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Investigation of Transfection Barriers Involved in Non-Viral Nanoparticulate Gene Delivery in Different Cell Lines

Stefanie Häfele and Regine Süss
Department of Pharmaceutical Technology and Biopharmacy, Freiburg Germany

1. Introduction

In order to improve the delivery efficiency of genetic material into cells both *in vitro* and *in vivo*, the development of effective non viral vectors for optimized gene transfer into target cells has become an important objective. Non-viral vector systems in particular, such as cationic lipids and polymers, have been widely investigated as to their suitability as a delivery system [1-4]. In cell lines, non-viral gene transfer mediated by cationic lipid/DNA aggregates has been accomplished efficiently showing no immunogenicity and low cytotoxicity [5]. Unfortunately, non-viral gene transfer into primary cells is still inefficient and results in low transgene expression *in vivo* [6]. In contrast to the transfection of most cell lines, which can be successfully performed using a variety of methods, the introduction of foreign DNA into primary cells requires careful selection of the gene transfer technique. Whereas viral strategies are involved in immunogenic risks, non-viral methods have proved to be inefficient for most primary cell types. This might be due to the fact that biological barriers have to be overcome in order to achieve successful gene delivery.

Therefore, knowledge about the uptake mechanism and the subsequent intracellular processing of non-viral gene delivery systems is important for the development of efficient gene delivery systems. Moreover, in understanding the internalization of particles into cells, distinct pathways might be targeted.

Multiple processes are thought to be involved in the cellular internalization of particles [7], whereas clathrin-dependent uptake is the one which has been investigated the most. However, other internalization pathways such as the caveolae-dependent pathway, macro-pinocytosis, phagocytosis and the non-clathrin-non-caveolae dependent pathway are possible ways for gene delivery and further processing in the cells as well [8].

The mode of internalization may affect the kinetics of intracellular processing as well as transfection efficiency. Depending on the uptake mechanism a variety of obstacles could be the reason for low transfection efficiency. Also depending on the mode of cellular uptake, internalization may lead to either lysosomal degradation and digestion, recycling back to the membrane, transcytotic transport across the epithelial barrier or delivery to other compartments.

Once having been released into the cytosol, additional barriers such as insufficient desaggregation of the complex, poor cytoplasmic transport, cytosolic digestion by means of

nucleases and finally low intra-nuclear DNA delivery have to be overcome. A promising strategy for increasing the efficiency of non-viral vectors is to target certain uptake pathways that improve the efficient delivery of particles. Such a strategy requires thorough investigation of the different internalization pathways and the subsequent intracellular events involved in each case.

In this work we concentrate on the first and second major barrier to improved transfection efficiency in human primary cells. Two different cell types were chosen because of their relevance in cardiovascular diseases: human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC). Both cells are involved in the unwanted re-narrowing (restenosis) of cardiac vessels after angioplasty and therefore are the primary targets in a strategy for cardiovascular gene-therapy.

The distinct uptake mechanism of cationic lipid/DNA aggregates had to be clarified in order to gain knowledge about further intracellular processing and other barriers which still had to be overcome. To distinguish between the different endocytic pathways involved in lipoplex internalization of the Rhodamine-labeled DC-30 lipoplexes (Rh-DC-30), both general and specific inhibitors of endocytic routes were monitored in the presence of lipoplexes and analyzed by means of flow cytometry. Table 1 represents an overview of the inhibitors used. To gain more insight into the intracellular fate of lipoplexes, investigation of their co-localization with a variety of molecules was carried out using spectral bio-imaging. Transferrin alexa fluor 488 (tf) was used as a marker for clathrin-mediated uptake [9-16], cholera toxin B alexa fluor 488 (chltx-B) was used as marker for internalization via caveolae and related membrane structures [8, 17], and FITC-dextran was used as a marker for macropinocytosis.

It was subsequently investigated whether the failure of the endosomal release of the lipoplex or the desaggregation of the complex was responsible for the low transfection efficiency. Therefore, plasmid DNA was additionally introduced into the cytosol by electroporation.

Inhibitor	Uptake route	Reference
Chlorpromazine (chlp)	Clathrin	[11]
Methyl-ß-cyclodextrin (mbCD)	Clathrin and caveolae	[22-24]
Filipin (fil)	Caveolae	[25-27]
Genistein (gen)	Caveolae	[28]
LY 29004 (Ly)	Macropinocytosis	[29, 30]
Wortmannin (wm)	Macropinocytosis	[29, 30]
Nocodazole (noco)	Microtubule depolymerization	[31]
Cytochalasin-D (cch-D)	Actin skeleton disruptor	[8]

Table 1. Inhibitors for the specific endocytic routes

2. Materials

DC-30 was purchased from Avanti Polar Lipids (Birmingham, AL, USA). The Cy-5- and Rh-DNA Labeling Kits, Transferrin Alexa Fluor conjugate 488, Cholera Toxin Subunit B Alexa Fluor conjugate 488 and DAPI were purchased from Molecular Probes (Leiden, The Netherlands). Cells were purchased from ATCC (American Type Culture Collection),

Manassas, USA and pEGFP from BD Clontech Germany, Heidelberg. Unless otherwise stated, all other chemicals were purchased from Sigma/Fluka (Deisenhofen, Germany) and were of analytical grade. Additional material is described in the appropriate method section.

3. Methods

3.1 Cell culture

Human vascular smooth muscle cells (HASMC) were cultivated in smooth muscle cell medium 2 (Promocell, Heidelberg, Germany) supplemented with 5 % (v/v) FCS and human aortic endothelial (HAEC) were maintained in endothelial cell medium MV (Promocell, Heidelberg, Germany) on 100 mm culture plates. For HAEC, tissue culture plates were first coated with sterile-filtered 2 % gelatine solution for 10 min and washed twice with PBS w/o calcium and magnesium before seeding. Cells were cultivated at 37 °C in a humidified atmosphere with 5 % (v/v) CO_2.

3.2 Plasmid preparation

The plasmid pEGFP carries the green fluorescent protein coding region under the control of the cytomegalovirus immediate-early promoter region. It was isolated from Escherichia coli (Stratagene, Amsterdam, NL) with the Maxi-Prep Kit from Qiagen (Hilden, Germany). Isolated DNA was stored in TE buffer (100 mM NaCl, 10 mM Tris-HCl) at a concentration of 1 mg/mL at -20 °C after its purity was verified by determining the ratio of absorbance at 260/280 nm and by gel electrophoresis.

3.3 DNA labeling

Cy-5-labeled and Rh-labeled DNA was prepared as described for the Mirus Labeling Kit (Molecular Probes, Leiden, Netherlands).

3.4 Lipoplex formation

Enhanced green fluorescent plasmid pEGFP (either labeled for uptake studies or unlabeled for transfection studies) was mixed with DC-30® in a lipid/DNA ratio (w/w) of 5:1 according to the following protocol: lyophilized DC-30® was redispersed in sterile transfection medium (TM; 250 mM saCcharose, 25 mM NaCl) at a concentration of 1 mg/ml and incubated for 30 min at room temperature. Plasmid DNA and the respective amount of DC-30®-dispersion were diluted separately into equal volumes of TM in order to achieve the desired lipoplex concentration in 200 µL of lipoplex preparation. The dilutions were combined discontinuously by pipetting the plasmid into the liposome solution, gently mixing and incubating for at least 30 min at room temperature to allow the formation of the lipoplexes.

3.5 In vitro transfection assay

Cells were seeded in a 24-well cluster dish at a density of 10^4 cells per well 24 h prior to the experiments and cultivated in the appropriate growth medium with serum. After 24 h in culture the cells were washed with 1 ml PBS and then 400 µL growth medium containing serum was added to the cells. 200 µL of freshly prepared lipoplexes were added to the cells. After incubating for 5 h at 37 °C (5 % (v/v) CO_2) the supernatants were removed and 1 ml of the appropriate growth medium was added to each well. Thereafter, the cells were cultured further for a total of 48 h at 37 °C, 5 % (v/v) CO_2. In the control experiments cells were incubated with 200 µL culture medium or 200 µL TM and treated the same way as the

lipoplex samples. Analysis was carried out by means of flow cytometry (see uptake and FACS analysis).

3.6 Electroporation experiments

Cells were cultivated to 80 % confluency as described above. Every 100 mm plate was trypsinated with 1 mL Trypsin/EDTA for 30 s (HAEC), and 90 s (HASMC) to detach the cells. Trypsinization was stopped by adding 3 ml culture medium with serum. After washing twice with ice-cold PBS, cells were redispersed in ice-cold electroporation buffer (100 mM Hepes, 137 mM NaCl, 4 mM Na_2HPO_4, 6 mM Dextrose) at a concentration of 10^7 cells/ ml. Electroporation experiments were carried out using a Gene Pulser II (BioRad Laboratories, Muenchen, Germany). 500 µL of cell suspension was poured in a chilled 0,4 cm gap cuvette. After adding 20 µg DNA (1 mg/mL TE-buffer pH 7,0; Tris 10 mM, EDTA 1 mM) the cuvette was placed on ice and electroporated with the following settings: for HAEC: 350 V, 750 µF; for HASMC: 500 V, 950 µF. After the shock, the cuvette stayed on ice for 10 min. Then, cells were transferred into a 100 mm culture plate, (gelatine-coated plates for HAEC, see section "cell culture") and incubated for 48 h at 37 °C and 5 % CO_2. Fluorescent pictures were taken with an Axiovert S 20 (Zeiss, Jena Germany, 20x).

As a positive control experiment, C3-Toxin plasmid was electroporated into the cells to see if DNA was delivered into the nucleus. Effective delivery was achieved when cells were rounded up and dead.

As cells were not vital enough for FACS analysis, fluorescent and non-fluorescent cells were counted via "Neubauer Zählkammer". Counting was repeated twice for every culture plate for at least 3 independent experiments.

3.7 Uptake studies and FACS analysis

Two days before the uptake experiment 10 000 HAEC and HASMC respectively were seeded onto a 24-well tissue culture plate. One hour before the experiment the culture medium was refreshed with 400 µL of cell specific medium containing 5 % FCS. Prior to the inhibition experiments the cells were incubated with the inhibitors (cytochalasin D (Cch-D, 10 µM) for 120 min, chlorpromazine (chlp, 56 µM), LY29004 (ly, 50 µM), wortmannin (wm, 50 nM) genistein (gen, 200 µM) and nocodazole (noco, 10 µM) for 60 min, filipin (fil, 5 µg/ml for HASMC, 10 µg/mL for HAEC) for 30 min and methyl-β-cyclodextrin (mbCD, 164 µM) for 15 min). Subsequently, the DC-30 based lipoplexes containing Cy5-labeled plasmid DNA were added and the cells were incubated at 37 °C for another 60 min. Then, cells were washed with PBS and detached with 200 µL trypsin/EDTA (0,5 mg/mL trypsin, 0,2 mg/mL EDTA) for 30 s (HAEC) or 90 s (HASMC) and harvested by centrifugation. The cell pellet was washed in one ml ice-cold PBS and resuspended again in 200 µL ice-cold PBS. Cell toxicity was measured by adding 2 µL 7-AAD (BD Biosciences), a fluorescent DNA-binding dye which only penetrates dead cells. Fluorescent positive cells were analyzed with a FACS Calibur using Cell Quest Pro software (BD Biosciences). 10 000 cells were measured for each sample. GFP was measured using fluorescence channel 1 (530+/-15 nm), Rh in fluorescence channel 2 (585+/-21 nm), 7-AAD in fluorescence channel 3 (661+/-8 nm) and Cy5 in fluorescence channel 4 (> 670 nm).

In order to determine the amount of lipoplex absorbed to the outside of the cell membrane, experiments were also performed at 4 °C. Furthermore, control experiments such as incubation only with Cy5-labeled DNA with buffer alone were performed.

3.8 Spectral bio-imaging

Cells were seeded onto 12-mm coverslips in 24-well plates 2 days prior to the experiment. Lipoplexes were prepared using DC-30® and Rh-labeled plasmid. Cells were then washed twice with PBS w/o calcium and magnesium and then incubated with 400 µL medium containing the lipoplexes. Cells were incubated simultaneously with the markers for the different pathways for 30 min to 2 h (tf alexa fluor 488 (10 µg/mL), chltx-B alexa fluor 488 (2 µg/mL) and FITC dextran 70 000, (1 µg/mL)). The amount of DNA was the same as that used in the uptake experiments. Prior to microscopic examination, the cells were fixed with 4 % paraformaldehyde (500 µL, 10 min, RT) and the coverslips were mounted on glass slides with 3 µL MobiGlow (MoBiTec, Goettingen, Germany), an antifading substance to reduce photobleaching effects.

Spectral bio-imaging was performed as described previously by Huth et al. [8] with a SpectraCube SD-200H system (Applied Spectral Imaging, Migdal HaEmek, Israel). An inverted fluorescence microscope (Axiovert S 100, Zeiss, Jena Germany) equipped with a high-pressure mercury lamp (HBO 100) for excitation and a triple bandpass filter set was used. All images were taken using a 100x/1.3 oil-immersion objective lens (Plan Neofluar, Zeiss, Jena, Germany). In the spectral range from 400 to 700 nm the objective lens shows only minimal fluctuations of transmission (85-90 %). The optical head attached to the microscope is composed of a Sagnac common-path interferometer and imaging optics including a cooled CCD camera (Hamamatsu C4880-85, Japan). Microscopic images were obtained with spectral bio-imaging 2.5 software (Applied Spectral Imaging). The acquisition time of a desired image varied from 30 to 90 s depending on the brightness of the fluorescence and the image size. Cells were first incubated with only one dye to get single-colored images. For further analysis images were then transferred to the SpectraView 1.6 software (Applied Spectral Imaging).

4. Results

4.1 Uptake experiments with inhibitors

For uptake experiments studied by means of flow cytometry, DNA was labeled with Cy-5. Cell experiments were performed at both 37 °C and 4 °C in order to distinguish cellular uptake from adsorption of lipoplexes to the outside of the cell membrane or their fusion with the cell membrane. It has been described that no energy dependent internalization process can take place at 4 °C [18]. As the size of the DC-30® lipoplexes was determined to be in the range of 300 and 500 nm [5], we assumed internalization to be an active process. The detection of positive fluorescent signal at 4 °C therefore refers to fusion or adsorption of lipoplex with the cell membrane. Fig. 1 represents the amount of fluorescent positive cells after one hour of incubation with lipoplexes at 37 °C.

The corresponding transfection efficiency is listed in Table 2 and shows that despite the fact that uptake of lipoplexes was successful in 24 % of HAEC, less than 1 % of HAEC were transfected. In HASMC, uptake reached as high as 80 %, whereas transfection was only 2 %.

Only 1 % of fluorescent cells were detected after incubation at 4 °C and therefore adsorption to the outside of the cell membrane or fusion with the cell membrane is negligible and the fluorescent positive cells as seen in Fig. 1 can be considered as resulting from cellular uptake (internalization) of the lipoplexes. Analysis of cytotoxicity confirmed results which were published previously [5].

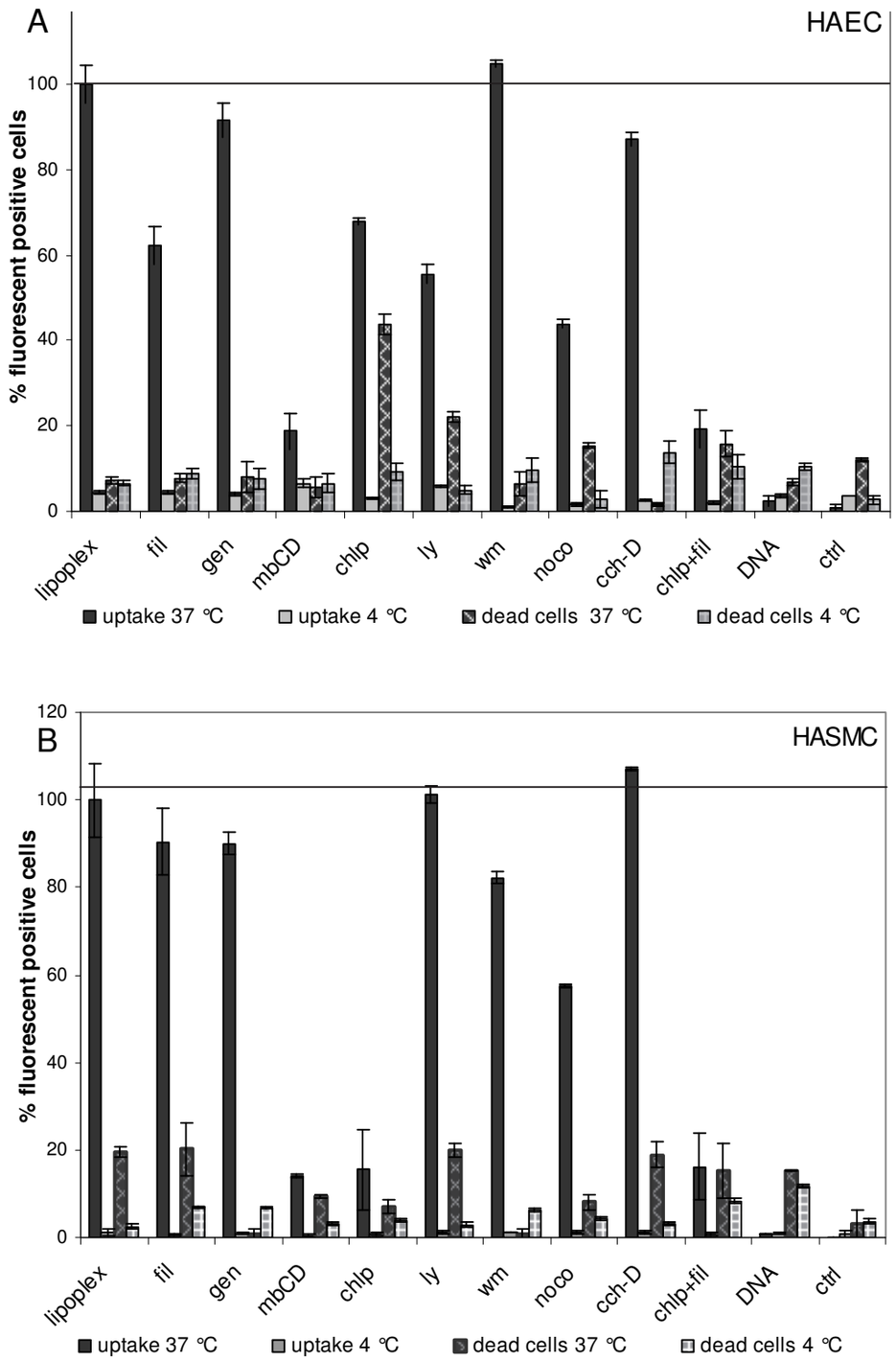

Fig. 1. Cells were incubated for one hour with Cy-5 labeled lipoplexes and analyzed by means of flow cytometry (Fl-4 channel). Prior to the measurement, every sample was additionally incubated with 7-AAD to determine the amount of dead cells (Fl-3 channel). Error bars indicate standard deviation (n=3 in 3 independent experiments).

Transfection method	Transfection agent	Transfection efficiency [%]	
		HAEC	HASMC
Non-viral cationic lipid-mediated DNA-delivery	DC-30 lipoplex	0,40 ± 0,02	2,01 ± 0,08
	DC-30 lipoplex + 100µM Chloroquine	0,70 ± 0,02	2,74 ± 0,14
	DC-30 lipoplex + 100 µM Chloroquine	0,11 ± 0,03	2,47 ± 0,22
Electroporation	DNA	43 ± 7	51 ± 8

Table 2. Influence of transfection method on transfection efficiency

Fig. 2 and Fig. 3 show the internalization of DC-30 lipoplexes by HAEC and HASMC in the presence of inhibitors of different uptake pathways. Again the incubation at 4 °C did not show any passive adsorption or fusion of DC-30® lipoplexes with the membrane in both cell types.

As depicted in Fig. 2 A the uptake of DC-30® lipoplexes by HAEC pretreated with filipin (fil) was reduced by about 40 % to 60 % relative to control values. However, another caveolae-inhibitor, genistein (gen), did not reduce lipoplex uptake significantly. Genistein affects tyrosine kinase [19], which associates with caveolae. In contrast, filipin affects 3-ß-hydroxycholesterols [20] and avoids the formation of caveolin coats ab initio. Filipin seems to intervene at the origin of internalization whereas genistein seems to inhibit further processing of caveosomes after budding out of the membrane.

The inhibitor methyl-ß-cyclodextrin (mbCD) is involved in cholesterol depletion of the plasma membrane and therefore influencing both cholesterol-rich domains (caveolae and clathrin-mediated endocytosis) and was shown to reduce lipoplex uptake significantly by 80 %.

Clathrin-dependent endocytosis was analyzed with chlorpromazine (chlp) which interacts with clathrin-coated pits and causes their loss from the surface membrane. Uptake was reduced by about 25 %. Combining the clathrin and caveolae-mediated endocytosis inhibitors chlorpromazine and filipin leads to a comparable reduction of lipoplex uptake with that achieved with mbCD.

Macropinocytosis is possibly involved in the uptake mechanism of DC-30® lipoplexes in HAEC. Pre-incubation with the macropinocytosis inhibitors LY29004 (Ly) and wortmannin (wm) only led to a significant reduction of uptake using Ly but not wortmannin.

Nocodazol (noco) acts as an inhibitor by depolymerizing microtubules and therefore prevents the transport vesicle (early endosome) from fusing with the late endosome in order to protect the early endosome´s content from digestion and degradation in the lysosomal compartment. Applying this inhibitor showed a 50 % reduction in fluorescent signal.

Incubation with cytochalasin D (Cch-D), a disrupter of the actin cytoskeleton, resulted in a reduction of about 10 % and did not seem to be strongly involved in the uptake or processing of DC-30® lipoplexes. In this context, the involvement of macropinocytosis, which is strongly actin dependent, and the different inhibition rates caused by LY and wortmannin does not clarify whether or not lipoplexes are internalized via macropinocytosis.

Fig. 2 B presents data on the internalization of DC-30® lipoplexes by HASMC. Cells pretreated with the caveolae-blocking reagents filipin and genistein showed only a slightly decreased uptake of lipoplexes. In contrast, upon incubation with the clathrin inhibitor

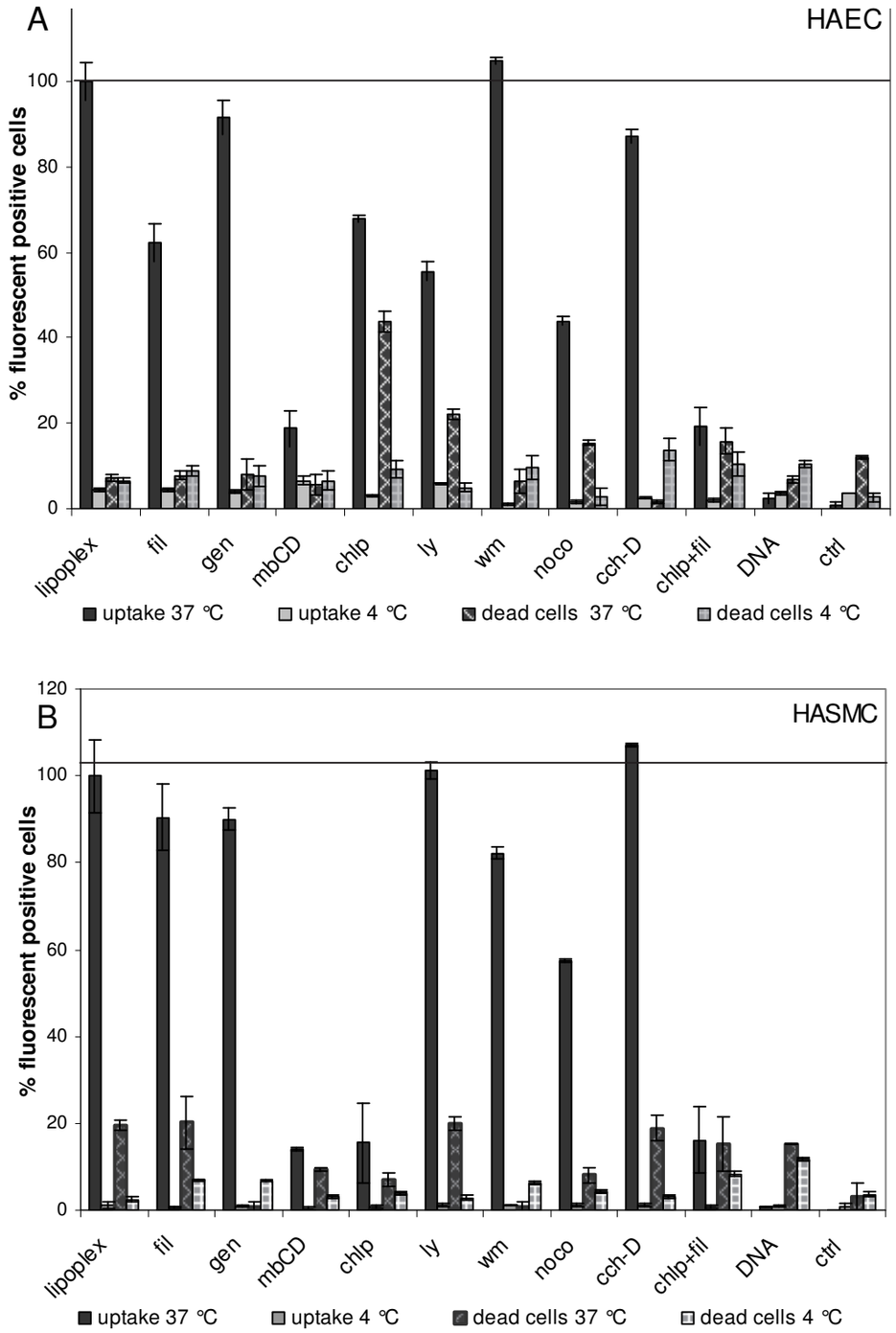

Fig. 2. After pre-incubation with different inhibitors (see Table 1) (x-range), HAEC (A) and HASMC (B) were incubated for one hour with Cy-5 labeled DC-30® lipoplexes and analyzed by means of flow cytometry (Fl-4 channel). Prior to measurement, every sample was also incubated with 7-AAD to determine the amount of dead cells (Fl-3 channel). The fluorescence intensity of lipoplexes without inhibitors was set to 100%. Error bars indicate standard deviation (n=3 in 3 independent experiments).

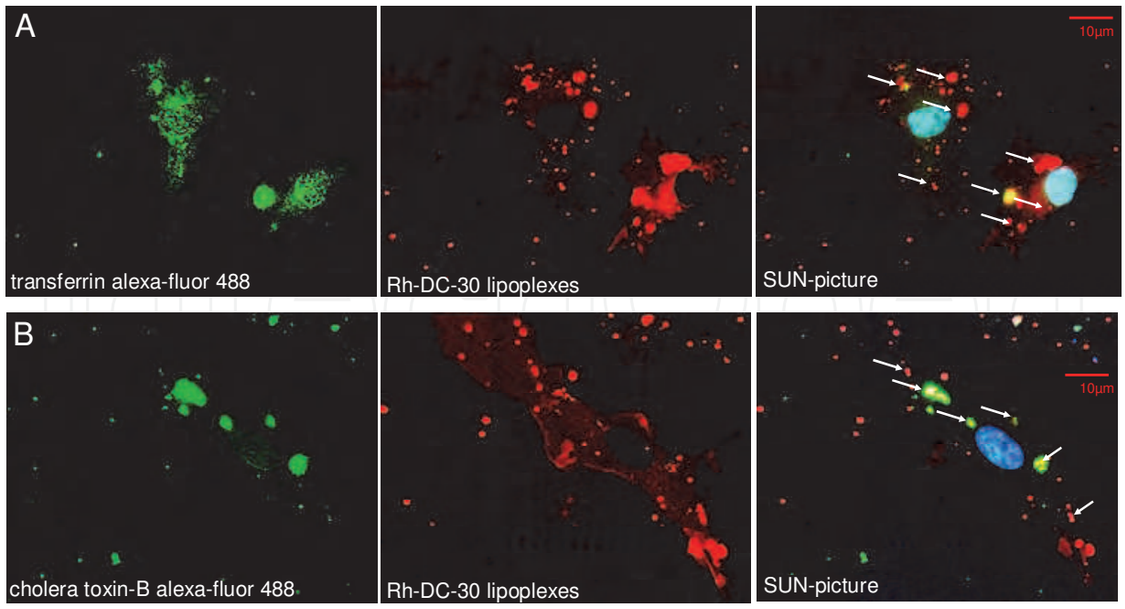

Fig. 3. HAEC were incubated for one hour with either (A) clathrin marker tf alexa-fluor 488 and Rh-lipoplexes or (B) caveolae marker choleratoxin-subunit B alexa-fluor 488 and Rh-lipoplexes. After subsequent DAPI staining of the nucleus, pictures were taken by spectral bio-imaging. I: localization of Rho-lipoplexes; II: localization of markers; III: overlay of single colour images.

chlorpromazine the highest reduction of uptake was observed (about 85 %). The same result could be achieved with mbCD, which is involved in both clathrin and caveolae-mediated pathways. Again, when combining the specific caveolae and clathrin inhibitors (chlp and fil), lipoplex uptake could not be further reduced. Macropinocytosis is also supposed to be involved in lipoplex uptake in these cells, but pre-treatment with wortmannin alone caused only a minor reduction (about 10 %) whereas Ly did not seem to effect the uptake mechanism.
On the other hand, uptake of DC-30® lipoplexes by HASMC pre-treated with nocodazole was diminished by about 45 %, which shows a correlation to the clathrin-blocking reagents. The actin-disrupting agent Cch-D did not cause a reduction of lipoplex uptake at all.

4.2 Microscopic uptake studies

The results obtained with uptake studies were confirmed using spectral-bio-imaging. Cells were first pre-incubated with the inhibitors chlormpromazine or filipin. Therefore, the internalization routes were examined via co-localization studies with markers for the clathrin- or caveolae-dependent pathways. The DNA of the lipoplex was labeled with Rhodamine (Rh). Transferrin alexa-fluor 488 was used as a clathrin marker, Choleratoxin-subunit B-Alexa Fluor 488 was a marker for internalization via caveolae-dependent endocytosis and FITC-dextran a marker for macropinocytosis. During preparation for the microscopic experiments, cells were additionally incubated with DAPI (nucleus staining) as a control. Co-localization of the lipoplex and pathway markers was determined by bio-spectral-imaging.
In HAEC after one h incubation time, co-localization of lipoplexes with tf-488 (Fig. 3A) as well as with chltx-B-488 (Fig. 3B) was detected which indicates uptake via clathrin- and

caveolae-dependent pathways. No co-localization with the macropinocytosis marker FITC-dextran 70,000 could be shown.
In HASMC, Rh-labeled lipoplexes only showed co-localization with tf-488 (Fig. 4), the marker for clathrin-dependent uptake.
In both cases, spectral bio-imaging studies confirmed the results achieved by flow cytometry.

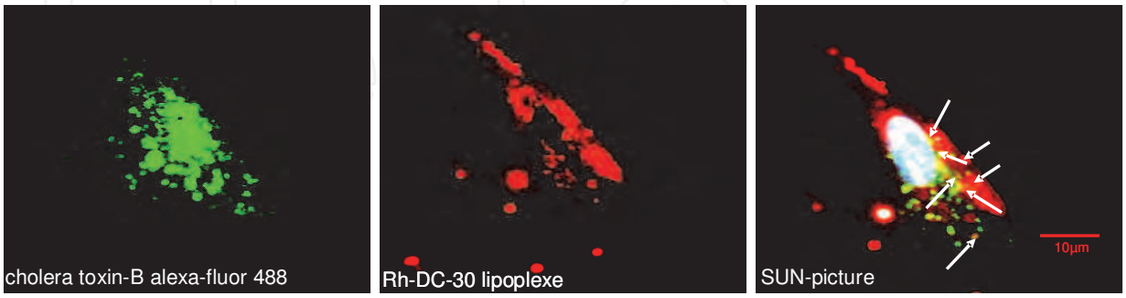

Fig. 4. HASMC were incubated for one hour with clathrin marker tf alexa-fluor 488 and Rh-lipoplexes. After subsequent DAPI staining of the nucleus pictures were taken by spectral bio-imaging. I: localization of Rh-lipoplexes; II: localization of tf alexa-fluor 488; III: overlay of single colour images.

4.3 Effects on transfection efficiency

As cellular internalization of lipoplexes was successfully achieved in both HAEC and HASMC by clathrin-dependent endocytosis, which results in the transport of lipoplexes via endosomes (Fig. 5), further investigations examined possible barriers after uptake.
For these experiments DNA was introduced into the cell by electroporation. With this method, endosomal release and disintegration of the DNA from the lipoplex are no longer involved. Basically, it can be determined that the introduced DNA is not degraded in the cytosol and is able to cross the nuclear membrane to enter the nucleus. Indeed, electroporation resulted in transfection efficiencies of 43 % in HAEC and 51 % in HASMC (Table 2, Fig. 6), indicating that transfection is restricted either by insufficient endosomal release or insufficient release of DNA from the lipoplex.
To investigate insufficient endosomal release, transfection experiments with DC-30® lipoplexes were accomplished with HAEC and HASMC as described before [5]. Additionally, prior to lipoplex incubation the cells were incubated with different concentrations of chloroquine, which represses lysosomal degradation by increasing the pH [21] to facilitate endosomal escape and therefore improve transfection efficiency. Addition of chloroquine did not lead to an increase in transfection efficiency (Table 2). This shows no insufficient endosomal escape of lipoplex.

5. Discussion

This study demonstrates, that the development of efficient non-viral gene delivery systems requires better understanding of the mechanism of the delivery and of the intracellular processing. Endocytosis is the major pathway of entry, but the involvement of specific endocytic pathways is still poorly defined. This study contributes to further clarify uptake mechanisms and barriers in gene delivery in primary cells.

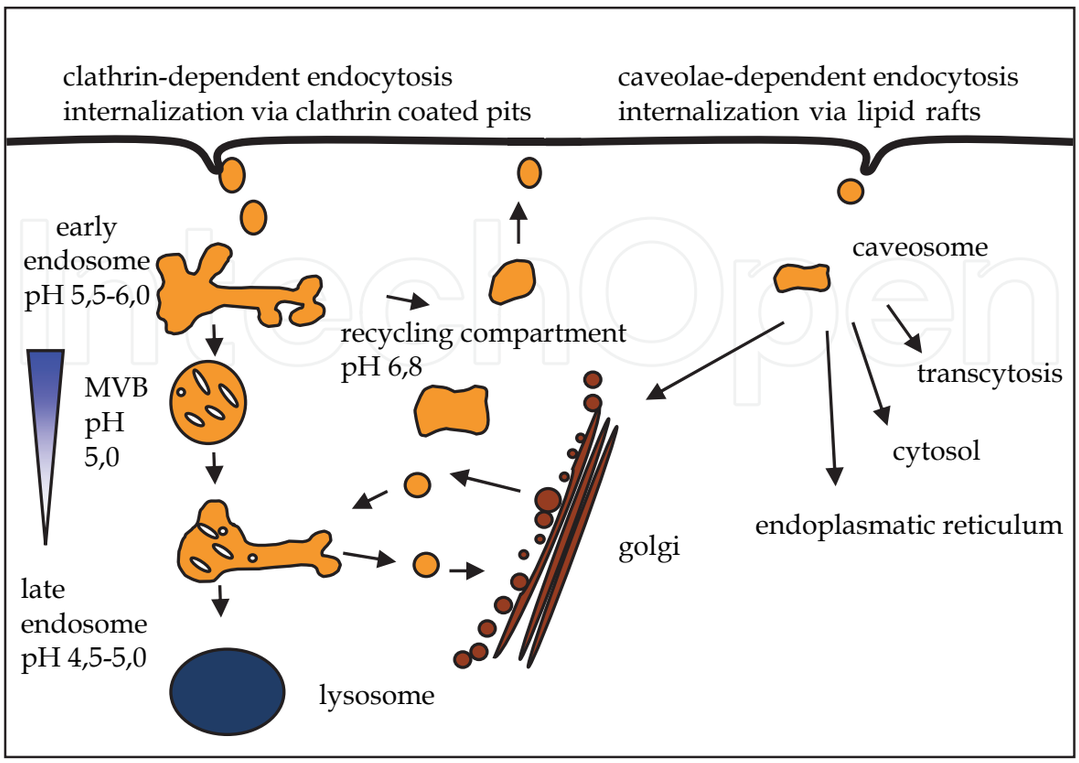

Fig. 5. Cartoon of the main cellular internalization routes of lipoplexes. On the left hand the clathrin-dependent pathway and on the right hand the caveolae-mediated uptake mechanisms are shown. Whereas the first pathway has been well investigated, caveolin-mediated internalization has not been examined in detail to date. The first step in the clathrin-dependent pathway is the budding of clathrin-coated vesicles resulting in early or sorting endosomes. Recycling back to the membrane happens in recycling endosomes. Early endosomes transform in MVB and subsequently late endosomes are formed which end up as lysosomes. Active exchange between the golgi apparatus and late endosomes is known. Particles, internalized via caveolae from so-called caveosomes offering various processing possibilities such as transcytosis, cytosolic release, delivery to the ER or transport to the golgi apparatus.

It showed, that poor transfection efficiency of primary human aorta and smooth muscle cells (HAEC and HASMC) is not due to insufficient delivery into the cell. Uptake of lipoplex reached 20 % in HAEC and 80 % in HASMC whereas transfection was less than 3 % in both cases. Furthermore, it was shown, that plasmid DNA, transferred directly into the cytosol of the cell by electroporation effectively transfected 40 to 50% of the cells. These data suggest, that DNA, once being present in the cytosol, is able to cross the nucleolae membrane and to show activity.

In the present study it was shown, that lipoplexes are partly internalized in HAEC via caveolae mediated endocytosis. The caveolae route is known to enable transport of nanoparticulate drug delivery systems into and/or through the cell without involvement of lysosomal compartments. In case of drugs being sensitive to acidic hydrolysis or enzymatic degradation, e.g., biomacromolecules such as polypetides or nucleic acids, it is important to avoid cellular routes that transport material to lysosomes. On the other side, uptake via

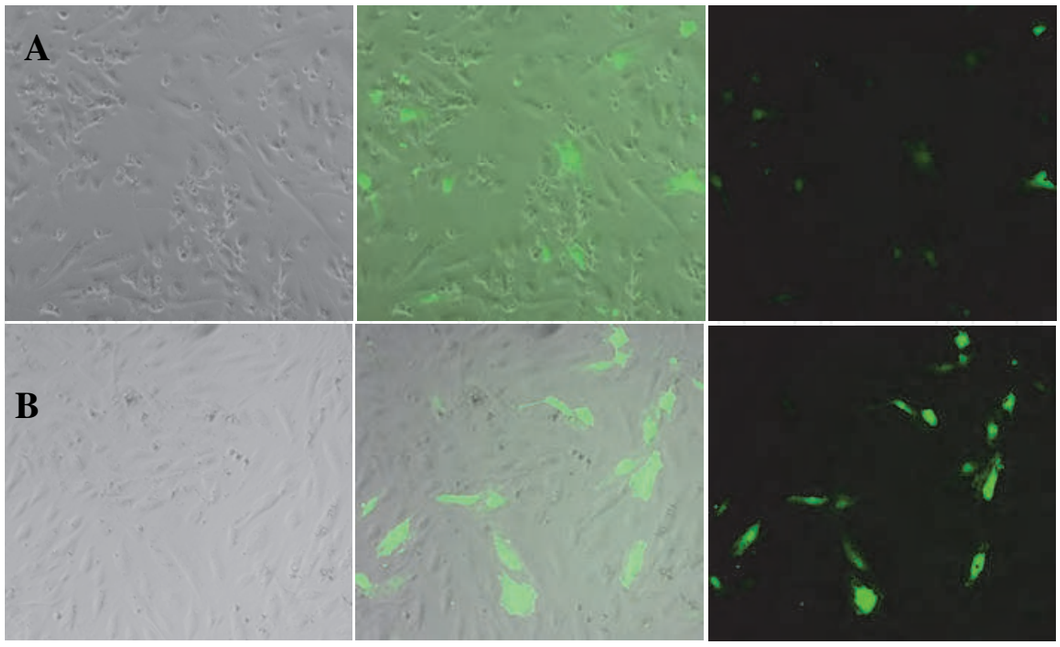

Fig. 6. HAEC (A) and HASMC (B) after electroporation of pEGFP and 48 h incubation. Left-handed: brightfield; Right handed: fluorescent picture (GFP), middle position: overlay of pictures.

caveolae does not automatically result in effective transfection as shown in this study. Although uptake in HAEC was partly achieved by the caveolae route, no transfection occurred. This is important to consider, because obviously it is not enough to focus the research on modifying particulate drug carriers, e.g., by attaching specific ligands, introducing surface charge or pH-sensitivity, or changing the particle size or elasticity in order to trigger the caveolae mediated endocytosis.

The use of different methods (flow cytometry, spectral bio-imaging, electroporation) and materials (markers and inhibitors of endocytosis pathways) contributed to the evidence that transfection of primary human aorta endothelium and smooth muscle cells was not effective due to insufficient dissociation of plasmid DNA from the lipid/DNA delivery system. This finding is of great importance for future drug deliver development in the field of gene therapy.

6. Acknowledgments

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7. Abbreviations

DC-30 (DC-Chol, 3ß-[(N´,N´-Dimethylaminoethane)-carbamoyl]-cholesterol-HCl and DOPE, Dioleoylphosphatidylethanolamine 3/7 (w/w); EGFP, enhanced green fluorescent protein; HASMC, human aorta smooth muscle cells; HAEC, human aorta endothelial cells; TM, transfection medium; DAPI, 4',6-Diamino-2-phenylindol-dihydrochlorid; mbCD, methyl-

beta-cyclodextrine; chlp, chlorpromazine; chtx-B, cholera toxin subunit B; tf, human transferrin; wm, wortmannin; gen, genistein; fil, filipin; noco, nocodazole; Ly, Ly294002 ; Rh, Rhodamine; cch-D, cytochalasin-D; ctrl, control; 7-AAD, 7-Amino-actinomycin

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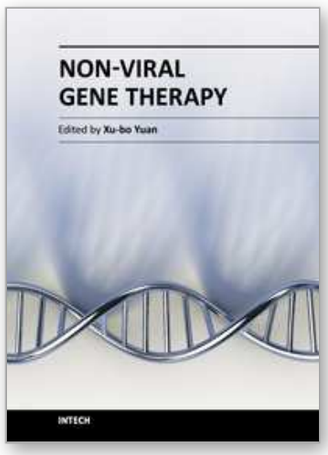

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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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Phone: +86-21-62489820
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