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Research paper

Albumin–protamine–oligonucleotide-nanoparticles as a new antisense delivery system. Part 2: cellular uptake and effect

Jörg Weyermann[a], Dirk Lochmann[a], Christiane Georgens[a], Andreas Zimmer[b,*]

[a]*Institute for Pharmaceutical Technology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany*
[b]*Department of Pharmaceutical Technology, Institute of Pharmaceutical Sciences, Karl-Franzens-University, Graz, Austria*

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Abstract

Antisense oligonucleotides have been used as a specific tool to inhibit the expression of disease associated genes for many years. Unfortunately, oligonucleotides are polyanionic macromolecules which have a weak permeability through biological membranes and are rapidly degraded by nucleases. The purpose of this work is to characterise a new drug delivery system developed by [V. Vogel, D.Lochmann, J. Weyermann, G. Mayer, C. Tziatios, J.A. van der Brock, W. Haase, D. Wouters, U.S. Schubert, J. Kreuter, A. Zimmer, D. Schubert, Oligonucleotide–protamine–albumin nanoparticles preparation, physical properties and intracellular processing, J. Controlled Rel. (in press)] [1] which allows an increased cellular uptake and an intracellular dissociation of the oligonucleotides. The new system based on nanoparticles (NPs) consists of human serum albumin, protamine sulphate and antisense-oligonucleotides (AlPrO). We tested these new nanoparticles on mouse fibroblasts which were stably transfected with a *N*-methyl-D-aspartate (NMDA) receptor (NR). This cell line enabled us to perform in vitro studies of cellular uptake, intracellular dissociation and effect of the antisense-oligonucleotide in a simple excitotoxicity model. We compared our findings with free oligonucleotides and a commercial available liposomal preparation (DOTAP). We found a 12-fold increased cellular uptake of oligonucleotides in comparison to free oligonucleotides while 100% of the cells were transfected. The AlPrO-NPs showed very low cytotoxic side effects during a 24 h application. We saw an antisense effect of about 35% in a functional assay as well as on the protein level (western blot). The results of the cell penetration and the antisense assay demonstrated that AlPrO nanoparticles are promising carriers for oligonucleotide administration.

Keywords: Nanoparticle; Antisense; Oligonucleotide; Liposomes; DOTAP; Albumin; Protamine

1. Introduction

Antisense-oligonucleotides are short single stranded DNA or RNA molecules (15–25 nucleotides). These oligonucleotides (ON) are effective blocking agents of protein expression (e.g. through translational arrest) with a high sequence specificity [2,3].

Unfortunately, oligonucleotide drugs are anionic macromolecules and, therefore, they have only a weak permeation through biological cell membranes resulting in a poor bioavailability. Beside this they are rapidly degraded by nucleases.

To overcome these limitations, structurally modified oligonucleotides were synthesized in which the phosphodiester backbone, the sugars or the heterocyclic bases were substituted or modified (for a review on this modifications see [4]).

However, these modifications possess major disadvantages, such as decreased mRNA hybridization, higher cytotoxicity and increased unspecific effects [5]. Thus, this work was focused on unmodified oligonucleotides which were delivered into cells as liposomes or nanoparticles in comparison to free ON.

Therefore, a strong need exists for delivery systems which protect the antisense drugs from enzymatic digestion and provide an enhanced transfer to the cytoplasm of

* Corresponding author. Address: Department of Pharmaceutical Technology, Institute of Pharmaceutical Sciences, Karl-Franzens-University, Schubertstraße 6, 8010 Graz, Austria. Tel.: +43 316 380 8881; fax: +43 316 380 9100.
E-mail address: andreas.zimmer@uni-graz.at (A. Zimmer).

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the target cells. These delivery systems might be liposomes, viral capsid structures or nanoparticles [6–8].

Cationic liposomes were investigated as DNA uptake enhancers, first for gene therapy and consequently also for antisense experiments. The first cationic lipid was introduced in 1987 [9]. Today there are many commercial products available. We used *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) as a reference for our nanoparticles because it has been proven to be a highly effective tool for the transfer of DNA oligonucleotides [10,11].

Nanoparticle as drug-carrier were developed in the early 1980s [12]. The use of the cationic peptide protamine to complex DNA was observed from nature where this principle is highly efficiently applied in sperm. Protamine is used in pharmaceutics for many years and is generally recognized as a save excipient. First attempts in delivering oligonucleotides with nanoparticles consisting only of protamine and oligonucleotide were described by Junghans et al. [13,14]. These particles showed two disadvantages. First of all they have the tendency to aggregate by the addition of salt [15]. And second they have a poor intracellular dissociation of the oligonucleotide [16]. To overcome these two problems Vogel et al. [1] developed a combination of human serum albumin (HSA), protamine sulphate (PS) and oligonucleotide which also form nanoparticles (AlPrO-NP). They were further characterized physicochemically by Lochmann et al. [17].

For the in vitro testing of the cellular uptake and the intracellular dissociation of the oligonucleotide we performed a new antisense model which is based on cell culture.

We used a cell line which stably expressed the functional recombinant *N*-methyl-D-aspartate (NMDA) receptor (NR) which is a ligand gated ion channel and which allows us the testing of antisense-oligonucleotides in a simple excitotoxicity model. The cell line was used before for the testing of synthesized ligands of the NR. Therefore, the mouse fibroblast cell line L(tk-) was co-transfected stable with cDNAs encoding the NR subunits NR1-1a and NR2A [18]. The antisense sequence was directed against the NR1 subunit which is essential for the expression of functional ion channels. This sequence was first described by Wahlestedt et al. [19] and showed their effectiveness in many other studies [20–22].

The cellular uptake of the different ON-preparations was quantified by a fluorescence method using fluorescence labelled oligonucleotides which were detected after lysis of the incubated cells. The intracellular localization of this labelled ON was investigated by confocal laser scan microscopy (CLSM).

The cytotoxic effects of the delivery systems and the ON were measured in a MTT assay. The basis for this colorimetric assays is the metabolic activity of viable cells. Because tetrazolium salts are reduced only by metabolically active cells, the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue colored formazan can be correlated to the viability of the cells [23,24].

The same cytotoxicity assay was used to determine antisense effects which leads in our model to a higher viability of the cells. To verify these results on protein level we performed western blotting.

In this report the characterization and the distinct effects of antisense-oligonucleotide formulations are presented in a functional in vitro model based on cell death.

2. Material and methods

2.1. Cell culture

From our earlier studies we used mouse L(tk-) cells (ATCC CCl1.3), which were stable cotransfected with NR1-1a and NR2A. For the transfection the dexamethasoninducible eukaryotic expression vectors pMSG NR1-1a and pMSG NR2A were used [25].

These cells were a generous gift from Ralf D. Steinmetz of the institute for Pharmaceutical Chemistry of the University of Frankfurt.

The NR expression and functionality was verified by RT-PCR, Western blotting, immunocytochemistry and fluo-4 calcium imaging. Stimulation of NR-ion channels with L-glutamate and glycine resulted in necrosis of cells within 1 h [18]. The cells were grown in minimal essential medium eagle (MEM, PAA, Cölbe, Germany) containing 10% fetal calf serum (FCS, Biochrom, Berlin, Germany) under cell culture conditions (37 °C, 5% CO_2). To avoid selection due to the NR background expression we added 100 mM of the open channel blocker ketamine (Sigma–Aldrich, Taufkirchen, Germany) to the growth medium.

2.2. Oligonucleotides

We used an unmodified ON which was synthesised by Biospring (Frankfurt, Germany). The antisense-oligonucleotide (AS-ON) was directed against the first 18 bases of the NR1 mRNA following the starting codon AUG. The scrambled control ON (SC-ON) contains the same composition of bases but in a randomised order. For our transfer studies we used 5′-fluorescein labelled oligonucleotides (FITC-ON) (Table 1).

2.3. Particle and lipoplex preparation

AlPrO-particles were prepared with different amounts of PS (see Table 2) and 1000 μg HSA (both obtained from

Table 1
Oligonucleotide sequences

AS-ON	5′-CAG CAG GTG CAT GGT GCA-3′
SC-ON	5′-ACG TTG GTC CTG CGG GAA-3′
FITC-ON	FITC-5′-CAG CAG GTG CAT GGT GCA-3′
TRITC-ON	TRITC-5′-CAG CAG GTG CAT GGT GCA-3′

Table 2
Content of AlPrO-NP

AlPrO-NP	HSA (μg/ml)	ON (μg/ml)	PS (μg/ml)
1: 1	1000	55.5	55.5
1: 1.5	1000	55.5	83.25
1: 2	1000	55.5	111
1: 3	1000	55.5	166.5

Sigma–Aldrich, Taufkirchen, Germany) in serum free cell medium. This mixture was vortexed for 5 s. Afterwards 55.5 μg ON was added to a final volume of 1 ml and again vortexed for 5 s. The resulting solution had a final ON-concentration of 10 μM.

For the liposome preparations DOTAP (Roche Diagnostics, Mannheim, Germany) was used to prepare lipoplex formulations according to the manufactures protocol.

All samples were formed in serum-free medium at a concentration of 10 μM ON and afterwards were diluted to final concentrations of 250, 500 nM, 1 and 2 μM with medium.

For the transfer studies and CLSM-pictures we prepared samples with FITC-ON according to the above described procedure.

2.4. Cytotoxicity assay

The cytotoxicity of DOTAP-liposomes and AlPrO-particles was determined with a MTT-assay. Furthermore, we used this assay to measure the excitotoxicity effects of our antisense-model. About 15,000 cells were set to each well of a 96-well plate (Nunc, Wiesbaden, Germany) and were grown for 24 h. One hundred microliter samples of the liposomal, respectively nanoparticle preparations, were added to each well and incubated for 4 h under cell culture conditions. The medium was refreshed and after another 20 h, 50 μl of a 0.25% (w/v) solution of MTT in PBS buffer (NaCl 136.9 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, pH:7.4) were added. Two hours later the cells were centrifuged at 400*g* and 4 °C (Eppendorf Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 4 min, washed twice with PBS and dissolved in a mixture of dimethyl-sulfoxide (Sigma–Aldrich, Taufkirchen, Germany), 5% (w/v) sodium lauryl sulphate (SDS) and 1% (v/v) 1 N hydrochloric acid. The viability of the cells was calculated from the absorption at 550 nm with a plate reader (FluoStar, BMG Labtechnologies, Offenburg, Germany).

2.5. Cell transfer

About 15,000 cells/well were cultured for 24 h in black 96-well plates (Nunc, Wiesbaden, Germany). The cells were incubated for 4 h with FITC-ON preparations (see Section 2.4). We also tested free oligonucleotides.

The cells were lysed with 100 μl/well SDS—lysis-buffer (37 mM Tris–HCl, pH 8, 1.5 MgCl2, 1.8% SDS, 25 U/ml benzonase, 2 μg/ml aprotinin and leupeptin, 2.4 mM phenylmethanesulfonyl fluoride (PMSF)). Fifty microliters lysate per well were taken off to determine the protein content by a commercially available bicinchonic acid (BCA) protein assay (Uptima, Montlucon, France). Fluorescence intensity was measured from the remaining 50 μl lysate (excitation 490 nm, emission 520 nm) using a fluorescence plate reader (FluoStar, BMG Labtechnologies, Offenburg, Germany).

2.6. CLSM imaging

L(tk-) cells were cultured in eight-well chamber slides (Nunc, Wiesbaden, Germany). DOTAP-liposomes, AlPrO-particles and free oligonucleotide were tested. The cells were incubated for 4 h with FITC-ON in a final concentration of 1 μM. The medium was changed and cells were incubated for 20 h in fresh medium. Cell membranes were co-stained with TRITC-conjugated concanavalin A (Sigma–Aldrich, Taufkirchen, Germany) for 2 min and fixed with 5% (w/w) aqueous paraformaldehyde solution. The samples were embedded in 10% Mowiol 4–88 (Hoechst AG, Frankfurt, Germany). Confocal microscopy was performed with a Leitz microscope (Leitz DM IRB, Wetzlar, Germany) and a True Confocal Scanner (TCS 4D, Leica, Heidelberg, Germany) equipped with a krypton–argon laser. Images of 40 subsequent confocal sections were recorded and rearranged to a section view visualizing the horizontal and vertical distribution of FITC-ON inside the cells. We also computed these 40 slices to a three-dimensional picture to give a more vividly impression.

2.7. Western blotting

10^6 Cells were cultured for 24 h in 25 cm^2 cell culture flasks (Nunc, Wiesbaden, Germany) The cells were incubated with AlPrO-NP preparations in serum-free medium for 4 h in a volume of 1 ml. The incubation mixture was replaced by culture medium and the protein expression was induced with 4 μM dexamethasone. After another 16 h the cells were washed with PBS-buffer, scraped off, again washed and centrifuged (4 °C, 7000*g*, 10 min). The resulting pellet was dried and the cells were lysed with 60 μl SDS—lysis-buffer (37 mM Tris–HCl, pH 8, 1.5 $MgCl_2$, 1.8% SDS, 25 U/ml benzonase, 2 μg/ml aprotinin and leupeptin, 2.4 mM PMSF). An aliquot of 5 μl was used to determine the total protein content of the samples. Same amounts of total protein (50 μg total protein per lane) were separated on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and visualized using a goat anti-NR1 (C-terminal) polyclonal IgG and an alkaline phosphatase conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, USA). For control reasons also actin was stained with a specific anti-actin polyclonal IgG to ensure equal total protein amounts on the blot. The spots were stained with nitroblue tetrazolium (NBT)

and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Applichem, Darmstadt, Germany) substrates. The membrane was scanned and quantified by Gel Scan V4.0. software (bioscitec, Frankfurt/M., Germany).

2.8. Physicochemical characterisation

The particle size and size distribution (polydispersity index) of the liposomes (DOTAP) and the AlPrO-NP were determined by a dynamic light scattering. Additionally, the surface charge (zeta-potential) was determined by measuring their electrophoretic mobility. Both measurement series were performed by using a Zetasizer HSA 3000 (Malvern, Herrenberg, Germany).

The loading capacity of the different preparations was determined by performing strong anion exchange high performance liquid chromatography (SAX HPLC). These methods were used as described before by Lochmann et al. [17].

3. Results and discussion

3.1. Cell transfer

The oligonucleotide cell transfer can be divided into two aspects, transfer efficiency and intracellular localisation.

3.1.1. Transfer efficiency

The transfer assay determined the mean fluorescence intensity in the cell lysate related to the number of cells determined by a BCA assay (shown in Fig. 1). The total protein content was found to be in a linear relation to the cell count (data not shown), so it was possible to correlate the cell number to the fluorescence intensity. We found that after 4 h of incubation of the cells with the different ON-formulations under serum free conditions, the uptake of the oligonucleotides was increased 6–10 times with DOTAP and the AlPrO-NPs reached a 6–12 times higher level compared to free ON. The AlPrO-NPs were found to be comparable to lipofection (DOTAP) regarding the transfer efficiency.

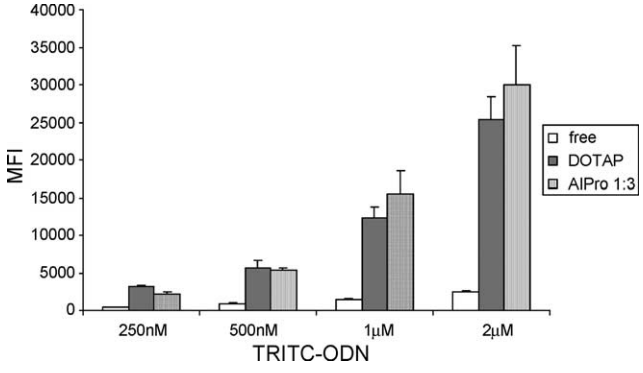

Fig. 1. Cell transfer efficiency: L(tk-) cells were incubated for 4 h with free FITC-ON, liposomes (DOTAP) and AlPrO 1:3. The cells were lysed and the fluorescence intensity (MFI) was determined (mean ± SD; $n = 4$).

3.1.2. Intracellular localisation

The cells were incubated with free FITC-ON (Fig. 2A and B), FITC-ON bound to DOTAP-liposomes (Fig. 2C and D) and entrapped in the AlPrO-NPs (Fig. 2E and F). The cell-membrane was co-stained by the TRITC conjugated plant lectin concanavalin A. Corresponding to the transfer assay, the cellular uptake of free ON was found to be negligible (Fig. 2A and B). This phenomenon confirms that the cellular uptake is a major problem in developing pharmaceutical applications of oligonucleotides [7].

The DOTAP-treated cells showed a heterogeneous intracellular distribution of the fluorescence [26]. In some cells no FITC-ON was detectable. The FITC-ON seems to be localized in certain distinct cell-areas (Fig. 2C and D).

The cells incubated with AlPrO-NP were transfected to 100%. There were no cells visible without an intense stained cytoplasm which belongs to the FITC-ON (Fig. 2E and F). We found a very low intensity of fluorescence in the nucleus. However, this is no disadvantage for the antisense approach because the translation of mRNA into a protein takes place in the cytoplasm. In contrast to the DOTAP experiment the FITC-ON delivered by AlPrO-NPs were more diffuse distributed over the whole cytoplasm and less concentrated in a certain area. This finding indicated a better intracellular dissociation of the oligonucleotides.

3.2. Cytotoxicity assay

3.2.1. Cytotoxic side effects of the carrier systems

The toxicity of DOTAP-liposomes and AlPrO-NPs was determined with two in vitro cytotoxicity sample series (Fig. 4). Metabolic active cells have the capacity to transform the MTT tetrazolium salt into the MTT formazan [23]. The measured absorption of the blue dye obtained by the MTT metabolism after lysis was correlated to the cell viability. Untreated cells were set as 100% viability reference standard (control), whereas cells treated with 1% Triton X-100 were used to define the sensitivity of the test assay (100% lysis). To exclude the effect of the different oligonucleotide sequences, we did not induce the expression of NR and, additionally, we tested different concentrations of the AS-ON as well as of the SC-ON formulations.

We found a cytotoxic effect for the DOTAP-treated cells. Up from 500 nM ON the cells had only about 50% of their former viability.

The AlPrO-treated cells showed only in high concentration of the NPs a moderate reduced viability, thus indicated that the AlPrO-NPs were more biocompatible for these cells with less cytotoxic effects of this delivery system itself.

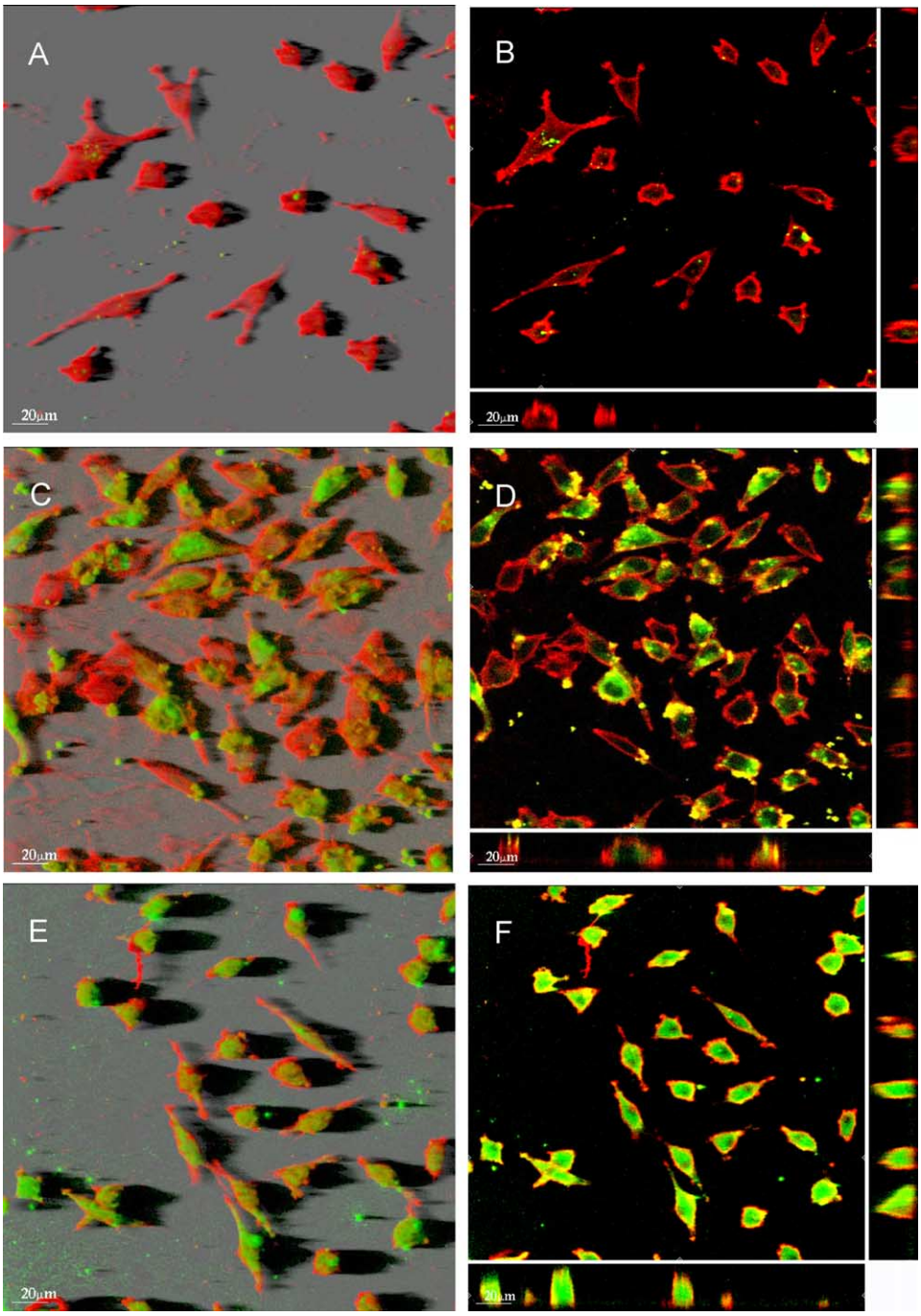

Fig. 2. CLSM-images: L(tk-) cells were treated with 1 μM FITC-ON for 4 h, the medium was refreshed and after another 20 h confocal laser scan microscopy was performed. (green fluorescence, FITC-ON; red fluorescence, cell membrane stained with TRITC-labelled concanavalin A). A, B: free FITC-ON; C, D: DOTAP-liposomes; E, F: AlPrO-NPs. A, C, E: 3D SFP-shadow projection. B, D, F: Section views, combined with xz section to demonstrate the intracellular uptake and distribution of FITC-ON.

3.2.2. Higher viability by antisense effects

Antisense effects were intensively investigated for cell proliferation and anticancer applications as well as for antiviral treatments [27,28]. Our approach leads to a reduced expression of the NR-protein effected through AS-ON and thereby a reduced excitotoxicity which can be measured by a higher MTT metabolism. This mechanism is in contrast to most other trials where a higher death rate is desirable, which also can be caused by unspecific effects.

In a first step we tried to optimize the AlPrO-NPs by varying the PS content and investigated which preparation had the highest antisense effect (Fig. 3A). We used different ratios of ON:PS (w/w) starting with 1:1 up to 1:3

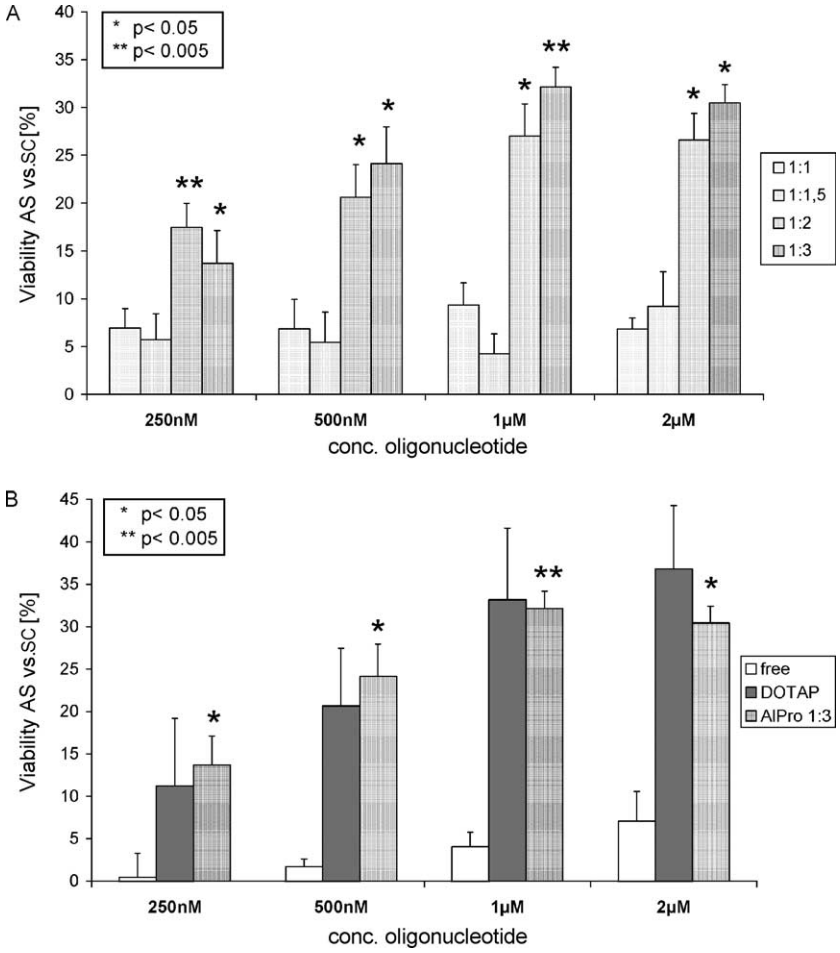

Fig. 3. (A) The viability of the AlPrO-NP treated cells in the excitotoxcity antisense model was determined with a MTT assay. The difference in viability between AS-ON and SC-ON treated cells was shown in the diagram. The static significance of this difference (AS versus SC) was checked with a student *t*-test (mean ± SD; $n = 3$). (B) Antisense-effects of different oligonucleotide preparations: The viability of the treated cells in the excitotoxcity antisense model was determined with a MTT assay. The difference in viability between AS-ON and SC-ON treated cells was shown in the diagram. The static significance of this difference (AS versus SC) was checked with a student *t*-test (mean ± SD; $n = 3$).

(Table 2), the HSA content was set to 1000 µg/ml. According to the physicochemical characterization of these preparations (Table 3) [17], we found a very low antisense effect of the 1:1 preparation which could be explained with the relative high negative zetapotential of the resulting NPs and, thus, a reduced uptake into the cells [26,29]. The 1:1.5 preparation showed a tendency to aggregation. These large particles of about 650 nm in diameter showed also an insufficient uptake and a low antisense effect as well [30]. The 1:2 ratio revealed an effect of about 28% higher viability. The AlPrO 1:3 preparation reached a higher viability of about 32% (AS-ON versus SC-ON) (Fig. 3A). A student *t*-test was performed to verify the significance of the differences between AS-ON and SC-ON treated cells. It can be discussed that the increase of antisense efficiency correlated to an increase of PS in the preparations, which can be attributed to a higher particle stability and a small monomodal size distribution (Table 3) [1,17,31].

These effects seems to be dose dependent, and was found to be maximal with 1 µM AS-ON. There was no further difference between the 1 and 2 µM ON preparation.

The second step was the comparison of our optimized AlPrO-system with free ON and DOTAP-liposomes (Fig. 3B). As we expected the free ON had only a small effect of about 5% on the viability of the AS-ON treated

Table 3
Physicochemical characteristics of the oligonucleotide preparations in buffered cell culture medium

	DOTAP	AlPrO 1:1	AlPrO 1:1,5	AlPrO 1:2	AlPrO 1:3
Zeta-potential (mV)	+5.6	−22.4	−18.9	−15.1	−12.3
Size average (nm)	215 ± 74	656 ± 25	331 ± 21	197 ± 4	285 ± 7
Polydispersity index	0.35	0.28	0.24	0.12	0.11
ON loading (%)	89.1	75.1	99.3	97.3	96.4

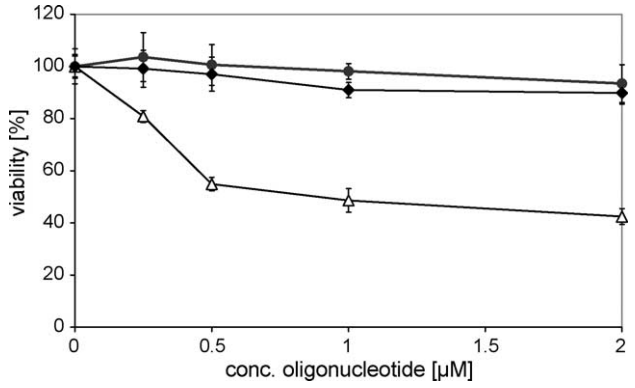

Fig. 4. Cytotoxic side effects of different oligonucleotide preparations: the viability of the treated cells was determined with a MTT assay (mean ± SD; $n=6$). (●) Free ON; (△) DOTAP; (♦) AlPrO.

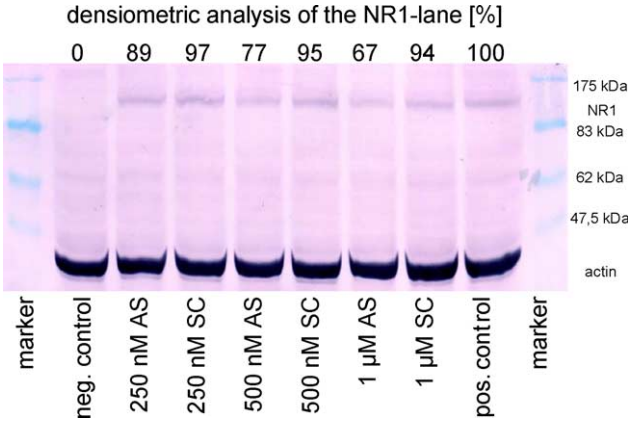

Fig. 5. Western-Blot for NR1 and actin of induced (pos. control), non induced (neg. control) and AlPrO-NP treated cells. Cells were induced with 4 µM dexamethasone for 16 h. Aliquots of solubilized cells (50 µg total protein) were probed with specific antibodies. The NR1-band was quantified by a densitometric volume analysis.

cells in relation to the SC-ON treated cells. With DOTAP we reached about 35% antisense effect (AS-ON versus SC-ON). Unfortunately, these differences are not statistically significant because of their high standard error of the mean. The higher variance in the DOTAP effects could be connected with the overlap of cytotoxic side effects of the delivery system and the NR mediated excitotoxic effect, which was used for the determination of the antisense effects (Fig. 4).

Summarizing these data it became clear that our new AlPrO-NP demonstrated a better effectiveness and a higher biocompatibility compared to cationic liposomes like DOTAP.

3.3. Western blotting

To verify our results from the functional assay which showed a higher viability of the AS-ON treated cells, we performed a western blot which allows the quantification of our target protein, the NR-protein.

Two different antibodies were applied to visualize the proteins. One was directed against the C-terminus of the NR1 and the other was directed against actin which was used as internal control to verify a constant protein content in all lanes. Naturally, this actin band is more intensive than our NR band because it is usually included in higher quantities into the cell. We investigated the antisense effect of three different concentrations of AlPrO-NPs (250 nM, 500 nM and 1 µM) AS-ON and SC-ON. Additionally, we used a negative control (0%) which was not stimulated with dexamethasone so that these cells did not express NR-protein. On the other side we used a positive control containing the maximal amount of the expressed NR-protein (set to 100%).

As a major result from the western blot analysis it was found that the AS-ON treated cells expressed less NR-protein depending on the AS-ON concentration. The SC-ON treated cells were nearly on the same protein level than the positive control (Fig. 5). These results confirmed our findings from the functional excitotoxicity model. Similar experiments were carried out for free ON and for DOTAP-liposomes (data not shown). Summarizing these experiments, we observed no detectible effect of free ON on the NR expression. The DOTAP-liposomes revealed a similar effect compared to AlPrO-NPs in western blotting.

4. Conclusion

We optimized the new delivery system for antisense-oligonucleotides developed by Vogel et al. [1] consisting only of three components: human serum albumin, protamine sulphate and oligonucleotides. These three components formed spontaneously nanoparticles (AlPrO-NPs) which are stable under isotonic conditions. We could show that by using this AlPrO-NPs the cellular uptake of oligonucleotides increased and that we could reach a transfer rate of about 100%.

No considerable cytotoxic side effects were observed for the NP preparations. After cellular uptake the oligonucleotides were distributed within the cytoplasm. The dissociated oligonucleotides showed an antisense effect in a functional excitotoxicity assay which could be correlated to the level of the target protein. These effects are comparable to a commercial available transfer reagent based on cationic lipids (DOTAP), with the advantage of a much lower cytotoxicity.

Summarizing the current results our new AlPrO-NP seems to be a highly effective non cytotoxic delivery system for antisense-oligonucleotides.

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