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Polycation-induced Cell Membrane Permeability Does Not Enhance Cellular Uptake or Expression Efficiency of Delivered DNA

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Abstract

Polycationic materials commonly used to delivery DNA to cells are known to induce cell membrane porosity in a charge-density dependent manner. It has been suggested that these pores may provide a mode of entry of the polymer-DNA complexes (polyplexes) into cells. To examine the correlation between membrane permeability and biological activity, we used two-color flow cytometry on two mammalian cell lines to simultaneously measure gene expression of a plasmid DNA delivered with four common nonviral vectors and cellular uptake of normally excluded fluorescent dye molecules of two different sizes, 668 Da and 2 MDa. We also followed gene expression in cells sorted based on the retention of endogenous fluorescein. We have found that cell membrane porosity caused by polycationic vectors does not enhance internalization or gene expression. Based on this single-cell study, membrane permeability is found to be an unwanted side effect that limits transfection efficiency, possibly through leakage of the delivered nucleic acid through the pores prior to transcription and translation and/or activation of cell defense mechanisms that restrict transgene expression.

Keywords

Polyplexes; lipoplexes; cationic vectors; gene delivery; flow cytometry; cellular uptake; membrane permeability; gene expression

INTRODUCTION

The transfer of genetic material into cells offers unique potential for disease and cell type-specific therapeutics. The success of the many nucleic acid therapeutic strategies is dependent upon the development of delivery vehicles that are capable of efficient cellular delivery while remaining nontoxic to the cells. This is still a challenge to the field. Non-viral synthetic vectors, such as cationic lipids and linear, branched and dendritic polymers have become popular

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SUPPORTING INFORMATION AVAILABLE

Flow cytometry control data for gene expression in the absence of propidium iodide and 2MDa tetramethylrhodamine dextran dye molecules for both Cos-7 and M4A4 cell lines. Cos-7 dextran uptake vs. gene expression and M4A4 propidium iodide and dextran uptake vs. gene expression. FACS fluorescein intensity vs. side scatter dot plots for all vectors. Flow cytometry control data for gene expression with FITC-labeled plasmid DNA in the absence of either propidium iodide or dextran. This material is available free of charge via the Internet at <http://pubs.acs.org>.

carriers due to their potential to overcome immunogenic concerns and high DNA loading capacity; however, many are also known to cause charge-dependent cytotoxicity due to disruption of both model and cellular membranes.^{1–5}

The creation of nanoscale membrane holes by polymeric materials has been studied extensively by our group. Atomic force microscopy (AFM) of supported lipid bilayers (SLBs) treated with various polymers revealed the dependence of electrostatics on the interaction – polycations showed high activity, while neutral polymers were inert.⁶ The diameter of the holes caused by generation 7 PAMAM dendrimer (G7-NH₂) in SLBs was measured to range from 15–40 nm and expanded with exposure time.⁷ As a more biologically relevant example, mammalian cells were monitored for the leakage of an endogenous enzyme, lactate dehydrogenase (LDH), as a function of polymer concentration.⁸ It was shown that branched polyethyleneimine (PEI), poly-L-lysine (PLL) and PAMAM dendrimers formed membrane pores large enough to allow release of a molecule as large as 140 kDa. Using patch clamp techniques, we have been able to determine a size range for these pores in 293A and KB cells through conductance changes (30–2000 pA) that occur across the membrane after exposure to these same polycationic materials. These currents are consistent with areas of 1–350 nm² (diameters of 1–21 nm).⁹ We have also probed the interaction mechanism between dendrimers and lipid vesicles through molecular simulation and isothermal titration calorimetry. These results show that the interaction is entropy-driven and support a dendrimer-encased vesicle model.^{10–12} This membrane-disrupting behavior of polycationic materials has been widely considered a negative side effect of polycationic nucleic acid delivery vehicles and a characteristic to be avoided in their design.^{13–15} However, membrane disruption could be considered a desirable property of cationic vectors if it led to enhanced cellular uptake of the DNA complexes and hence, enhanced gene expression.

Cellular uptake is a crucial step in efficient transfection, and its mechanism is closely related to the intracellular trafficking and fate of the nucleic acid cargo. Endocytosis has often been accepted as the means of uptake of nonviral vectors;^{16–18} however, there is conflicting evidence. The size of the nanoparticles introduced to the cell can influence the mechanism of internalization, as shown in a study by Rejman et al. where latex particles smaller than 200 nm were exclusively taken up via clathrin-mediated endocytosis while larger particles used a caveolar-mediated route.¹⁹ Ogris et al. compared PEI polyplexes of less than 150 nm to larger aggregates of > 500 nm.²⁰ Smaller polyplexes were internalized quickly through receptor-mediated routes, but larger, more slowly internalized complexes provided the highest gene expression. Lipid-DNA complexes (lipoplexes) were thought to enter cells by fusing with the lipid bilayer and releasing their contents into the cytoplasm.^{21, 22} Recently, decreased activity following cholesterol depletion suggested lipoplexes are uptaken via caveolar-dependent mechanisms; yet common caveolae inhibitors, such as filipin and cytochalasin D, had little effect on internalization.¹⁸ In contrast, phagocytosis was implicated in the uptake of LipofectACE lipoplexes in epithelial cells.²³ It was reported that PEI polyplexes are uptaken via both clathrin-dependent and clathrin-independent routes based upon a study using pathway inhibitors.^{24, 25} These and other findings demonstrate the complexities of nonviral complex internalization and how the most effective mechanism, that which leads to gene expression, remains poorly understood. It is likely that many concurrent mechanisms are at play, the most effective of which may be followed by a small, active population that must be probed carefully.

The use of noninvasive, non-endocytic methods of internalization are highly desirable in the field of gene delivery due to difficulties with endosomal escape and subsequent degradation of the cargo before it reaches the nucleus. Thus, many have tried to circumvent endocytotic uptake mechanisms through mechanical means such as electroporation²⁶ or microinjection^{27, 28} or through agents thought to aid in membrane permeation.^{29, 30} Lai et al. revealed that among a size distribution of latex polymer beads, only the ones smaller than 25 nm followed a preferred

non-endocytic, non-degradative pathway.³¹ The ability of polycations to promote cell membrane pore formation led to the hypothesis that passive diffusion of polyplexes across the compromised membrane might enhance transfection through bypassing uptake pathways resulting in lysosomal degradation. The fact that complexes at excess polymer ratios are more effective at delivering the DNA cargo further supported this claim, since the unbound polycation present at these ratios, or the cationic polyplexes themselves,³² could serve as the permeabilizing agent. If so, we may have overlooked an important mechanism of cellular uptake and continuing to focus on pathway inhibitors, as is common to the field, would prevent identifying the active population.

Designing experiments to gain understanding of the mechanisms of cell transfection is challenging, because any indicator (whether fluorescent or inhibitory) can affect the mechanisms themselves. Therefore, we chose to focus our investigation on one specific hypothesis – cell membrane permeability induced by polycationic gene delivery vectors is positively correlated with gene expression. To test this hypothesis while limiting perturbation of the system as much as possible, we used both two-color flow cytometry and fluorescence-activated cell sorting (FACS). Our study is unique in that its conclusions are based on quantification and correlation of membrane leakiness and transfection on a single-cell basis rather than bulk measurements, which can obscure small active populations. Transfection of a cyan fluorescent protein (CFP)-encoding plasmid allowed for expression in single cells to be tracked with cellular uptake of normally excluded dyes, propidium iodide (PI) and a large dextran, in two mammalian cell lines (Cos-7 – African green monkey kidney and M4A4 – human breast adenocarcinoma). In parallel, we also compared the expression of CFP in fluorescein-leaking and non-leaking cells as a permeability marker flowing in the other direction. To isolate the internalization step from the rest of the transfection process, membrane permeability to the dyes and plasmid DNA (pDNA) uptake were followed simultaneously using flow cytometry. We have found that the permeability induced by cationic nucleic acid delivery agents does not enhance gene expression or cellular internalization, confirming that the formation of nanopores by polycationic vectors is an undesirable side effect that should be minimized through vector or polyplex design. These results also suggest that this characteristic of polycations may be triggering a cellular response which inhibits the transfection process, such as disruption of internalization mechanisms (i.e. clathrin lattice formation, receptor clustering at the cell surface, or lipid mobility) or up-regulation of cytosolic nucleases.

MATERIALS AND METHODS

Materials

Propidium iodide, 2 MDa tetramethylrhodamine dextran and fluorescein diacetate (FDA) (Invitrogen; Carlsbad, CA), 4.7 kbp cyan fluorescent protein-encoding pAmCyan1-C1 DNA (Clontech; Palo Alto, CA), trypsin-EDTA, Dulbecco's Modified Eagle's Medium (DMEM), and phosphate buffered saline (PBS) (all from Gibco; CA) were used as received. Cos-7 and M4A4 cell lines were purchased from ATCC (Manassas, VA) and maintained according to the recommended protocol. jetPEITM transfection reagent was purchased from Genessee Scientific (San Diego, CA) and Lipofectamine 2000TM from Invitrogen. Plasmid DNA fluorescence labeling was performed using the fluorescein *LabelIT* TrackerTM system from Mirus Bio (Madison, WI) according to the protocol using a 0.25:1 (v:w) ratio of dye per μg of DNA. DNA concentrations were quantified by absorbance at 260 nm, using a molar extinction coefficient of $6,600\text{ cm}^{-1}\text{ M}^{-1}$. The XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay was performed using the Cell Proliferation Kit IITM from Roche Applied Science (Indianapolis, IN). The LDH (lactate dehydrogenase) assay was performed using the CytoTox-ONE Homogenous Membrane IntegrityTM kit from Promega (Madison, WI).

PAMAM Dendrimer Synthesis and Characterization

Generation 5 (G5-NH₂) and Generation 7 (G7-NH₂) PAMAM dendrimers were purchased from Dendritech Inc. (Midland, MI). Lower molecular weight impurities and trailing generations were removed from G5-NH₂ by dialysis with a 10,000 MWCO membrane against deionized water for four days, exchanging washes 7 times. G7-NH₂ was dialyzed with a 50,000 MWCO membrane for three days with four water changes. The number average molecular weight and polydispersity index or PDI of the dendrimers were determined by gel permeation chromatography using a double detection system (refractive index and static light scattering) to be 27,336 g/mol and 1.018 for G5-NH₂ and 101,200 g/mol and 1.175 for G7-NH₂. Potentiometric titration was conducted to determine the average number of primary amines per G5-NH₂ (112) and G7-NH₂ (403). All of the primary amines are expected to be protonated at pH 7.4; therefore, the charge of each dendrimer is equal to its number of primary amines.³³

Dynamic Light Scattering

Polypeplexes were formed by adding an equal volume of polycation solution to 100 μ L of 20 μ g/mL pDNA in DNase/RNase-free water to result in a +/- charge ratio of 10. Lipoplexes were formed according to the manufacturer's protocol by diluting 6 μ L of Lipofectamine 2000TM reagent into a total volume of 100 μ L with DNase/RNase-free water and adding this to 100 μ L of 20 μ g/mL pDNA solution (a 1 μ g DNA: 3 μ L reagent ratio). The solutions were mixed well and then incubated at room temperature for 20 min. Hydrodynamic diameter of the complexes was then measured at 25 °C on a Malvern Zetasizer Nano ZS (Worcestershire, UK) with a 4 mW He-Ne laser operating at 633 nm with a 173° scattering angle. Correlation functions were analyzed by cumulant fit to the data of three measurements of three runs each, with sizes reported as the z-average from a unimodal distribution. The complex solutions were then diluted into 2 mL of pre-warmed serum-free medium (SFM) and size measured again at 37 °C, both immediately and after 1 h incubation.

Cell Transfection

Two mL of 1.0×10^5 cells/mL Cos-7 or M4A4 cells were seeded in 6-well plates in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37 °C under 5% CO₂ overnight. Polypeplexes were formed by adding an equal volume of polymer solution to 100 μ L of 20 μ g/mL pDNA in DNase/RNase-free water to result in a +/- charge ratio of 10. Lipoplexes were formed according to the manufacturer's instructions as stated above in the *Dynamic Light Scattering* section. The solutions were mixed well and then incubated at room temperature for 20 min. Meanwhile, the complete medium was aspirated from the cells, they were washed with PBS and then 2 mL of SFM was added to each well. The polypeplex or lipoplex solution was added to the respective well, mixed thoroughly and incubated at 37 °C and 5 % CO₂. After 3 h, the media were replaced with complete media for the remaining 45 h of the transfection. For cell sorting experiments, the procedure was scaled up in T-75 flasks to a cell density of 2.7×10^5 cells/mL in 15 mL of medium for each condition.

XTT and LDH Assays

Cell viability after exposure to complexes formed using all four delivery vectors was determined using two standard protein assays which assess the metabolic activity of the cells (XTT) and release of cytosolic enzymes (LDH). 3×10^4 cells in 150 μ L were seeded in 96-well plates as above. Polypeplexes were formed by adding an equal volume of vector solution to 7.5 μ L of 20 μ g/mL pDNA in DNase/RNase-free water to result in +/- charge ratios from 0 to 30. Since Lipofectamine 2000TM is proprietary, the recommended ratio of 3 μ L of reagent: 1 μ g of pDNA was considered +/- = 10 and the other ratios were determined accordingly. The solutions were mixed well, then incubated at room temperature for 20 min before being added to the pre-rinsed cells in 150 μ L of SFM. Samples of media only (low control) and lysis buffer

(high control) were also included for comparison. After 3 hours of incubation at 37 °C, 100 µL of cell supernatant was transferred to another 96-well plate for the LDH assay and the remaining 50 µL was aspirated from each well for the XTT assay. The cells were then rinsed with 1X PBS before a mixture of 50 µL PBS and 30 µL of XTT reagent were added to each well, and the plate was incubated for 3 h at 37 °C. For the LDH assay, 100 µL of LDH reagent was added to the 100 µL of supernatant in each well and the plate incubated at 37 °C for 30 min. After incubation, A₄₉₂-A₆₉₀ for each plate was measured on a Biotek Synergy HT plate reader (Winooski, VT) with 10 s of shaking. Data is reported as the average and standard deviation of three wells of the same passage and DNA complex solution.

Flow Cytometry

Cells were stained with PI or tetramethylrhodamine dextran through addition of 40 µL of 50 µg/µL dye in DNase/RNase-free water to 2 mL of cell medium which had been first incubated with the polyplexes or lipoplexes for transfection for about 10 min. After the incubation, cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 2000 rpm for 5 min. Resulting cell pellets were then resuspended in PBS. The fluorescence signals of individual cells were assessed with a Coulter EPICS/XL MCL Beckman-Coulter flow cytometer, and data were analyzed using Expo32 software (Beckman-Coulter, Miami, FL). The data is reported as the average and standard deviation of three wells from the same passage and DNA complex solution. To confirm anti-correlation of the data, Pearson's correlation coefficients and their respective significances for a one-tailed test were determined using Microsoft Office Excel 2007 (Redmond, WA).

Fluorescence Activated Cell Sorting (FACS)

FDA staining was performed as follows: the medium was removed, and 3 mL of 25 µg/mL FDA/H₂O solution was added to each T-75 flask. After thirty minutes, the supernatant was aspirated and replaced with 10 mL of SFM. Polyplex or lipoplex solution (1 mL) was added and incubated at 37 °C and 5% CO₂ for another 120 min. When sorting based on PI or dextran fluorescence, the DNA complexes and dye were incubated for three hours as in the analysis procedure. After the incubation, cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 2000 rpm for 5 min. Resulting cell pellets were then resuspended in PBS. Sorting was performed based on either uptake of PI/dextran or cell loss of fluorescein with a BD FACSVantage SE, and data were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA). The sorted cells were maintained in complete media on ice until seeded into plates for further assay.

XTT Assay of PI/Tetramethylrhodamine dextran Sorted Cells

After sorting based on dye uptake, Cos-7 cells were seeded into a 96-well plate at 150 µL of 3×10^4 cells in complete medium. The next day, medium was removed, and 50 µL of PBS and 30 µL of XTT reagent were added to each well. The cells were incubated at 37 °C and 5% CO₂ for three hours and then A₄₉₂-A₆₉₀ was measured. Data is reported as the average and standard deviation of six wells from the same population of sorted cells. Statistical significance of the comparison between metabolic activity of the two populations (dye-positive and dye-negative) was performed using the Student's t-test in Microsoft Office Excel 2007 (Redmond, WA).

Analyzing Transfection of Fluorescein Sorted Cells

After sorting based on fluorescein retention, Cos-7 cells were seeded into a 96-well plate at 150 µL of 5×10^4 cells in complete medium. The cells were incubated at 37 °C and 5% CO₂ for 48 hours before being trypsinized with 0.25% trypsin-EDTA, centrifuged at 2,000 rpm for 5 min and resuspended in PBS for flow cytometry analysis using the Coulter EPICS/XL MCL

Beckman-Coulter flow cytometer (Beckman-Coulter, Miami, FL). Data is reported as the average and standard deviation of three separate sorting occasions on cells of the same passage number. Statistical significance of the comparison of gene expression between the two populations (dye-positive and dye-negative) was performed using the Student's t-test in Microsoft Office Excel 2007 (Redmond, WA).

Cellular Internalization of Polyplexes

Polyplexes were formed as explained above in the *Cell Transfection* section using fluorescein-labeled plasmid DNA. After three hours of incubation of the polyplexes with 2×10^5 Cos-7 cells in SFM at 37 °C and 5% CO₂ in the presence of 100 µg/mL propidium iodide or 2 MDa tetramethylrhodamine dextran, cells were trypsinized with 0.25% trypsin-EDTA, centrifuged at 2,000 rpm for 5 min, rinsed twice with PBS to remove surface-bound polyplexes and finally resuspended in PBS. Cellular uptake of the pDNA was monitored using flow cytometry of 10,000 events. Data is reported as the average and standard deviation of three wells from the same passage and DNA complex solution. Statistical significance of correlation of the data was performed using Pearson's correlation coefficients and the one-tailed test in Microsoft Office Excel 2007 (Redmond, WA).

RESULTS

Polyplex Size

Dynamic light scattering was used to measure the hydrodynamic diameter of the polyplexes and lipoplexes at the conditions used for the transfection experiments. Regardless of the vector used to package the pDNA, all complexes were 50–90 nm in water; however, their flocculation properties varied upon introduction to serum-free medium (Table 1). Lipofectamine 2000-DNA complexes, although largest of the series in water, were most stable to physiological salt conditions. jetPEI polyplexes aggregated from 248 to 691 nm during one hour of exposure to SFM. The two dendrimer formulations were nearly identical in size in water (55 and 57 nm for G5-NH₂ and G7-NH₂, respectively), but larger aggregates resulted from charge-shielding by the medium in the case of G7-NH₂. This may be due to differences in the DNA packaging of the two generations. For example, G7-NH₂, with a diameter of 8.0 nm, may be more amenable to DNA wrapping than the 5.3 nm diameter G5-NH₂ as proposed by Chen et al.³⁴ The charge density being greater in the case of generation 7 may also contribute to polyplex size and surface charge variation.

XTT and LDH Assays

Two common bulk assays were used to determine a reasonable working concentration of polycation which was not toxic to the cells for the flow cytometry experiments. The XTT assay follows the conversion of a tetrazolium salt to a formazan dye by metabolically active cell mitochondria. The LDH assay, however, tracks the leakage of the endogenous enzyme lactate dehydrogenase from cells with damaged membranes. The amount of enzyme released from the cells is proportional to the generated fluorescence of the resorufin product. The percent cytotoxicity as a function of +/- ratio of the complexes for both assays is shown in Figures 1 and 2. Interestingly, there is no clear trend in cytotoxicity by the delivery vectors, other than the consistently benign nature of Lipofectamine 2000. jetPEI caused more permeability to LDH at higher +/- ratios in M4A4 cells (Figure 2b) compared to the other polycations and other cell line, but its effect on mitochondrial activity was not as pronounced (Figure 1b). At this point, we are unsure of the cause of the spike in metabolic activity we are seeing with some agents in some cell lines, and unfortunately this question is beyond the scope of this paper. The complexes formed with all four DNA delivery vectors were demonstrated to be nontoxic (> 80% viability) up to +/- = 10 through both assays; therefore, all further experiments were

carried out at this ratio, so the observed dye uptake or release could be ascribed to an increase in cell membrane permeability as opposed to general lysis due to cell death.

Uptake of Propidium Iodide

Two-dimensional flow cytometry plots simultaneously display two channel fluorescence characteristics of single cells (Figure 3). Selecting gated regions based on the control samples results in four quadrants: double-negative (bottom left; cells were not transfected nor permeant to the dye), double-positive (top right; cells transfected and were permeant to the dye), dye positive (top left; cells were permeant but did not transfect) and CFP positive (bottom right; cells transfected but were not permeant). As shown in Figure 3 and Table 2, gene expression and membrane permeability to PI was cell line- and polycation-dependent. jetPEI and Lipofectamine 2000 did not demonstrate significant pore formation in the Cos-7 cell line, but there was some permeability to propidium iodide in M4A4 cells (an increase of 1.5 % and 0.9 %, respectively, over the DNA-only control). Although both dendrimer samples induced porosity to PI in both cell lines, G7-NH₂ polyplex-treated cells showed a greater effect than those treated with G5-NH₂ polyplexes at the same charge ratio. An anti-correlation was seen between transfection and PI uptake across both cell lines, reflected in the absence of cells in the double-positive quadrant. This anti-correlation was confirmed to be statistically significant at a 95% confidence level for all vectors in both cell lines, except for Lipofectamine 2000 polyplexes in Cos-7 cells which showed no strong correlation between permeability to PI and gene expression (Supplemental Information).

Uptake of 2MDa Tetramethylrhodamine Dextran

As was the case with PI, membrane permeability to the large dextran was cell line and polycation dependent, with the dendrimers producing the most porosity over the DNA-only controls (Figure 4). G5-NH₂ and G7-NH₂ polyplex-treated samples showed similar membrane permeability to the dextran in Cos-7 cells, while in M4A4 cells, the higher generation dendrimer demonstrated higher dye uptake. Comparing Tables 2 and 3 shows that cells were equally or less permeable to the large dextran than propidium iodide, indicating that not all of the pores formed by the polyplex or lipoplex treatment were of sufficient size for the uptake of this dye. An anti-correlation between transfection and dextran uptake was again demonstrated across both cell lines and was confirmed to be statistically significant at a 95% confidence level for all delivery vectors (Supplemental Information).

Determination of Cell Viability of Dye Positive Cells

After sorting based on PI or 2 MDa dextran uptake, cells were analyzed for mitochondrial activity using the XTT assay. Dendrimer G7-NH₂ was chosen as a representative example, due to it having the most pronounced effect on membrane integrity in the Cos-7 cell line, evidenced by the greatest uptake of the large dextran (Table 3). The results, shown in Figure 5, demonstrate that there was no significant difference in formazan production by dye-positive and dye-negative cells, as confirmed statistically at the 99% confidence level. As mentioned above, we do not currently understand why the polyplex-treated cells showed more production than the cells only control.

Leakage of Fluorescein

Cos-7 cells were loaded with fluorescein diacetate, treated with DNA complexes formed with each vector, then sorted based on fluorescein fluorescence intensity. Forty-eight hours later, transfection was analyzed through flow cytometry. Figure 6 is an example of the gating used to sort, which reveals two clear populations (cells that retained fluorescein and those that did not). These gates were kept constant throughout the experiment. Figure 7 shows the comparison of gene expression between cells which retained and cells which lost fluorescein through

percent of the population for jetPEI, G5-NH₂ and G7-NH₂. [Lipofectamine 2000 was not included in this graph, because the population of fluorescein-permeant cells was too low to collect (see Supplemental Information for all fluorescein intensity vs. side scatter plots)] There was no significant difference in transfected percentage between these two populations for either jetPEI, G5-NH₂ or G7-NH₂, at the 99% confidence level.

Cellular Internalization of Polplexes

Correlation of membrane permeability to the dyes versus uptake of the polyplexes is shown in Figures 8 and 9 and Table 4. As in the expression studies, Lipofectamine 2000 and jetPEI-delivered complexes did not induce any cell membrane porosity to either PI or the large dextran over the DNA-only negative control. In fact, only G7-NH₂ polyplexes created significant enough membrane damage to allow uptake of the dextran. Both dendrimer polyplex-treated samples allowed uptake of PI in many cells – 24.6 and 31.1 % for G5-NH₂ and G7-NH₂, respectively. pDNA internalization was detected upon three hours exposure to all polyplexes. In the case of propidium iodide, all delivery agents provided similar percentages of pDNA uptake (5.2, 4.6, 6.3 and 7.1 % for jetPEI, Lipofectamine 2000, G5-NH₂ and G7-NH₂, respectively) demonstrated by events in the upper right and bottom right quadrants. In the case of tetramethylrhodamine dextran, the pDNA uptake varied more from 15.8 to 19.2 to 66.6 to 73.3 % for the same order of delivery agents. Despite the presence of some cells possessing both pDNA fluorescence and dye, an anti-correlation between membrane permeability to either dye and pDNA internalization was shown and confirmed to be statistically significant at a 95% confidence level for all vectors (Supplemental Information).

DISCUSSION

The mechanism involved in lipoplex and polyplex cellular uptake is not well-understood and may vary as a function of delivery vector and cell line. Studies using selective inhibition of certain pathways have concluded everything from phagocytosis²³ to clathrin-mediated endocytosis^{18, 35, 36} to caveolar uptake via lipid rafts^{24, 36} are involved, which most likely reflects the adaptive nature of these complexes to finding a way inside or response of these mechanisms to the probes used in their measurement. It has also been noticed that cellular uptake does not necessarily lead to gene expression, as was the case with PEI and poly (dimethylaminoethyl methacrylate) (pDMAEMA) polyplexes internalized via the clathrin-mediated route.³⁷ Nanoscale holes have been discovered in both model and cell membranes caused by polycations often used to deliver nucleic acids.^{6–8, 38} Therefore, it became important to understand whether these pores lead to enhanced transfection through uptake of the DNA via a non-degradative pathway or if they simply represent a mechanism of toxicity.

To answer this question, we used a single-cell fluorescence method, flow cytometry, to detect membrane permeability and gene expression simultaneously. Characterizing individual cells is the only way to truly correlate these properties, as bulk assays provide an averaged level of activity and can mask the characteristics of active, or inactive, subpopulations. This single-cell analysis is an important distinction between this study and others in the field. Two different dye molecules were used to indicate the presence of nanopores: 668 Da propidium iodide (diameter ~1 nm) and 2 MDa tetramethylrhodamine dextran (diameter ~ 40–60 nm).^{39, 40} These dyes were chosen based on their exclusion from intact cell membranes and their different sizes. Cellular uptake of the dextran will reflect the presence of membrane pores with a larger diameter than those which enable uptake of propidium iodide. In an effort to prevent electrostatically driven aggregation or another interaction between the permeability probe and the polyplexes and lipoplexes, we did not pre-incubate the dyes with the DNA complexes before introduction to the cells. Instead, the complexes were added to the media approximately

10 min prior to the PI or dextran, although we acknowledge that 10 min may not be long enough to confidently exclude any effect the dye may have on cellular processes.

Transfection efficiency has been repeatedly shown to increase with complex charge (+/-) ratio; however toxicity usually accompanies this trend. Therefore, we used common bulk assays – XTT and LDH - to identify a high +/- ratio without significant cytotoxicity for our flow cytometry studies. Across all three cell lines and polycations, +/- = 10 complexes maintained >80% cell viability, so this ratio was chosen. This means that in all subsequent flow cytometry experiments (although we excluded obvious cellular debris from the gated population) there were up to 20% cells with compromised membranes resulting from toxicity, and these cells were PI- and dextran-positive. However, we also expected to detect cells experiencing transient membrane porosity following polyplex treatment, since our group has previously shown the transient nature of polycation-induced cell membrane pores through enzyme leakage assays.⁸ We also sought to confirm that not all dye-positive cells were dead/dying through the comparison of XTT results for permeant and non-permeant populations after sorting. The results, shown in Figure 4, demonstrate similar metabolic activity of both populations, proving that many dye-positive cells are experiencing transient porosity and are, therefore, capable of gene expression. This important result validates that the correlation conclusions presented are not a consequence of the dye-positive population being dead or inactive.

There was consistently less membrane permeability to the larger dextran compared to PI. This is evidence that not all of the pores formed by these complexes are of sufficient size to allow diffusion of the 2 MDa dextran into these cells. The DNA complexes were found to possess a wide distribution averaging 50–90 nm in diameter in water; however, colloidal flocculation occurs when they are introduced to serum-free media as in the transfection experiments, and the aggregates grow with time. Depending on how long it takes these complexes to be internalized by the cell, they could range from 150 nm to over a micrometer in diameter. Since most of the nanoscale pores induced by the positively charged vectors are too small to allow passive diffusion of the dextran, it is likely that internalization of larger polyplexes and lipoplexes via this mechanism is also restricted.

The permeabilization markers, propidium iodide and tetramethylrhodamine dextran, are present soon after introduction of the polyplexes and lipoplexes in order for their internalization to accurately reflect membrane pores the complexes could use for passive diffusion. It is important, however, to understand whether these dye molecules are affecting transfection. Therefore, expression of the CFP gene has been measured in the absence of either dye in both cell lines to establish a baseline for transfection efficiency, and this data is provided in Supplemental Information. The results show that incubation of polyplexes and lipoplexes with propidium iodide does not affect gene expression in the Cos-7 cell line, but does decrease expression in M4A4 cells. This could be due to its positive charge competing for cell surface recognition and/or DNA binding. Incubation of polyplexes and lipoplexes with the large dextran had no effect on dendrimer-mediated transfection but slightly lowered expression levels for jetPEI and Lipofectamine 2000 vectors in the Cos-7 cell line. The dextran's presence lowered gene expression for all delivery agents except G5-NH₂ in M4A4 cells. These controls demonstrate the difficulty associated with any experiment using a probe, since no probe can be truly isolated from the system. We were careful to add reagents in such a way as to minimize interactions between DNA complexes and the permeability markers. Although there were no clear trends as to if or how these dye molecules might be interacting with our system of cells and DNA complexes, it is important to acknowledge that results obtained in their presence should be interpreted carefully. Since this study does not aim to determine all possible mechanisms of internalization of these agents but instead the correlation of one property – membrane leakiness – to uptake and gene expression, the conclusions are valid.

Cells easily take up fluorescein diacetate (FDA) within 30 min. FDA is quickly hydrolyzed into fluorescein by cytosolic enzymes and this molecule does not easily permeate cells unless they have lost membrane integrity. However, we noticed that over time, the dye will slowly leak even from healthy untreated cells. Therefore, in order to track membrane permeability to molecules inside the cell, we had to modify the procedure used with PI and dextran. By detecting the membrane leakage to fluorescein at an early timepoint, we were able to distinguish polycation-induced permeability from natural slow diffusion of the dye out of the cell. Thus, we sorted cells after 2 hours of treatment, incubated them for 48 more hours and then analyzed the transfection with flow cytometry. We saw more cells leaked fluorescein after treatment than took up either PI or dextran. This indicates that fluorescein, although similar in size to propidium iodide (332 Da vs. 668 Da), is a more sensitive marker of membrane permeability, possibly due to concentration gradient differences between molecules flowing into and out of a cell. It may also be possible that the process of gene transfection is somehow contributing to exocytosis of this dye molecule. However, since the flow of molecules into cells through these membrane pores is the process that would enable polyplex or lipoplex uptake, the PI and dextran experiments more closely reflect this correlation.

After sorting based on fluorescein retention, the cells were analyzed for gene expression using flow cytometry, and the comparisons between permeant and non-permeant cells are shown in Figure 7. Delivery using jetPEI afforded the highest expression, as was previously seen in the Cos-7 cell line (Figures 3, 4 and Tables 2, 3); however, there was no significant difference in transfection between cells that retained fluorescein and those that did not. Although Lipofectamine 2000 polyplex treatment did not induce enough porosity to collect a reasonable number of cells for further assay, this result was consistent for the other three polycationic vectors, which suggests that cell membrane permeability to fluorescein has no effect on gene expression.

The lack of correlation between membrane permeability to these dyes and gene expression suggests that either polyplexes/lipoplexes are not being internalized via passive diffusion through the pores or that internalization via this mechanism results in a pathway that does not lead to efficient transfection. If there is uptake through these pores, these complexes would avoid lysosomal degradation and hence, be an attractive option for delivery if they could be trafficked properly throughout the cell. In that case, expression may be enhanced through the addition of nuclear localization signal (NLS) peptides^{41, 42} to the DNA delivery vectors or the addition of Pluronics, which activate cell trafficking machinery to increase nuclear delivery⁴³.

To determine whether poor internalization through the nanoscale holes or inefficient trafficking once inside the cell was leading to the lack of correlation, we performed similar flow cytometry experiments using polyplexes formed with a fluorescein-labeled plasmid. By rinsing the cells well after incubation with the polyplexes and then measuring fluorescein and PI or dextran inside the cells simultaneously, we were able to quantify individual cell characteristics. Again, the dendrimer polyplex-treated samples induced the most membrane porosity, and the number of cells experiencing porosity to the larger dextran was dramatically reduced compared to the smaller PI. It is important to emphasize that since the flow cytometry measurements in this DNA internalization study were obtained soon after dye exposure while the previous gene expression study results were obtained after 48 hours, comparing PI and/or dextran uptake percentages between the two experiments is not valid. As shown in supplemental information, dye uptake between labeled and unlabeled pDNA-treated samples is similar when measured at the same time point, but neither PI nor dextran are retained fully by the cells over time. The implication is that the percentage of permeable cells shown in Tables 2 and 3 are those which have retained fluorescence during the whole 48 hours.

All polycations increased the number of cells internalizing pDNA over the pDNA-only control, although there is evidence that PI is inhibiting polyplex uptake. The percentage of cells demonstrating fluorescein-pDNA fluorescence was consistent in the presence of propidium iodide across all four delivery agents, as shown in Figure 8. When the same experiment was performed substituting the charge-neutral dextran for positively-charged PI, more cells possessed labeled pDNA, as demonstrated most dramatically for G7-NH₂ where 72.8 % of cells internalized pDNA compared to 6.7 % when PI was simultaneously present. This result suggests that although we provided the DNA complexes with a 10 min head start on internalization, the charged propidium molecule may bind to the cell surface and block cellular uptake of the remaining, also positively charged, polyplexes and lipoplexes. PI was shown to bind to a trypsin-like protease, guanidinobenzoate, on leukemia cells and thus, inhibit its activity.⁴⁴ However, if PI is blocking uptake through binding receptors, this is further evidence of membrane permeability not being (and receptor-mediated endocytosis being) an important mechanism of cellular internalization of these agents. Another possibility is that PI competitively displaces the cationic vector. Propidium, like ethidium, intercalates between the base pairs of DNA which may disrupt the secondary structure of the nucleic acid, thereby weakening or releasing the complex with the vector. Preventing this is why we specifically did not pre-incubate the DNA complexes with the dye molecules. It is interesting to note that the gene expression for Cos-7 cells in the absence and presence of PI was within error (Figure 3 and Figure S1 of supporting information), so although internalization may be hindered in the presence of PI, gene expression is not. This result supports the existence of a different uptake pathway leading to efficient expression of the plasmid cargo, one that does not employ membrane permeation or negatively charged receptors that could be blocked by PI.

In the case of the dendrimer polyplex-treated samples, it is evident that cellular internalization of the pDNA cargo is much more efficient than gene expression through comparison of Cos-7 data from Figures 3 and 4 to Figures 8 and 9. The percentage of gene-expressing cells was significantly lower than the percentage of cells showing internalized pDNA. Knowledge of the many intricate steps involved between uptake and expression, such as endosomal escape, trafficking to the nucleus, DNA release and nuclear internalization, supports this result and suggests that one of these events downstream of uptake is the transfection barrier. In the case of jetPEI, which has been shown to be an excellent endosomal escape artist^{2, 45}, gene expression was high in relation to uptake (27.9% compared to 15.8%, respectively for the dextran case). Perhaps this is a result of its exceptional ability to quickly be released from degrading vesicles. Again, the lower internalization of all polyplexes and lipoplexes in the presence of propidium iodide did not, however, lead to lower gene expression.

An anti-correlation was observed between internalization of the polyplexes and lipoplexes and cell membrane permeability to both sizes of dye. This result indicates that diffusion of polyplexes and lipoplexes through nanoscale membrane holes induced by cationic delivery vectors is not an important mechanism of cellular entry. There is a small number of G7-NH₂ polyplex-treated cells that exhibited both dye and pDNA uptake, which may reflect a larger degree of membrane disruption caused by this dendrimer, compared to the other polycations. Generation 7 PAMAM dendrimer alone has been shown to induce more damage to SLBs and cell membranes than generation 3 or 5.⁶⁻⁸ However, the anti-correlation between PI and dextran internalization and gene expression reminds us that these DNA molecules do not lead to efficient transfection, which suggests that any complexes entering via this mechanism are not trafficked properly to the cell nucleus.

Although a lack of correlation between membrane permeability and gene expression simply points to another route of cellular entry of these delivery complexes, the anti-correlation is unexpected. This result suggests that the formation of defects in the plasma membrane, although non-cytotoxic, somehow discourages transfection of DNA incorporated by other

mechanisms. It is possible that the damaged membrane is unable to properly self-assemble receptors or lipid rafts used for clathrin-mediated endocytosis due to lateral diffusion barriers. Our group is currently investigating this option through Fluorescence Recovery After Photobleaching (FRAP) experiments. The anti-correlation could also be due to loss of the delivered DNA, either through leakage out of the pores into the medium or activation of DNA degrading enzymes (DNases). It has long been known that membrane damage, and subsequent Ca^{2+} influx, can trigger DNA breakdown and release.^{46, 47} These enzymes could degrade the foreign DNA before it is able to be transcribed and translated to the gene of interest, thus resulting in a lack of expression in those cells which show uptake of PI and dextran. Testing these hypotheses is beyond the scope of this work but should be explored to fully explain the anti-correlation between membrane permeability and gene expression.

CONCLUSION

The transfection of therapeutic genes into cells is a complex process, consisting of DNA packaging, cell targeting, avoiding degradation, cellular uptake, intracellular trafficking and releasing of the material. The mechanism of uptake has garnered much attention as evidence exists for polyplexes and lipoplexes entering cells through many different pathways and these pathways are closely associated with the trafficking of the nucleic acid cargo throughout the cell. Polycationic vectors have been shown by our group to induce membrane permeability and thus, it was hypothesized that transfection of their polyplexes would be enhanced via uptake through these pores, leading to a non-degradative endpoint. Herein, we have demonstrated using a single-cell technique, flow cytometry, that gene expression and cell uptake of two different sized dyes are anti-correlated, even at polymer concentrations that do not lead to cytotoxicity. We have also shown that expression is not correlated with cellular release of fluorescein. Since cellular internalization of pDNA was also anti-correlated with membrane permeability, passive diffusion through cell membrane nanopores is now known to not be an important mechanism of uptake of polyplexes and lipoplexes. The anti-correlation results also suggest that the membrane pores actually have detrimental effects on the biological properties of the cell, despite no evidence of cytotoxicity via traditional assays. Understanding the role of this membrane disruption on cellular internalization and gene expression lends insight into the complicated transfection pathway and directs the design of future delivery vehicles that do not exhibit this effect on cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. XTT assay results for Cos-7 (a), and M4A4 (b) cell lines at increasing +/- ratio of JetPEI ♦, Lipofectamine 2000 ■, G5-NH₂ ▲, and G7-NH₂ ×.

Figure 2. LDH assay results for Cos-7 (a), and M4A4 (b) cell lines at increasing +/- ratio of JetPEI ♦, Lipofectamine 2000 ■, G5-NH₂ ▲, and G7-NH₂ ×.

Figure 3. Flow Cytometry data for Cos-7 cells treated with propidium iodide and transfected with CFP-encoding pDNA and various polycations at $\pm$ charge ratio = 10.

Figure 4.

Flow Cytometry data for Cos-7 cells treated with 2MDa tetramethylrhodamine dextran and transfected with CFP-encoding pDNA and various vectors at $\pm$ charge ratio = 10.

Figure 5. Absorbance of the formazan produced in XTT assay indicating metabolically active Cos-7 cells after sorting based on exclusion — or uptake ▨ of propidium iodide (a) or 2MDa tetramethylrhodamine dextran (b).

Figure 6.

(a) Forward scatter versus side scatter plot of Cos-7 cells loaded with fluorescein diacetate then treated with G7-NH₂ polyplexes at ± 10 for 2 hrs. The gate was selected in an attempt to excluded cellular debris with low forward scatter. (b) Fluorescein fluorescence intensity versus side scatter for the same cells. Sorting gates (R2 and R3) were chosen to include most of the two populations without overlap. Collected cells were only those in R2 or R3 which originated from R1 in (a).

Figure 7. Percent of Cos-7 cell population exhibiting gene expression when treated with polyplexes of various polycationic vectors 48 hrs after sorting for fluorescein retention — or leakage ▨.

Figure 8. Flow cytometry data for Cos-7 cell membrane permeability to propidium iodide versus plasmid DNA internalization.

Figure 9.

Flow cytometry data for Cos-7 cell membrane permeability to 2 MDa tetramethylrhodamine dextran versus plasmid DNA internalization using various vectors for delivery.

Table 1

Hydrodynamic diameter of polyplexes formed in DNase-/RNase-free water at ± 10 with the four different DNA delivery vectors and after exposure to serum-free medium.

	Diameter (nm) in H ₂ O	Diameter (nm) in SFM – 0 min	Diameter (nm) in SFM – 60 min
jetPEI	65.83 $\pm$ 5.37	248.3 $\pm$ 23.3	691.0 $\pm$ 76.7
Lipofectamine 2000	86.33 $\pm$ 5.34	167.9 $\pm$ 13.3	199.6 $\pm$ 12.2
G5-NH₂	55.28 $\pm$ 4.99	221.7 $\pm$ 17.0	853.8 $\pm$ 82.8
G7-NH₂	57.29 $\pm$ 2.00	277.7 $\pm$ 56.0	1228 $\pm$ 107

Table 2

Percentage of cells possessing CFP or propidium iodide fluorescence based on flow cytometry, representing gene expression and cell membrane permeability, respectively. Values given are averages and standard deviations of three separately prepared samples of the same passage.

		Cells Only	DNA Only	jetPEI	Lipofectamine 2000	G5-NH ₂	G7-NH ₂
Cos-7	+Expression -Permeability	0.1 ± 0.1	0.2 ± 0.1	40.3 ± 3.5	17.6 ± 1.4	4.3 ± 0.9	2.0 ± 0.5
	-Expression +Permeability	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.1 ± 0.6	2.2 ± 0.7
	+Expression +Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	+Expression -Permeability	0.0 ± 0.0	0.1 ± 0.1	2.3 ± 0.4	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.4
M4A4	-Expression +Permeability	1.2 ± 0.4	1.7 ± 0.6	3.2 ± 0.9	2.6 ± 0.6	15.2 ± 4.3	16.8 ± 3.9
	+Expression +Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Percentage of cells possessing CFP or 2 MDa tetramethylrhodamine dextran fluorescence based on flow cytometry, representing gene expression and cell membrane permeability, respectively. Values given are averages and standard deviations of three separately prepared samples of the same passage.

Table 3

		Cells Only	DNA Only	jetPEI	Lipofectamine 2000	G5-NH ₂	G7-NH ₂
Cos-7	+Expression -Permeability	0.0 ± 0.0	0.1 ± 0.0	27.9 ± 3.5	13.2 ± 1.7	0.9 ± 0.4	4.2 ± 0.2
	-Expression +Permeability	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.7 ± 0.3	0.4 ± 0.1
	+Expression +Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	+Expression -Permeability	0.1 ± 0.0	0.0 ± 0.0	8.2 ± 1.1	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
M4A4	-Expression +Permeability	2.4 ± 0.5	1.2 ± 0.5	1.3 ± 0.2	1.2 ± 0.6	2.0 ± 0.7	4.2 ± 1.1
	+Expression +Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 4

Percentage of Cos-7 cells, based on flow cytometry, possessing FITC or propidium iodide/2 MDa tetramethylrhodamine dextran fluorescence, representing pDNA internalization and cell membrane permeability, respectively. Values given are averages and standard deviations of three separately prepared samples of the same passage.

		Cells Only	DNA Only	jetPEI	Lipofectamine 2000	G5-NH ₂	G7-NH ₂
PI	+ DNA Uptake - Permeability	2.8 ± 0.1	2.9 ± 0.8	5.2 ± 2.3	4.6 ± 1.8	6.2 ± 3.2	6.7 ± 3.3
	- DNA Uptake + Permeability	1.1 ± 0.1	1.9 ± 1.0	1.9 ± 0.3	1.9 ± 0.7	24.6 ± 4.4	31.1 ± 5.3
	+ DNA Uptake + Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1	0.4 ± 0.6
Dextran	+ DNA Uptake - Permeability	1.3 ± 0.4	1.6 ± 1.1	15.8 ± 6.4	19.2 ± 1.2	66.5 ± 2.1	72.8 ± 2.6
	- DNA Uptake + Permeability	0.4 ± 0.2	1.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.2	0.6 ± 0.3	4.2 ± 2.9
	+ DNA Uptake + Permeability	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.0	0.5 ± 0.5