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Research paper

Fluorescence *in situ* hybridization to monitor the intracellular location and accessibility of plasmid DNA delivered by cationic polymer-based gene carriers

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ABSTRACT

Information about the intracellular trafficking of exogenous DNA delivered by nonviral gene delivery systems is of major importance for optimization of such gene carriers. We used fluorescence *in situ* hybridization (FISH) as a tool to visualize polyplex-delivered pDNA inside cells. This avoids the need to directly label DNA inside the polyplexes, which may influence their cellular behavior and fate. Using FISH the introduced plasmid DNA could be detected in the cytosol and nucleus of different cell lines. The FISH probe itself did not interact with cells nor different polymers used for condensing the DNA. We further demonstrate differences in accessibility of polyplex-delivered DNA when different polymers were used for DNA complexation. Therefore, FISH is a valuable tool to detect location and accessibility of exogenous plasmid DNA delivered in the cell by cationic polymers.

1. Introduction

The introduction of exogenous DNA into the nucleus of diseased cells is a key step in gene therapy. As DNA itself is poorly taken up by cells, gene carriers are needed that can facilitate the transport of DNA into the cell. Both viral vectors and synthetic vectors have been used for this purpose [1,2]. Synthetic or nonviral vectors generally consist of cationic structures (e.g. polymers, peptides or lipids) that can electrostatically interact with DNA to form small complexes with a net positive surface charge. These small, condensed structures are efficiently taken up by cells. Nevertheless, transfection efficiencies with cationic polymers or lipids have been poor and leave much room for optimization. This requires a good understanding of the intracellular fate of gene carriers. For effective transfection, nonviral gene carriers need to be taken up by cells after which the DNA has to be delivered into the nucleus [1]. Somewhere during this process, the condensed DNA needs to be released from the gene carrier. The timing of dissociation is critical as premature release of DNA may cause degradation of the DNA [3] whereas tardy or no dissociation may severely hamper nuclear import and transcription of the transgene [4]. Sensitive techniques that allow the monitoring of the intracellular fate of nonviral gene carriers and their associated DNA are therefore important as it enables to identify bottlenecks in the process of gene delivery.

Several, mainly fluorescence-based methods have been developed to investigate the intracellular trafficking of the DNA, involving non-covalent as well as covalent DNA-labeling methods. Non-covalent DNA-labeling methods include the use of DNA-intercalating agents like ethidium homodimer [5] and YOYO-1 [6] and methods based on electrostatic complexation of the fluorescent probes with DNA. Covalent linkage of fluorescent tags to the DNA prior to complexation with its carrier has been used as well [7]. However, both methods have serious limitations, which may cause artifacts and misinterpretation of the obtained results. Upon dissociation and/or degradation of gene delivery complexes inside the cell, fluorescent tags may be released and redistributed throughout the cell, not representing the location of the DNA of interest. Furthermore, the effect of attaching labeled compounds to the DNA may change its physicochemical properties and thereby induce changes in its intracellular fate [8]. Finally, emission of fluorescent probes is usually greatly influenced by its microenvironment (pH, tissue-density) as well as the concentration of fluorescent probes.

The above mentioned shortcomings of direct DNA-labeling techniques to visualize the intracellular location of exogenous

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DNA may be prevented by using post-transfection labeling procedures, where the DNA is labeled *in situ* after being delivered inside the cells. Fluorescence *in situ* hybridization can be used for this purpose [9]. FISH is a cytogenetic technique which is mostly used to detect and locate specific sequences on chromosomes using fluorescent probes [10] but can also be used to detect the presence of exogenous DNA inside cells or tissues [11]. For example, Gussoni et al. studied the presence of exogenous genes in rat brain tissue after local administration of a viral vector, whereas Dean monitored the intracellular location of plasmid DNA microinjected into the cytosol of cells [12,13].

The aim of this study is to test whether FISH is also a suitable method to monitor the location and accessibility of exogenous DNA delivered inside the cell by different polymeric carriers (polyplexes). A digoxigenin (DIG)-labeled probe complementary to the plasmid DNA was used in combination with fluorescently labeled anti-DIG antibodies to visualize the cellular location and accessibility of the plasmid DNA. Specific hybridization of the DIG-labeled DNA probe and subsequent detection of this probe with fluorescently labeled antibodies allows sensitive visualization of the location of specific DNA within the cell using confocal laser scanning microscopy (CLSM). The results show that FISH not only gives information about the location of polyplex-delivered exogenous DNA within the cells at different time points after transfection, it also provides clues about the accessibility of DNA complexed with different cationic polymers.

2. Materials and methods

2.1. Cells and cell culture

COS-7 African green monkey cells were grown in DMEM (Gibco BRL, Breda, The Netherlands) complemented with heat-inactivated 5% fetal calf serum (FCS) and 25 mM Hepes. OVCAR-3 cells were grown in the same medium, but then supplemented with 10% FCS. NIH/3T3 cells were grown in RPMI 1640 medium (Gibco BRL, Breda, The Netherlands) complemented with 10% FCS and 4.5 g/l L-glutamine. Cells were cultured at 37 °C in a 5% CO_2 humidified air atmosphere. All cultures were supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml).

2.2. Polyplex preparation

pDMAEMA-co-AEMA was synthesized as described before [14] and when required labeled with rhodamine-B-isothiocyanate (Aldrich). The plasmid DNA (pDNA) used in this study was an expression plasmid encoding the firefly luciferase under the control of the human cytomegalovirus promoter (pLuc; Plasmid Factory, Bielefeld, Germany). PDMAEMA- and pDMAEMA-co-AEMA-rhodamine B polyplexes were prepared to obtain a final concentration of 1 μg pDNA/well in the optimal polymer/DNA N/P ratio of 5/1. Polyplexes were prepared by adding 37.5 μg/ml pDMAEMA or pDMAEMA-co-AEMA-rhodamine B to 50 μg/ml pDNA in a volume ratio of four to one (polymer to DNA). Linear 22-kDa PEI (ExGen 500, Fermentas) was complexed with plasmid as described by the manufacturer at an N/P of 6/1. pDAMA polyplexes were made at a 12/1 N/P ratio. Polyplexes were incubated for 30 min at room temperature prior to addition to cells.

2.3. Cell transfection

For each cell line, 10^4 cells per well were seeded on a glass 16-well chamber slide (Costar) 24 h before transfection to reach 60–70% confluency during the transfection period. Prior to transfection the culture medium was refreshed with 100 μl complemented DMEM medium, containing 10% FCS. The cells were incubated for 1 h with 100 μl polyplex dispersions. Subsequently, the cells were washed and refreshed with complemented medium containing 10% FCS. The cells were cultured for 1, 4 or 24 h after transfection.

For the probe polyplex accessibility study, cells were kept at 4 °C prior to addition of polyplexes. After addition of pDMAEMA-co-AEMA-rhodamine B-based polyplexes the cells were incubated for 60 min at 4 °C and subsequently washed with ice-cold medium to remove the unbound polyplexes.

2.4. Fluorescence in situ hybridization

After various incubation times (1, 4 and 24 h) cells were washed three times with PBS, incubated with 100 μl hypotonic buffer (0.075 M KCl) and fixed with 100 μl methanol/acetic acid (4/1) for 30 min at room temperature. Subsequently, the cells were dehydrated in an alcohol solution series (50%, 70%, 95% and 100%) for 5 min each and air dried. To synthesize the DIG-labeled dUTP probe 1 μg of pDNA (the same DNA used for transfection of the cells) was labeled using DIG-Nick Translation Mix as described by the manufacturer (Roche Applied Science). This resulted in a probe with a length varying between 200 and 750 bp. The probe was purified using a Qiaquick PCR purification kit (Qiagen). The DIG-labeled probe at a concentration of 7 μg/ml was denatured in a hybridization solution (50% formamide, 2% Denhardt's solution (Sigma), 10% dextran sulfate, 10% herring sperm DNA (1 mg/ml, Sigma) in 1× SSC (sodium chloride–sodium citrate buffer), and 30 μl probe for 30 min at 70 °C. Prior to the addition of the probe-hybridization solution (20 ng probe/well), the DNA in the cells on the chamber slide was denatured in 70% formamide in 2× SSC for 12 min at 80 °C. Subsequently, the slide was covered with Parafilm and incubated in a humidified box overnight at 37 °C. After hybridization the cells were washed with 2× SSC, followed by three separate incubations in 50% formamide in 2× SSC for 5 min at 43 °C. Hereafter, the cells were washed stringently for 5 min in 0.1× SSC at 60 °C and washed with PBS. To detect the cell-associated probes cells were stained using the Fluorescent Antibody Enhancer Set for DIG detection according to manufacturer's protocol (Roche Applied Science). The nuclei were stained with 2 μM TO-PRO-3 iodide (1 mM, Molecular Probes) in PBS for 25 min at room temperature. Hereafter, the cells were mounted in FluorSave™ Reagent (Calbiochem) and covered with glass. The slides were examined using a Leica TCS-SP CLSM and analyzed using Leica TCS-SP Power Scan software (Leica Microsystems, Rijswijk, The Netherlands).

3. Results

3.1. Detection of pDNA inside cells with FISH

Plasmid DNA complexed with the cationic polymers PEI, pDMAEMA or pDAMA was detected inside transfected OVCAR-3 cells with fluorescent *in situ* hybridization using a DIG-labeled probe. The probe was synthesized by DIG-Nick Translation using the same plasmid template as was used for transfections. The probe was purified and denatured before incubation with transfected cells, which were fixed and permeabilized to allow penetration of the DIG-labeled probe. The probe hybridized to pDNA was detected using a FITC-conjugated antibody against DIG. FISH proved to be specific for the plasmid DNA, because it did not hybridize with chromosomal DNA in the cell (Fig. 1a). In addition, the probe did not exhibit electrostatic binding with the cationic polymers used for gene delivery, since no FISH signal was observed in OVCAR-3

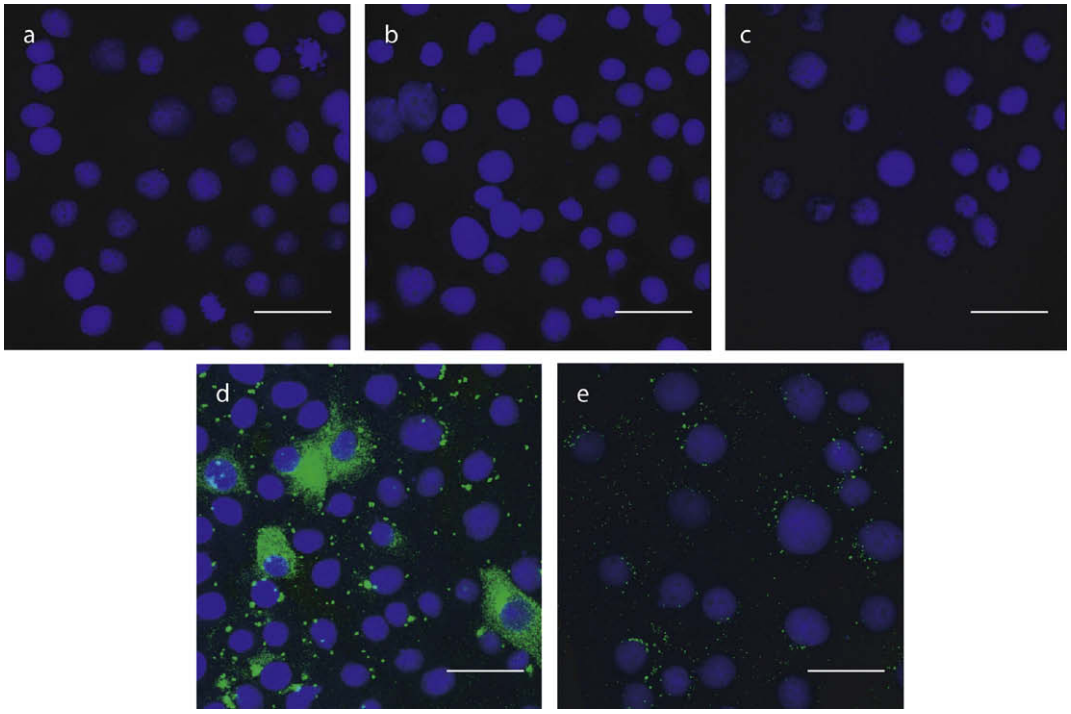

Fig. 1. Determination of the specificity of FISH probes. OVCAR-3 cells were incubated for 1 h at 37 °C in the presence of PEI (b) or pDMAEMA (c) polymer or pEI- or pDMAEMA-based polyplexes (d and e, respectively) or in the absence of polymers or polyplexes (a). Cells were washed and further incubated for 24 h at 37 °C prior to fixation and incubation with the FISH probe. Nuclei were stained with TO-PRO-3 (blue). Bar represents 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells incubated with PEI (Fig. 1b) or pDMAEMA (Fig. 1c) polymers alone. However, bright fluorescent spots were mainly observed inside the transfected cells after OVCAR-3 cells were incubated with PEI-complexed pDNA for 1 h, followed by a 24-h incubation at 37 °C in polyplex-free medium (Fig. 1d) or with pDMAEMA-based polyplexes (Fig. 1e).

3.2. FISH detection of pDNA inside transfected cells is dependent on the accessibility of pDNA for the FISH probe

To establish whether FISH is able to detect polyplex-condensed pDNA and to test if the polymer and plasmid DNA localize together in the cell, rhodamine-labeled pDMAEMA (pDMAEMA-co-AEMA-rhodamin B) was used to complex the pDNA into polyplexes. This polymer has the same DNA binding properties as regular pDMAEMA and is able to transfect cells with comparable efficiency as pDMAEMA [15]. OVCAR-3 cells were incubated with rhodamine-labeled pDMAEMA-based polyplexes for 4 h at 4 or 37 °C, fixed and incubated with pDNA-specific DIG-probes followed by the detection with FITC-labeled anti-DIG antibodies. No FISH signal was detected when cells were incubated with pDMAEMA polyplexes at 4 °C, despite binding of the polyplexes to the OVCAR-3 cell membranes as judged by the presence of punctuate rhodamine fluorescence at the cell surface, indicating that the pDNA inside the polyplexes is not available for the DIG-probe at 4 °C (Fig. 2a). In contrast, pDNA inside pDMAEMA-based polyplexes became accessible for FISH after cellular uptake at 37 °C (Fig. 2b). This was visualized by the co-localization of rhodamine-labeled pDMAEMA with the anti-pDNA FISH signal, resulting in yellow spots, as well as free pDNA detection throughout the cytosol in green (Fig. 2b).

To investigate whether the degree of accessibility of polyplex-delivered pDNA varies between different cell lines, FISH was performed on OVCAR-3, NIH/3T3 and COS-7 cells after incubation with pDMAEMA-based polyplexes. All tested cell types showed high accessibility of pDNA after cellular uptake of the polyplexes. The highest degree of co-localization was observed in OVCAR-3 cells (Fig. 3a). NIH/3T3 cells incubated with polyplexes showed primarily rhodamine fluorescence and only slight levels of FITC fluorescence, indicating that pDMAEMA-based polyplexes are efficiently internalized by these cells, but the pDNA is only poorly accessible for the FISH probe 24 h after addition of the polyplexes to the cells (Fig. 3b). In contrast, COS-7 cells showed high levels of FISH signal, which did not co-localize with the rhodamine fluorescence, indicating that in these cells, most pDNA delivered by polyplexes has been released form the complexes (Fig. 3c).

3.3. Polyplexes prepared with polymers with increasing pDNA condensing capacities give decreasing FISH signals

As previous experiment showed that after polyplex transfection detection of pDNA inside cells is dependent on the accessibility of the DNA for the FISH probe, we tested different polyplexes prepared with polymers with different DNA condensing capacities. pDAMA has been shown to bind strongly to pDNA resulting in a low dissociation rate and as a consequence showed poor transfection efficiency [4]. To see whether there is a negative correlation between pDNA condensing capacity of the polymer and the accessibility of polyplex-delivered pDNA for FISH probes after cellular uptake, OVCAR-3 cells were incubated for 24 h at 37 °C with pDAMA, pDMAEMA and PEI-based polyplexes, all containing the same amount of pDNA. The amount of FISH signal was higher when cells were transfected with PEI-based polyplexes compared to pDMAEMA-based polyplexes (Fig. 4b and c). Interestingly, hardly any FISH signal was observed when pDAMA was used to transfect cells, indicating poor accessibility of pDNA delivered by pDAMA polyplexes (Fig. 4a).

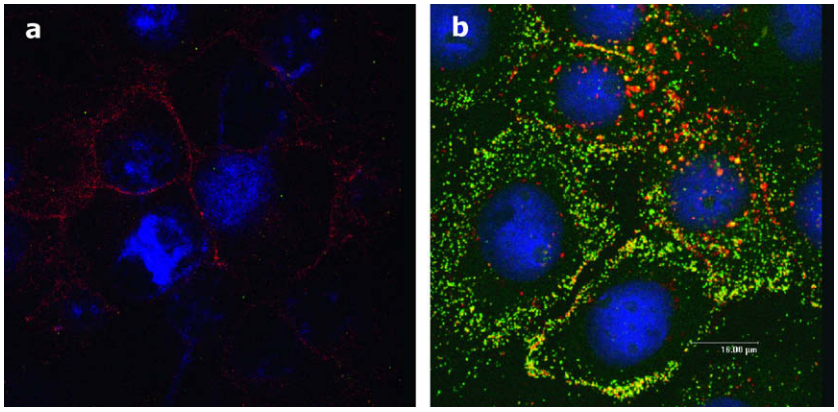

Fig. 2. Accessibility of pDNA for FISH. (a) Rhodamine-labeled pDMAEMA-based polyplexes incubated with OVCAR-3 cells for 4 h at 4 °C. (b) OVCAR-3 cells were incubated with rhodamine-labeled pDMAEMA-based polyplexes for 1 h at 37 °C, washed and further incubated for another 4 h at 37 °C. Accessible pDNA was stained using a pDNA-specific DIG-labeled probe and FITC-labeled anti-DIG antibodies as described in Section 2. Nuclei were stained with TO-PRO-3 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

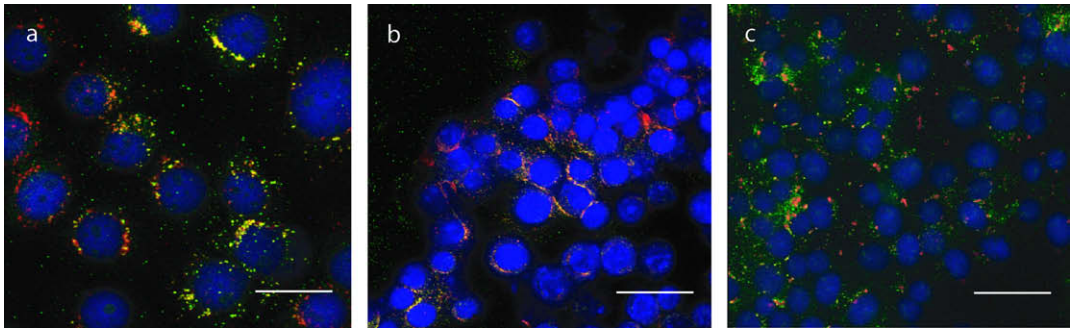

Fig. 3. Co-localization of rhodamine-labeled pDMAEMA with pDNA by FISH in different cell types. Cells were incubated for 1 h at 37 °C with pDMAEMA-based polyplexes, washed and further incubated for 24 h at 37 °C prior to fixation. (a) OVCAR-3 cells. (b) NIH/3T3 cells. (c) COS-7 cells. Nuclei were stained with TO-PRO-3 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

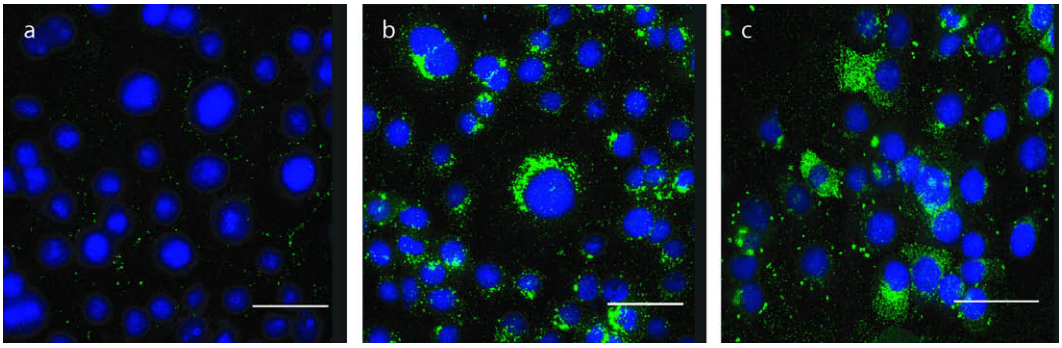

Fig. 4. pDNA condensing capacity of different polymers for FISH accessibility in OVCAR-3 cells. Cells were incubated for 1 h at 37 °C with different polyplexes, washed and further incubated for 24 h at 37 °C prior to fixation. (a) pDAMA. (b) pDMAEMA. (C) PEI. Nuclei were stained with TO-PRO-3 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. FISH can detect trafficking of polyplex-associated DNA towards the nucleus in time

The intracellular route of pDNA delivered by PEI and pDMAEMA-based polyplexes was determined in more detail. pDMAEMA is able to condense DNA and transfect cells, although transfection efficiency is less than for PEI-based polyplexes. We previously observed that both uptake and gene expression occurred at a lower pace after incubation of cells with this polymer (pDMAEMA) than with PEI [16]. Here, we have used FISH to monitor the intracellular location of both pDMAEMA- and PEI-based polyplexes in time. pDMAEMA-based polyplexes were incubated with OVACR-3 cells. After 4 and 24 h addition of the polyplexes, cells were fixed and labeled with FISH. After 4 h the FISH signal was detected as punctuated fluorescence signal throughout the cytosol (Fig. 5a), whereas after a 24-h incubation period the DNA localized mainly around the nucleus (Fig. 5b).

The route of PEI-based polyplexes was followed for 1 h after which the polyplexes were removed and already in quite a few cells DNA was detected inside the nucleus (Fig. 6a), comparable of that with pDMAEMA polyplexes after 4 h of trafficking (Fig. 5a). In cells that were incubated for 24 h pDNA was detected

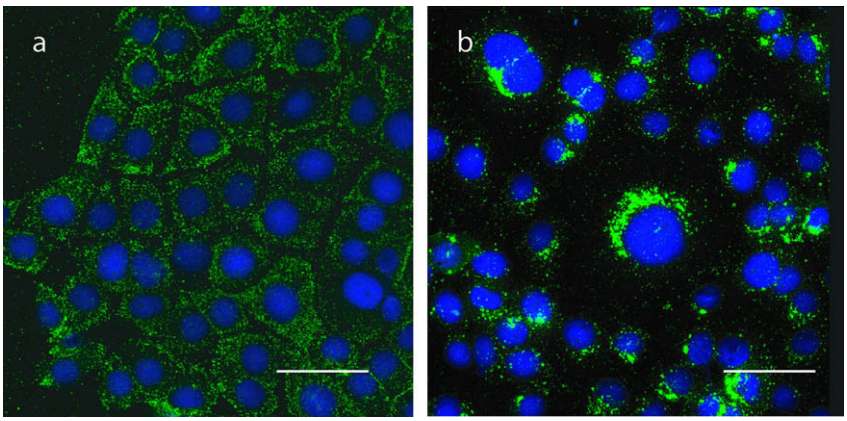

Fig. 5. Trafficking of pDMAEMA polyplex-associated pDNA in OVCAR-3 cells. Cells were incubated for 1 h at 37 °C with pDMAEMA-based polyplexes after which unbound polyplexes were removed and further incubated at 37 °C prior to fixation. (a) Location of pDNA throughout cytosol 4 h after removal of polyplexes. (b) pDNA detection around nucleus 24 h after removal of polyplexes. Nuclei were stained with TO-PRO-3 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

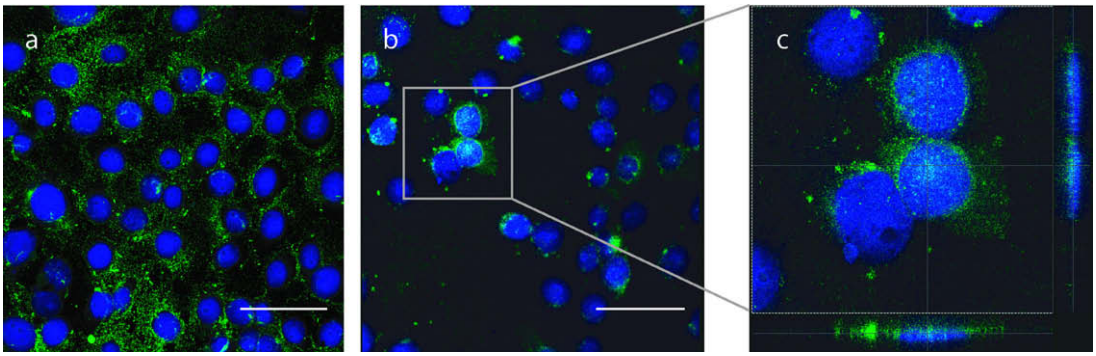

Fig. 6. Trafficking of PEI polyplex-associated pDNA in OVCAR-3 cells. Cells were incubated for 1 hr at 37 °C with PEI-based polyplexes after which unbound polyplexes were removed and further incubated at 37 °C prior to fixation. (a) Location of pDNA throughout cytosol 1 h of incubation after removal of polyplexes. (b) pDNA detection in and around nucleus 24 h after removal of polyplexes. (c) Confirming detection of pDNA inside the nuclei by CLSM layer stacks. Nuclei were stained with TO-PRO-3 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mainly in or around the nucleus, indicating transport of polyplexes towards the perinuclear region after 24 h (Fig. 6b).

To confirm that the DNA is inside the nucleus after a 24-h incubation period a stack of sections through the cell was taken by CLSM and represented from the top and from the side. From these cross-sections it is clear that FISH fluorescent label co-localizes with the nucleus (Fig. 6c).

4. Discussion

With the determination of gene expression the endpoint in gene delivery is established. However, such an endpoint assay does not provide valuable information about the critical steps preceding gene expression. Not much is known about the key requirements for efficient gene delivery with nonviral carriers. It is still unclear where in the process of cellular uptake, polyplexes dissociate to release the DNA for transcription. Premature dissociation may lead to DNA degradation, whereas slow dissociation may hamper nuclear uptake and subsequent transcription of the complexed DNA. Here, we have used fluorescence *in situ* hybridization to compare the accessibility of DNA complexed with different cationic polymers after cellular uptake and, at the same time, determine the intracellular location of the gene complexes. We first set out to test the specificity of the FISH probe to exclude interaction of the probe with either endogenous DNA or with the cationic polymer. No fluorescence was detected when cells were incubated with only polymer without pDNA. When cells were incubated with PEI- or pDMAEMA-complexed with pDNA clear fluorescent signal was observed inside the cell after FISH treatment.

Low levels of FISH signal inside the cell after transfection could be caused by low uptake rates, DNA degradation or tight condensation of the DNA by the polymer. When DNA is complexed too tightly by the polymer it could be that the pDNA is inaccessible for the FISH probe to bind to the DNA. To test probe accessibility to condensed DNA, cells were incubated with rhodamine-labeled pDMAEMA polyplexes at 4 °C. At this temperature polyplexes can bind to cells, but are not internalized. Rhodamine-label was used to visualize the polyplexes and to ascertain that the polyplexes were not washed away from the cell membrane. Results showed that most rhodamine-labeled pDMAEMA polyplexes were detected outside the cell when incubated at 4 °C, whereas no FISH signal could be observed. These results demonstrate that the FISH probe is not able to bind DNA that is tightly complexed by a cationic polymer. Conversely, after the incubation of rhodamine-labeled pDMAEMA polyplexes at 37 °C intracellular FISH signal could be detected indicating that the DNA inside polyplexes becomes accessible to the FISH probe after cellular uptake of the polyplexes (Fig. 2).

To investigate if the pDNA and pDMAEMA polymer remain associated inside the cell, rhodamine-labeled pDMAEMA-co-AEMA polymer was used to transfect OVCAR-3, NIH/3T3 and COS-7 cells. Polymer and pDNA associations were detected as co-localized

spots, as well as intracellular free polymer (red) and free dissociated pDNA (green) after 24 h incubation. Highest polyplex dissociation occurred in COS-7 cell where most pDNA was detected in free form. Large numbers of perinuclear location of polyplexes was observed in OVCAR-3 and NIH/3T3 cells. This indicates that the polyplexes remain mostly associated in the cytosol and probably prolong the half-life of the DNA by protection against digestion by nucleases. The observed differences in the degree of FISH signals after transfection of different cell types with the same polyplexes indicate that big differences exist in intracellular processing of polyplexes between different cell types. These differences in intracellular processing may lead to differences in transfection efficiencies as was previously demonstrated by us [16].

In a previous study, we have shown that the polymer pDAMA could efficiently condense pDNA into small polyplexes, but that these polyplexes were not able to transfect cells in culture, despite cellular uptake. Ethidium bromide displacement studies revealed that the interaction of the pDAMA polymer with pDNA was strong, which could be an explanation for poor transfection efficiency [4]. Here, we further investigated the accessibility of pDAMA-condensed pDNA inside cells using FISH. Only faint FISH signal could be detected in cells 24 h after transfection with pDAMA polyplexes. Also after longer incubation periods of 48 and 72 h only faint fluorescence was observed (data not shown). This is in sharp contrast with the high FISH signal detected in cells transfected with pDMAEMA and PEI polyplexes (Fig. 4). The lack of FISH signal observed here confirms the strong interaction of pDAMA with pDNA, even after cellular uptake and processing of these polyplexes. Taken together, these results show that FISH can be used to determine the accessibility of polyplex-delivered pDNA inside cells, which may be predictive for the transfection efficiency. In case of pDAMA, FISH shows that the lack of transfection with pDAMA polymers is most likely due to their strong DNA condensing properties.

Finally, it was demonstrated that FISH can be used to monitor the location of polyplex-delivery pDNA inside cells in time. Binding and uptake studies of PEI- and pDMAEMA-based polyplexes with FACS showed that cellular binding of the particles was the same, but that PEI polyplexes were taken up more rapidly than pDMAEMA polyplexes and that the amount of internalized DNA remained higher for PEI (data not shown). To determine if there are also differences in cellular localization with the different polymers we assessed the intracellular location of pDNA with FISH. In OVCAR-3 cells the location of PEI and pDMAEMA-polyplexed pDNA was perinuclear after 24 h for both polymers and already 1 h after incubation with PEI polyplexes pDNA seems to localize inside the nucleus, indicating that the PEI polymer can rapidly facilitate transport into the nucleus [17]. This is in accordance with previous results in which we observed that PEI-transfected cells express the reporter gene within 30 min after polyplex addition [16]. The nuclear localization of pDNA was confirmed by a stack formation in which high fluorescent signal was observed to co-localize with the nucleus (Fig. 6). The nuclear localization indicates that the polymers were able to protect the plasmid DNA from degradation by nucleases in the cytoplasm and deliver the DNA into the nucleus. This is in agreement with previous studies where PEI polyplexes were microinjected into the cytoplasm of cells, which resulted in gene expression, indicating that PEI can facilitate nuclear uptake of plasmid DNA [18,19]. Interestingly, it seems that also rhodamine-labeled pDMAEMA was found inside the nucleus, but this has to be confirmed by CLSM stacks. If so, this indicates that the polymer is able to facilitate transport of DNA into the nucleus, likely during mitosis. During cell division the nuclear envelope (NE) is broken down and reassembled in each daughter cell on completion of mitosis. Previously, nuclear localization of PEI, both in the presence and absence of DNA, was observed. Godbey et al. observed nuclear localization of polyplexes after 3.5–4.5 h [17]. The mechanism of nuclear uptake of PEI polyplexes was not elucidated, but they put forward that it could be caused by interaction with lipids inside the cytoplasm or with the membrane of endocytic vesicles. The lipids and membrane components would provide the particle with a phospholipid coating, which could facilitate nuclear import via fusion with the NE. This mechanism of nuclear localization of pDMAEMA-based polyplexes needs to be further analyzed.

In conclusion, we have shown that FISH is a powerful tool to study both location and accessibility of polyplex-delivered exogenous DNA inside transfected cells. This technique can be used to obtain valuable information about the intracellular fate of polyplexes necessary to improve nonviral gene delivery systems.

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