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Intracellular Trafficking Pathways Involved in the Gene Transfer of Nanostructured Calcium Phosphate-DNA Particles

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Abstract

Nano-structured calcium phosphate (NanoCaP) particles have been proven to be a powerful means of non-viral gene delivery. In order to better understand the mechanisms through which NanoCaPs-mediated mammalian cell transfection is achieved, we have sought to define the intracellular trafficking pathways involved in the cellular uptake and intracellular processing of these particles. Previous work has indicated that NanoCaP-DNA complexes are most likely internalized via endocytosis, however the subsequent pathways involved have not been determined. Through the use of specific inhibitors, we show that endocytosis of NanoCaP particles is both clathrin- and caveolae-dependent, and suggest that the caveolae mechanism is the major contributor. We demonstrate colocalization of NanoCaP-pDNA complexes with known markers of both clathrin-coated and caveolar vesicles. Furthermore, through the use of quantitative flow cytometry, we present the first work in which the percent internalization of CaP-DNA complexes into cells is quantified. The overall goal of this research is to foster the continued improvement of NanoCaP-based gene delivery strategies.

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Keywords

nanoparticles; calcium phosphate; non-viral gene delivery; clathrin-mediated endocytosis; caveolae-mediated endocytosis

Introduction

Non-viral gene therapy remains an attractive alternative to viral approaches for the treatment of acute and chronic disease [1]. Non-viral carriers possess several advantages over viral approaches with respect to toxicity, immunogenicity and DNA carrying capacity, as well as tissue-specific targeting [2-4]. Several non-viral vectors are currently in development [1, 5-16]. Among these, calcium phosphate (CaP) remain known for their tremendous potential as non-viral gene delivery vectors [17-22]. Previously, our group demonstrated the excellent potential of nano-structured calcium phosphate particles, or NanoCaPs, for use in non-viral gene delivery applications. Our system of NanoCaPs synthesis is novel and well-controlled, and results in consistently high transfection efficiency [23]. Multiple pathways have been identified for endocytosis, and differing mechanisms by which these pathways deliver transgenes are known to exist [24, 25]. In this work, we seek to identify which endocytic mechanisms govern cellular uptake and subsequent intracellular processing of NanoCaPs. Our goal is the development of an even more effective CaP-based gene delivery agent, capable of overcoming the various extracellular and intracellular trafficking barriers associated with non-viral gene delivery [4, 26].

Endocytosis is currently regarded to be the primary method by which non-viral gene delivery vectors are internalized [25]. There are multiple endocytic pathways including phagocytosis, clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis and clathrin-/caveolae-independent endocytosis. Recently, several authors have reported the significance of both clathrin- and caveolae-mediated pathways in lipid [27, 28] and polymer [27, 29-32] based gene delivery. However, although the results of several studies indicate endocytosis as the means of internalization for CaP-DNA complexes [20, 33], the specific involvement of clathrin- and caveolae-mediated pathways in CaP-mediated gene delivery has never been assessed. Clathrin-mediated endocytosis, which is the most well-characterized uptake mechanism, is a multi-step process typically initiated by the strong binding of a ligand to a specific receptor on the cell surface. Receptor binding induces clustering and the eventual dissociation of clathrin-coated pits (approximately 100-150nm) from the plasma membrane. Depolymerization of the clathrin-coats subsequently ensues and depending on the cargo, these un-coated vesicles (referred to as early endosomes, pH = 5.9-6.0), then either (1) mature to late endosomes (pH = 5.0-6.0) and ultimately to lysosomes, or (2) release their cargo, and are trafficked to their target organelle [25, 34]. Caveolae are small (approximately 50-80nm), uncoated invaginations of the plasma membrane rich in cholesterol and glycosphingolipids [35]. It is hypothesized that upon internalization, caveolae either fuse with larger, more complex, preexisting membrane vesicles, deemed caveosomes, or are trafficked to early endosomes [36]. Macromolecules trafficked via the former route are not subject to acidification.

In this study, we assess the roles of clathrin- and caveolae-mediated endocytosis in both the cellular uptake as well as in the subsequent intracellular trafficking of our NanoCaPs-pDNA complexes. Using specific chemical inhibitors, we determine the involvement of these pathways in CaP-mediated gene delivery. Results of these studies are outlined in this manuscript.

Materials and Methods

Materials

All chemicals, inhibitors, fluorophores, transfection reagents, and cell culture media used in these experiments were purchased as described below: ACS grade calcium chloride dihydrate (100.0%), biological grade sodium chloride (99.0%), ACS grade sodium phosphate tribasic dodecahydrate (98.0%), USP grade dextrose monohydrate (100.0%) and molecular biology grade HEPES free acid (100.0%) from Fisher Scientific (Pittsburgh, PA); SigmaUltra grade potassium chloride (99.0%), HPLC grade filipin III (>85%), phenylarsine oxide (PAO) ($\geq 97\%$) and chlorpromazine hydrochloride ($\geq 98\%$) from Sigma Aldrich (St. Louis, MO); pCMV luciferase (5mg/ml) from Aldevron, LLC (Fargo, ND); *LabelIT*[®] Fluorescein Labeling Kit from Mirus Bio (Madison, WI); Alexa Fluor 555 transferrin, Alexa Fluor 555 cholera toxin subunit B (CTB) (recombinant), Dulbecco's minimum essential medium (DMEM) and penicillin/streptomycin (P/S) from Invitrogen (Carlsbad, CA); MTT cell growth assay kit from Chemicon (Temecula, CA) and characterized fetal bovine serum (FBS) from Hyclone (Logan, UT). The cell lines used in these experiments, specifically, the COS-7 African green monkey kidney fibroblast-like cells and the HeLa human epithelial cells, were both obtained from the American Type Culture Collection (ATCC) (Manassas, VA).

Synthesis of the NanoCaPs/pDNA complexes

Nanoparticles of CaP complexed with pDNA were synthesized as described previously [23]. Briefly, 125.0 μ l of a calcium precursor solution, comprising 6.25 μ l (1 μ g/ μ l) of pDNA (pCMV luciferase), 15.53 μ l of 2M CaCl₂ and 103.22 μ l of deionized water, was added to an equal volume of a HEPES Buffered Saline Solution (HBS), pH 7.5. A calcium-to-phosphate ion ratio (Ca/P) of 130 was used for all experiments. The calcium precursor was added to the phosphate precursor solution at an addition rate of 13.4 μ l/sec as the phosphate precursor was concurrently vortexed at 500rpm.

Luciferase reporter gene expression

HeLa and COS-7 cells were cultured in 75 cm² flasks at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS and 1% P/S. All cells were maintained at subconfluence and passaged every 2-3 days. A day prior to transfection, cells were seeded in 24-well plates at a concentration of 3.0 $\times 10^4$ cells/well. On the day of transfection, both HeLa and COS-7 cells were pre-treated with various concentrations of either filipin (concentrations ranged from 0.25 μ g/ml-1.50 μ g/ml) or phenylarsine oxide (PAO) (concentrations ranged from 0.025 μ M-0.200 μ M), for either 1 hour or 10 minutes, respectively, in fresh serum free medium. The NanoCaPs/pDNA complexes were then synthesized as described above and added to the wells (15.0 μ l/well). Incubation was continued for an additional hour before the medium was replaced with fresh FBS-supplemented medium. Twenty-four hours later, these cells were harvested and analyzed for both luciferase expression, using a standard luciferase assay system (Promega, Madison, WI) as well as for total protein content using a standard bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc, Rockford, IL).

Cell viability assessment: MTT assay

The cytotoxic effects of PAO and filipin on both HeLa and COS-7 cells were assessed using a MTT cell viability colorimetric assay. Cells were seeded in 24-well plates at a concentration of 3.0 $\times 10^4$ cells/well. 24 hours post-seeding, cells were treated with either filipin or PAO, for either 2 hours or 1 hour and 10 minutes, respectively, in fresh serum free medium. This incubation time represented the total amount of time the cells were treated with the inhibitors in the transfection experiment described above. Subsequently, the

medium was replaced with fresh FBS-supplemented medium and the cells were incubated for an additional 22 hours. Cell viability was then assessed using a MTT assay in the following manner. First, the medium was replaced with 250µl of fresh serum medium per well and 25µl per well of MTT dissolved in PBS. Second, the plates were incubated in a humidified incubator at 37°C for 4 hours. Third, 250µl of an isopropanol/0.04N HCl solution was added to each well. Fourth, the plates were incubated at room temperature (~25°C) for 30 minutes under constant shaking. Finally, the absorbance was measured at both a test wavelength of 570nm, and a reference wavelength of 690nm, using a Perkin Elmer Victor 3V (Waltham, MA) plate reader. Cell viability was then expressed as percent cell viability relative to untreated cells, which served as the control.

Statistical Analysis

The data were analyzed to test for mean viability and transfection efficiency differences among the concentration groups using a one way multivariate analysis of variance (MANOVA). The univariate post hoc tests were performed using the Student-Newman-Keuls procedure. Significance was considered to be less than $P=0.05$.

Cellular uptake studies

Flow cytometry was used to assess the level of NanoCaPs/pDNA internalization in both HeLa and COS-7 cells. In this experiment, cells were seeded in 12-well plates at a density of 6.0×10^4 cells/well, one day prior to transfection. 24 hours post-seeding, the cells were pretreated with either filipin (1.25µg/ml used for both cells lines) or PAO (0.15 and 0.20µM for HeLa and COS-7 cells, respectively) for either 1 hour or 10 minutes, respectively, in fresh serum free medium. Following the pretreatment, cells were transfected with the NanoCaPs/fluorescein-labeled-pDNA complexes, prepared as described above, for an additional hour (30.0µl particles added/well). pCMV Luciferase was fluorescently labeled with fluorescein (492nm excitation and 518nm emission) using a Mirus *LabelIT*[®] Fluorescein Labeling Kit (Madison, WI). Subsequently, the serum free medium was removed. The cells were washed with serum free medium, harvested by treatment with trypsin/EDTA and were then re-suspended in ice cold phosphate buffered saline (PBS). The cells were then centrifuged and treated with trypan blue (TB) (0.04%) (MP Biomedicals, Irvine, CA). TB was used to quench the extracellular fluorescence of non-internalized particles [27, 37]. The cells were subsequently washed twice with ice cold PBS, re-suspended in PBS and analyzed using a FACSort[™] flow cytometer (Becton Dickinson, San Jose, CA, USA). Ten thousand gated, viable cells were collected for each sample [27, 29]. All subsequent calculations were performed using WinMDI (Version 2.9) (Scripps Research Institute, La Jolla, CA, USA).

Confocal microscopy

Confocal microscopy was used to visualize colocalization between the internalized NanoCaPs/fluorescein-labeled-pDNA particles and the pathway markers Alexa Fluor 555 transferrin (Invitrogen, Carlsbad, CA) and Alexa Fluor 555 cholera toxin B (Invitrogen, Carlsbad, CA). For this, HeLa and COS-7 cells were seeded on glass Lab-Tek[™] Chamber Slides (Nalge Nunc International, Rochester, NY, USA) at a cell density of 3.8×10^4 cells per chamber (4 wells/chamber) a day prior to transfection. 24-hours post seeding, the cells were simultaneously treated in serum-free medium with either Alexa Fluor 555 transferrin (6.4µg/ml) or CTB (1.3µg/ml) and the NanoCaPs/fluorescein-labeled-pCMV Luciferase complexes (28.2µl per well) for 4 hours. Subsequently, the cells were washed twice with serum-free medium and fixed with 3.7% paraformaldehyde. Gel/Mount (aqueous mounting medium with anti-fading agents) (Biomed, Foster City, CA), was then added to reduce photobleaching effects. Each slide was then sealed with a coverglass (Fisher Scientific, Pittsburgh, PA). All slides were protected from light and stored at 4°C until further analysis.

Cells were then visualized using a confocal microscope (Olympus IX81). Laser 1, which was used for the NanoCaPs-fluorescein-labeled-pDNA complexes, had an excitation and emission wavelength of 488 and 520nm, respectively. Laser 2, which was used for the endocytic trafficking markers, had an excitation and emission wavelength of 543 and 612nm, respectively. Laser 3, which was used for differential interference contrast (DIC) microscopy, had an excitation wavelength of 543 nm. All images were taken using a 60× oil PLAPON objective lens (numerical aperture 1.42) and captured using Fluoview FV1000 software. Images were scanned using sequential scanning to eliminate “cross-talk” between the fluorophores [38]. An optimal section thickness of 0.41 μm was determined by the software.

Results

Endocytosis mechanisms involved in NanoCaPs-pDNA gene transfection

In our effort toward developing more effective and efficient CaP gene delivery vectors, we set out to determine the specific means of cellular uptake and intracellular processing of NanoCaPs-pDNA complexes. First, *in vitro* transfection experiments with specific inhibitors were conducted to determine which endocytic mechanisms might be relevant to the transfection of NanoCaPs-pDNA complexes. A luciferase reporter assay was used in the assessment of transfection efficiency.

Phenylarsine oxide (PAO) is a potent inhibitor of clathrin-mediated endocytosis, while filipin inhibits the raft/caveolae pathway. As shown in Fig. 1a, treatment of HeLa cells with 0.025-0.200 μM PAO reduced gene transfection by 48-75% whereas treatment of HeLa cells with 0.75-1.50 $\mu\text{g/ml}$ of filipin reduced gene expression by 64-98% (Fig. 1b). There was a statistically significant difference between these treated groups and the controls ($P < 0.05$). There was no statistical difference in gene expression between the control cells (i.e cells transfected with the NanoCaPs-pDNA complexes without the presence of an inhibitor) and HeLa cells treated with either 0.25 or 0.50 $\mu\text{g/ml}$ of filipin ($P > 0.05$).

Inhibition was also observed for PAO and filipin treated COS-7 cells. Gene expression in COS-7 cells was inhibited when treated with either 0.150 μM (63% decrease) or 0.200 μM (43% decrease) of PAO (Fig. 1c). Also, treatment of COS-7 cells with 0.75-1.50 $\mu\text{g/ml}$ of filipin decreased gene expression by 52-90% (Fig. 1d). There was a statistically significant difference between these treated groups and the controls ($P < 0.05$). There was no statistical difference between the control cells and cells treated with either 0.25 or 0.50 $\mu\text{g/ml}$ of filipin ($P > 0.05$). These results show that gene transfection of the NanoCaPs-pDNA complexes is both clathrin- and caveolae-dependent in both HeLa and COS-7 cells. The implication of these results will be described further in the discussion section. Furthermore, as shown in Figs. 1a-1d, all four dimethyl sulfoxide (DMSO) controls (i.e. cells transfected with solutions comprising NanoCaPs-pDNA complexes and DMSO without an inhibitor) yielded gene transfection efficiencies comparable to that of their respective controls. Thus, the observed decreases in transfection were not due to the presence of DMSO in the inhibitor solutions.

Differentiation between specific inhibition and cytotoxic effects

Because both PAO and filipin interfere with the vital processes of the cell, high concentrations of either inhibitor can lead to cytotoxic effects. Therefore, a series of MTT cell viability colorimetric assays were conducted to determine whether the aforementioned decreases in transfection were indeed due to specific inhibition, or rather reflected a cytotoxic effect. As shown in Figs. 1a and b, viability of HeLa cells treated with either PAO (0.025 μM -0.200 μM) or filipin (0.25-1.50 $\mu\text{g/ml}$) was $\geq 86\%$. A similar trend was observed

for COS-7 cells (Figs. 1c and d). Viability of COS-7 cells treated with 0.025-0.200 μ M PAO ranged from 91 to 98%. Also, COS-7 cells treated with 0.25-1.25 μ g/ml of filipin were approximately 85%-100% viable. Cytotoxicity was only observed following treatment of COS-7 cells with 1.50 μ g/ml of filipin (cells were approximately 65% viable). Thus, when this data is viewed in conjunction with the work of von Gersdorff et al [29], we infer that specific inhibition was observed when HeLa cells were treated with (a) 0.025 to 0.200 μ M of PAO and (b) 0.75 to 1.50 μ g/ml of filipin and when COS-7 cells were treated with (a) 0.150 and 0.200 μ M of PAO and (b) 0.75 to 1.25 μ g/ml of filipin. All other data points were excluded because they either yielded gene transfection efficiencies which were not statistically significant from that of the control ($P>0.05$), or because inhibitor treatment resulted in cytotoxicity.

For simplicity, one concentration of filipin and PAO from each concentration range (and from each cell line) mentioned above which yielded specific inhibition was selected for all further analyses requiring the inhibitors (i.e. the cellular uptake portion of this assessment). The selected concentrations were 1.25 μ g/ml of filipin for both cell lines, and 0.15 μ M and 0.20 μ M of PAO respectively for the HeLa and COS-7 cells.

Mechanisms of cellular uptake of NanoCaPs-pDNA complexes

Flow cytometry was used to quantitatively determine the percentage of HeLa and COS-7 cells positive for the internalization of the NanoCaPs-pDNA complexes. This test method was also used to assess the contribution of the clathrin- and caveolae-dependent pathways to the internalization of the CaP nanoparticles (NanoCaPs). With respect to the former, when quantified, approximately 93% of HeLa (Fig. 2a) cells and 87% of COS-7 (Fig. 2b) cells were positive for the uptake of the NanoCaPs-fluorescein-labeled-pDNA particles (Fig. 2). With respect to the latter, both pathways were involved in the uptake of the NanoCaPs-pDNA complexes (Fig. 3). As depicted in Fig. 3, treatment with filipin strongly suppressed the internalization of NanoCaPs-pDNA complexes in both HeLa and COS-7 cells, with observed percent cellular uptakes of approximately 11% and 22%, respectively, relative to the positive control. By contrast, PAO had a moderate effect on internalization. Cellular uptake of the nanoparticles decreased to 47% in HeLa cells and 38% in COS-7 cells, when treated with PAO. Thus, both pathways were involved in the uptake of NanoCaPs-pDNA complexes.

Qualitative colocalization analysis

As shown above, the cellular uptake of the NanoCaPs-pDNA complexes in both cell lines is both clathrin- and caveolae-dependent. In order to visualize the subsequent trafficking of these particles within the cell itself, we utilized confocal microscopy. Transferrin and cholera toxin B (CTB) are known to be sensitive and effective markers for the caveolae and clathrin pathways, respectively. We utilized an Alexa Fluor 555 conjugate of transferrin and an Alexa Fluor 555 conjugate of CTB, together with fluorescein-labeled-pDNA for colocalization studies. Fig. 4d (image of transfected HeLa cell) shows a high degree of fluorescent overlap between the NanoCaPs-fluorescein-labeled-pDNA particles (Fig. 4a) and Alexa Fluor 555 transferrin (Fig. 4b). These data suggest that a large portion of the NanoCaPs-pDNA complexes were found in endosomes containing transferrin. Colocalization was also observed for HeLa cells treated with both the NanoCaPs-fluorescein-labeled-pDNA particles (Fig. 4e) and Alexa Fluor 555 CTB (Fig. 4f). Similar observations were made for the COS-7 cells (Figs. 5a-h). It is notable that diffuse fluorescence of fluorescein was observed in the nuclei of the cells, suggesting successful trafficking of the pDNA to the nucleus. This phenomenon was observed in all of the imaged samples.

Quantitative colocalization analysis

In the previous section, we noted qualitatively that colocalization was observed between the NanoCaPs-fluorescein-labeled-pDNA complexes and the corresponding markers for two endocytic pathways in both HeLa and COS-7 cells. In this section, we provide quantitative results to support and extend our observations. The overlap coefficient according to Manders was used to quantify colocalization [38], and this data is shown in Table 1. For HeLa cells, the extent of colocalization were determined to be approximately 0.81 and 0.87 for cells treated with Alexa Fluor 555 transferrin and Alexa Fluor 555 CTB, respectively. In the case of COS-7 cells, the degree of colocalization was approximately 0.82 for cells treated with either Alexa Fluor 555 transferrin or Alexa Fluor 555 CTB.

Discussion

Efficient transfection of CaP based gene delivery vectors occurs when the carrier successfully overcomes existing barriers to entry and processing. To date, however, the mechanisms governing the cellular uptake of CaP-based gene transfer systems are poorly understood. In order to engineer progressively more effective CaP carrier systems, we sought to extend our knowledge of the mechanisms/pathways involved in mediating both the efficient uptake, as well as the intracellular processing of nanoparticles of CaP. The present work is the first report of such a mechanistic study aimed at identifying and understanding which pathways mediate the successful delivery of CaP-based gene delivery agents.

Luciferase gene transfection experiments were conducted to determine the significance of the clathrin- and caveolae-mediated trafficking pathways on the gene transfection efficiency of NanoCaPs-pDNA complexes in HeLa and COS-7 cells. Both endocytic pathways were determined to mediate gene expression, with the latter endocytic pathway contributing more strongly. Specifically, transfection efficiency was strongly inhibited by filipin, with observed decreases of 96% and 80%, respectively, in HeLa and COS-7 cells (Figs. 1b, d). Treatment with PAO in HeLa and COS-7 cells respectively yielded a 74% and 43% decrease in gene expression (Figs. 1a, c). This data confirms the involvement of endocytosis in the intracellular processing of CaP-based gene delivery vectors as reported in the literature [18, 19].

Furthermore, the involvement of clathrin- and caveolae-dependent pathways in the uptake of the NanoCaPs-pDNA complexes was both qualitatively and quantitatively assessed using confocal microscopy. In this study, HeLa and COS-7 cells were transfected with NanoCaPs-fluorescein-labeled-pDNA complexes and were treated with either Alexa Fluor 555 CTB or Alexa Fluor 555 transferrin. In both cell types, colocalization was visually observed (Figs. 4d and h and 5d and h). Quantitatively, a high degree of colocalization was recorded between the NanoCaPs-fluorescein-labeled-pDNA complexes and both Alexa Fluor 555 CTB and Alexa Fluor 555 transferrin with Manders coefficients in the range of 0.8 and above as shown in Table 1.

Cellular uptake was also quantitatively assessed using flow cytometry. Overall, 93% of HeLa (Fig. 2a), and 87% of COS-7 (Fig 2b) cells, were positive for the internalization of NanoCaPs-pDNA complexes. In earlier work, Loyter et al. treated murine Ltk^{-Aprt} cells with CaP-fluorescently-labeled-DNA complexes. These authors qualitatively observed that virtually all of the treated cells were positive for the internalization of CaP-DNA complexes [39]. Based on our search of the literature, we conclude that ours is the first work in which the percent internalization of CaP-DNA complexes into cells has been quantified. Furthermore, we have assessed the independent contributions of clathrin- and caveolae-dependent pathways on the internalization of NanoCaPs-pDNA complexes. Inhibition of caveolae-dependent endocytosis (using filipin) strongly decreased the cellular uptake of

NanoCaPs/pDNA particles. Treatment of both cell lines with filipin decreased cellular uptake by approximately 89% and 78% (Fig. 3). Internalization of the nanoparticles was moderately affected by PAO treatment (which inhibits clathrin-dependent uptake), with observed decreases in internalization by approximately 53% and 62%, respectively, in HeLa and COS-7 cells (Fig. 3). Previous studies involving CaP-based gene delivery vectors have shown that the cellular uptake of these particles occurs via endocytosis [19, 33, 39]. However, these studies used general inhibitors of endocytosis acting non-specifically via energy depletion [25], and did not address the distinct contributions of the individual endocytic pathways. Thus, ours is the first assessment showing that the cellular uptake of CaP-based gene delivery systems is mediated by both clathrin- and caveolae-dependent endocytosis as observed in both HeLa and COS-7 cell lines, with the latter pathway impacting cellular uptake more strongly.

Previously, we reported that the synthesized NanoCaPs-pDNA complexes are primarily between 25-50nm [23]. Recent authors have reported on the relationship between particle size and the internalization route, but with conflicting results. For instance, Rejman et al demonstrated that small latex particles (<200nm) were internalized exclusively via clathrin-mediated endocytosis, whereas the cellular uptake of larger particles (approximately 500nm) occurred via caveolae-dependent endocytosis [40]. Interestingly, Grosse et al. reported different results. In their assessment, they determined that small (≤ 100 nm), intermediate (100-200nm) and large (>200nm) PEI complexes were internalized via caveolae-dependent endocytosis, clathrin-dependent endocytosis and macropinocytosis, respectively [41]. von Gersdorff et al. observed that the cellular internalization of small PEI-based polyplexes (approximately 100nm) occurred via both caveolae- and clathrin-mediated endocytosis whereas the uptake of large complexes (>1 μ m) was found to be both clathrin- and caveolae-independent and occurred primarily via a third pathway [29]. Despite these conflicting results, and based on the aforementioned size of the NanoCaPs-pDNA complexes, our cellular uptake results support the work of von Gersdorff et al. Nevertheless, more research needs to be done before any definitive conclusions can be drawn about the significance of vector size in non-viral mediated gene delivery.

Escape from the endosomal/caveosomal compartments is paramount to achieving efficient gene transfection. In clathrin-mediated endocytosis, clathrin-coated vesicles (endosomes), upon loss of the clathrin coat, undergo continuous acidification from the initial neutral pH at the cell surface to the pH found in lysosomes (pH 4-5) [42]. By contrast, caveolar vesicles and caveosomes (which are pre-existing compartments rich in both cholesterol and glycosphingolipids) possess a neutral pH [43, 44]. Currently, the proposed mechanism of escape for CaP-based gene delivery vectors considers only uptake via clathrin-mediated endocytosis. The current hypothesis suggests that CaP-pDNA particles successfully overcome this barrier to transfection via endosomal disruption. Specifically, it is believed that the continuous acidification of the endosomal compartments aids in the dissolution of CaP [45]. This dissolution results in the swelling and eventual osmotic rupture of the endosomal compartment [46]. The diffuse fluorescence of fluorescein-labeled-pDNA observed in the cell nuclei (Figs. 4a, e and 5a, e) confirm the successful trafficking of pDNA to this compartment. It is therefore plausible that NanoCaPs successfully rupture the endosomal compartments prior to the trafficking of these complexes to lysosomes. Further studies need to be conducted to confirm this hypothesis. Also, further work needs to be done to elucidate the mechanism of escape for nanoparticles trafficked via the caveolae-dependent pathway. It remains unknown whether the NanoCaPs-pDNA complexes housed within caveolae fuse with endosomes, or if these particles are trafficked to early endosomes [36]. In that latter case, it is hypothesized that they would follow the intracellular trafficking route described above. Thus, the intricate nature of the clathrin- and caveolae-dependent pathways makes elucidation of the endocytic trafficking mechanism of the NanoCaPs/pDNA

complexes more complex. Nevertheless, these intricacies make following the fate of CaP-based gene delivery vectors along the trafficking route an attractive area of study.

Conclusion

The present study demonstrates that the cellular uptake as well as the subsequent gene expression of the NanoCaPs-pDNA complexes in HeLa and COS-7 cells was mediated by both clathrin- and caveolae-dependent endocytosis. We corroborate these findings with confocal microscopy data confirming colocalization of the complexes with markers of both pathways. Prior to our study, emphasis has been directed toward clathrin-mediated endocytosis as the probably means for CaP-DNA internalization. We conclude however that effort should be redirected toward understanding the mechanisms mediating CaP trafficking via caveolae-dependent endocytosis. A more detailed understanding of the intracellular routing of these endocytic mechanisms will further the development of new, more effective and efficient NanoCaPs-based gene delivery strategies.

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Fig. 1. Transfection efficiency and cellular viability of HeLa (a, b) and COS-7 (c, d) cells, treated with specific endocytic inhibitors

Filipin and PAO treatment yielded decreases in gene expression for both cell types. Cell viability was $\geq 80\%$ for all treatment groups with the exception of COS-7 cells treated with

1.50 μ g/ml of filipin (which yielded a cell viability of 65%). Data of transfected cells not subject to inhibitor treatment was set to 100% (control). The DMSO control represented cells transfected with solutions comprising the NanoCaPs/pDNA complexes and DMSO without an inhibitor. Transfection efficiencies are presented as % transfection efficiency $\pm$ SEM (n=6). Cell viabilities are presented as % cell viability $\pm$ SEM (n=6).

Fig. 2. Cellular uptake of the NanoCaPs/pDNA complexes without pre-treatment with a specific inhibitor

HeLa (a) and COS-7 (b) cells were transfected with the NanoCaPs/fluorescein-labeled-pDNA particles. The % cellular uptake was quantified using flow cytometry.

Fig. 3. Cellular uptake of NanoCaPs/pDNA complexes in the presence of specific endocytic inhibitors

HeLa and COS-7 cells were pre-treated with either PAO or filipin for 10 minutes or 1 hour, respectively. These cells were later transfected with the NanoCaPs/pDNA particles for an additional hour. Trypan blue was used to quench the fluorescence of extracellularly bound particles. The % cellular uptake was quantified using flow cytometry. Cellular uptake of the nanoparticles in HeLa cells after pre-treatment with PAO and filipin diminished to approximately 47% and 11%, respectively. In COS-7 cells, internalization of the complexes decreased to about 38% and 22% following pre-treatment with PAO and filipin, respectively. Data of transfected cells not subject to inhibitor treatment was set to 100%. Values are given as average % cellular uptake $\pm$ SEM (n=6).

Fig. 4. Colocalization of NanoCaPs/fluorescein-labeled-pDNA complexes and specific uptake pathway markers in HeLa cells

Confocal microscopy was used to visualize the fluorescence of the internalized NanoCaPs/fluorescein-labeled-pDNA particles (a,e) and Alexa Fluor 555 transferrin (b) and Alexa Fluor 555 CTB (f). The corresponding DIC images (c,g) as well as the merged images of all three channels are presented (d,h).

Fig. 5. Colocalization of NanoCaPs/fluorescein-labeled-pDNA complexes and specific uptake pathway markers in COS-7 cells

Confocal microscopy was used to visualize the fluorescence of the internalized NanoCaPs/fluorescein-labeled-pDNA particles (a,e) and Alexa Fluor 555 transferrin (b) and Alexa Fluor 555 CTB (f). The corresponding DIC images (c,g) as well as the merged images of all three channels are presented (d,h).

Table 1

Quantitative determination of the extent of colocalization observed in HeLa and COS-7 cells between the NanoCaPs/pDNA particles and the endocytic pathway markers. The assessment was done using the entire series of images (image slices) collected for each sample. In this analysis, three different regions were imaged for each sample (n=3).

Cell Line	Pathway Marker	Overlap
HeLa	Transferrin CTB	0.81 $\pm$ 0.02
		0.87 $\pm$ 0.01
COS-7	Transferrin CTB	0.82 $\pm$ 0.03
		0.82 $\pm$ 0.04