Lipofectamine 3000 was lower at all time points. There were statistical differences in aFI values between the inlet, middle, and outlet areas of the chambers at different time points and treatments (Figure 4B); however, these aFI values reflect the uneven distribution of cells within the chambers. Generally, the pattern of GFP expression in cells at the inlet, middle, and outlet areas of the chambers was similarly trending downward, indicating that exposure of cells to the NPXs may be higher at the inlet and decreasing towards the middle and the outlet.



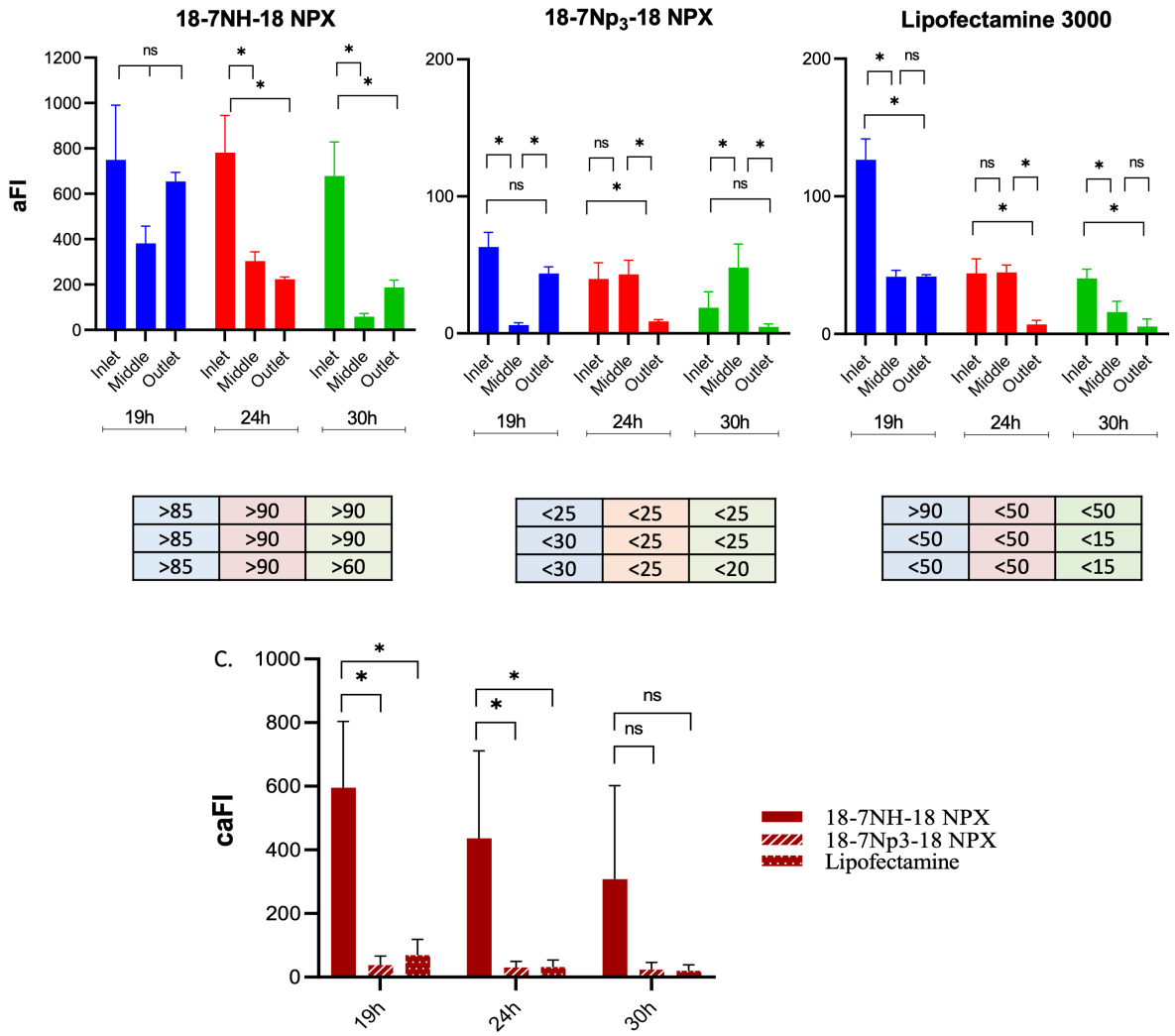


Figure 4. GFP expression in A7 astrocytes after treatment with gemini NPXs at 19, 24, and 30 h after the 5 h NPX perfusion.

A. Confocal microscopic images of GFP expression in A7 astrocytes in the culture chambers. Images were captured at the culture chamber's inlet, middle, and outlet—bars 50 µm.

Chamber 1: untreated control; Chamber 2: treatment with 18-7NH-18 gemini NPXs, 5 h perfusion at 14 kPa; Chamber 3: 18-7Np$_3$-18 gemini NPXs 5 h perfusion at 14 kPa; Chamber 4: commercial transfection agent Lipofectamine 3000 at 5 h perfusion at 14 kPa.

B. FI and TE evaluation of A7 astrocytes after treatment with NPXs; aFI was calculated from FI measured at three selected 0.5×10^{-3} cm^2 areas with the highest cell density in the confocal microscopic images at the inlet, middle, and outlet of the culture chambers using the Measurement function of the ZEN 3.2 software and reported as mean ± s.d. (n=3) aFI values for each treatment. Statistical analysis indicates differences within the different areas of the culture chambers ($p<0.05$) at different time points and treatments; however, the uneven distribution of cells within the chambers contributes to these differences, and at this level of analysis, the decreasing trend in FI over time is the notable information.*

C. The caFI calculated for the whole chambers from the mean aFI values of the inlet, middle, and outlet of the culture chambers for 18-7NH-18 NPXs, 18-7Np$_3$-18 NPXs, and Lipofectamine 3000 treatment at 19, 24, and 30 h. Treatment with 18-7NH-18 NPXs was statistically higher than 18-7Np$_3$-18 NPXs and Lipofectamine 3000 ($p<0.05$) at 19 h and 24 h but not at 30 h.*

To better compare GFP expression between treatments, an overall evaluation of the FI in the whole chambers was carried out by comparing the combined average fluorescence intensity values (caFI) calculated from the aFI values determined at the inlet, middle, and outlet of the culture chamber (shown in Figure 4B) (Figure 4C). The caFI of cells transfected with 18-7NH-18 NPXs were 595 ± 209, 436 ± 275, and 308 ± 294 at 19, 24, and 30 h, res-

pectively. The cells transfected with 18-7Np$_3$-18 NPXs showed < 25% TE with caFI of 38 ± 29, 30 ± 19 and 24 ± 22, at 19, 24 and 30 h, respectively, and with Lipofectamine 3000 initially at 19 h TE was > 90% declining to < 50% by 24 h, with caFI of 70 ± 49, 32 ± 22, 21 ± 18 at 19, 24 and 30 h, respectively. Generally, the GFP expression in all chambers was highest at 19 h ($p < 0.05$) and then maintained at similarly lower intensities at T=24 and T=30 h after perfusion (Figure 4 C). At 19 and 24 h, 18-7NH-18 NPXs had significantly higher GFP expression compared to 18-7Np$_3$-18 NPXs and Lipofectamine (caFI values $p < 0.05$) (Figure 4 C).

Data from the flow cytometric analysis of the cells from our previous work[11,12] showed TE of 18-7NH-18 NPXs (5:1 ρ± ratios) and Lipofectamine™ 3000 treated cells at 24 h as 5.6 ± 0.4% and 8.4 ± 0.5%, respectively, and 18-7NH-18 NPXs, 18-7N(p$_3$)-18 NPXs (5:1 ρ± ratios) and Lipofectamine™ 3000 treated cells at 48 h as 10.7 ± 0.46, 15.4 ± 0.7%, and 12.2 ± 0.4%, respectively,[11,12] which overall was significantly lower than TEs in the microfluidic plates (Figure 5).

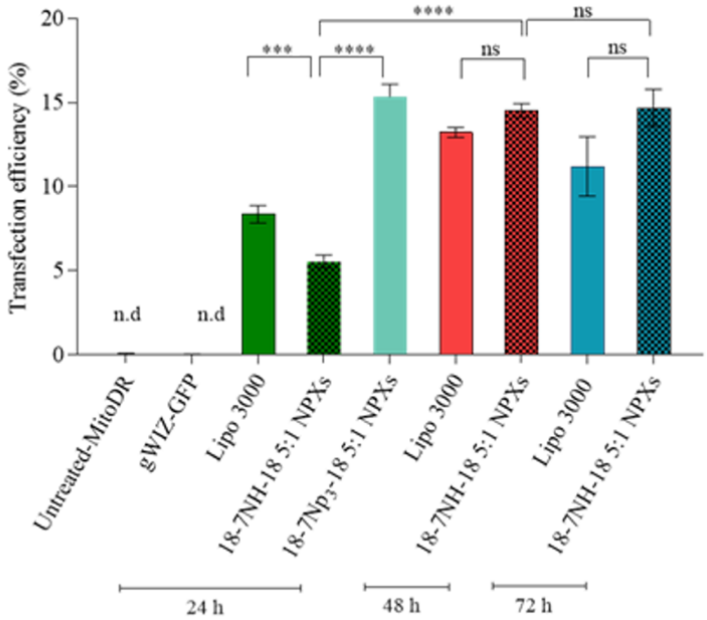


*Figure 5. Transfection studies in A7 astrocyte monolayers in traditional culture plates. TE was evaluated by flow cytometry at 24, 48, and 72 h. TE of 18-7NH-18 NPXs vs 18-7Np$_3$-18 NPXs and Lipofectamine 300 at 24 h *** p=0.0003; **** p<0.0001; and TE of 18-7NH-18 NPXs at 24 h vs 48 h **** p<0.0001.Selected data of the NPXs of gemini:plasmid = 5:1 charge ratios relevant in the current study are compiled and graph modified from the studies by Narsineni et al.[11,12]*

Similarly, confocal microscopic images of A7 astrocytes in 2D monolayers transfected under static conditions with the same NPXs showed lower TE at 24 h and 48 h after treatment.[11] However, the 18-7N(p$_3$)-18 NPXs at 5:1 ρ± ratio performed significantly better ($p < 0.0001$) compared to 18-7NH-18 NPXs at a similar ratio, whereas there was no significant difference between 18-7NH-18 NPXs and Lipofectamine 3000.TE of 18-7NH-18 gemini NPXs and 18-7Np$_3$-18 gemini NPXs in A7 astrocytes after 5 h treatment in serum-free media followed by subsequent incubation with complete media.

Microfluidic techniques are versatile and can be employed for the production of NPs, especially for gene therapy and vaccines, and as tools for culturing cells under controlled conditions and screening the effect of various drugs on cellular function.[13,14]

In one of the earlier studies, Li et al. fabricated simple 1 cm x 60 µm x 900 µm micro-channel structures in a silicone PDMS mold bonded with a glass bottom and cultured COS-7 cells after pre-coating the surfaces with 0.01% (w/v) poly-L-lysine. In this static system, they showed the dose dependency of TE using Lipofectamine™ 2000 carrying GFP-encoding

plasmid in COS-7 cells. In comparison with the cell culturing method in traditional plates, the microfluidic system consumed much less solvent, reagent, and cells, and it was suggested that miniaturization of the cell culture had future advantages.[15]

In a setup closer to the microfluidic plate and cell culture controls used in this study, Raimes et al. evaluated the transfection of mouse embryonic stem cells (mESCs) with Lipofectamine complexed with GFP plasmid in a controllable perfused microfluidic device designed for homogeneous culture conditions of adherent cells. The device also had a two-position valve to automate the injection of the transfection reagent. It was shown that TE was significantly higher in the microfluidic device compared to monolayer culture (34 vs. 17.2%).[16] The higher TE was explained by the five-fold higher surface area-to-volume in the microfluidic versus the monolayer plate, which allows faster diffusion of NPs and higher TE and FI even with a lower dose of NPs.

Similarly, Luni et al. also showed that the efficiency of iPSC reprogramming in a microfluidic device was increased compared to traditional well-plate methods due to an increased concentration of endogenous signaling molecules.[17]

There are also several examples of using microfluidic techniques for cell growth and transfection with various microfluidic devices designed to enhance gene delivery actively.

Loo et al. used a microfluidic transduction device with a mechanotransfection technique to achieve membrane poration in cells upon flowing through microchannels fabricated with ridges based on volume exchange for convective transfection (VECT).[18]

Jarrell et al. made a microfluidic device to deliver eGFP-mRNA or Cas9-sgRNA for genome editing to primary human T lymphocytes as a proof-of-concept for a transfection technology for gene-modified cell therapy or chimeric antigen receptor-expressing T-cells (CAR-T) therapy. The device is based on so-called microfluidic vortex shedding with or without an electric field (μVS or eμVS), where the cell+RNA mixture flows between six rows of micrometer-scale circular posts at regulated pressure-generating vortexes that disturb the cellular membranes and allow intracellular mRNA delivery with less than about 10% decrease in cell viability.[19,20]

Another example is a microfluidic device that uses dielectrophoresis (DEP) and electrotransfection of single cells.[21] This nanochannel electroporation (NEP) technology is suitable for the large-scale delivery of DNA or RNA into single-aligned cells nanoporated in the electric field. The DEP-NEP platform produced a 73% TE (93% if calculated based on successfully loaded cells on the nanochannels) and high cell viability.

These studies indicate that the low-volume microfluidic environment provides a more optimal setting for the interaction of cells with NPs and other agents. In our study, we also found that after optimization of cell density and culture conditions, the TE after treatment with 18-7NH-18 NPXs was >90%. However, there were some differences in the different locations within the culture chamber. Surprisingly, the 18-7Np$_3$-18 NPXs, which showed higher TE in the monolayer plate, showed lower TE here. This could be explained by the fact that the 18-7Np$_3$-18 NPXs carrying the p$_3$ cell adhesion peptide moiety may bind to the polyornithine-coated surfaces of the culture chamber and potentially decrease the availability of NPs to interact with the cells. This was confirmed by a separate experiment where we dosed labeled NPs across the polyornithine-coated surface (without cells) and found an increase in the fluorescence background on the surface after 5 h of perfusion of the NPXs (results not shown).

In this study, we showed that the microfluidic culture technique could have advantages but also face some limitations. For example, using chamber surface coating may be needed for some cell types (such as astrocytes in our study), which may lead to some unexpected effects. In our case, adhesive peptide-modified 18-7Np3-18 NPXs have shown this limitation due to the coating, probably due to the presence of the p3 cell adhesion peptide moiety that might be attached to the coating surface, whereas other types of NPXs did not show this limitation. This limitation might be theoretically overcome by pre-saturating chamber surface areas not occupied by cells with peptides prior to dosing with NPXs or applying different chamber coatings, such as polylysine, gelatin, collagen I, or fibronectin. This was beyond the scope of this study but would be part of future

studies aiming for a more detailed evaluation of the efficiency of drug delivery systems with bio-adhesive moieties.

In vitro, 2D cell culture studies are the most common initial method for most biological and pharmacological assessments. Significant efforts are being made towards improved methods, especially with kinetic capabilities, that may predict in vivo effects, such as 3D cultures and microfluidic techniques. The main critique for the 2D monolayer model is that cells growing in this kind of culture differ significantly in shape, gene, and protein expression compared to cells in vivo. Therefore, many studies were carried out to develop a better cell culture that is more representative of the conditions in the body. The 3D culture was one of the solutions, and it is worth mentioning that our group succeeded in developing such systems.[22,23] Although the cell conditions in 3D culture more closely resemble the cell conditions *in vivo*, drug delivery system interaction with cells can show many differences when comparing the static drug delivery in the 2D and 3D methods. Therefore, a microfluidic cell culture system, such as in this study, serves to simulate the dynamic exposure of the cells to the delivery system *in vivo*. This study could pave the way for more work in the use of the microfluidic technique towards better in vitro-in vivo correlation studies.

Conclusions

The CellASIC ONIX2 microfluidic system is a computer-controlled setup for live monitoring of cellular functions and changes upon treatment. For this transfection screening study, the M40S-03 microfluidic plate for mammalian cells was used to characterize A7 astrocytes cultured under various conditions, including different cell loading, culturing, and transfection modalities. After optimization of the cell concentration, loading method, perfusion pressure during culturing, and NP perfusion, high cell viability could be achieved. Screening three different NP systems, 18-7NH-18 NPXs, 18-7Np$_3$-18 NPXs, and Lipofectamine 3000, showed that TE differences between different delivery systems can be assessed. In this study, TE values were overall significantly higher for all NPs compared to the 2D environment, and the highest FI was observed with 18-7NH-18 NPXs, whereas in 2D monolayers transfected under static conditions, 18-7N(p3)-18 NPXs performed significantly better.

Our experiments with microfluidic devices for cell growth and transfection studies indicate that the low-volume microfluidic environment provides a more optimal setting for interacting cells with NPs and other agents. Although the microfluidics-supported technique is an excellent tool for the controlled growth and treatment of cells and live evaluation of the kinetics of gene expression, it is not yet confirmed whether there is a better *in vitro-in vivo* correlation compared to the monolayer plate system. The CellAsic system provides other plate configurations where tissue spheres can be grown in a 3D environment, which could be another modality to further simulate *in vivo* conditions.

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