Cellular Uptake and Intracellular Pathways of PLL-g-PEG-DNA Nanoparticles

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Polycationic molecules form condensates with DNA and are used for gene therapy as an alternative to viral vectors. As clinical efficacy corresponds to cellular uptake, intracellular stability of the condensates, and bioavailability of the DNA, it is crucial to analyze uptake mechanisms and trafficking pathways. Here, a detailed study of uptake, stability, and localization of PLL-g-PEG-DNA nanoparticles within COS-7 cells is presented, using FACS analysis to assess the involvement of different uptake mechanisms, colocalization studies with markers indicative for different endocytic pathways, and immunofluorescence staining to analyze colocalization with intracellular compartments. PLL-g-PEG-DNA nanoparticles were internalized in an energy-dependent manner after 2 h and accumulated in the perinuclear region after >6 h. The nanoparticles were found to be stable within the cytoplasm for at least 24 h and did not colocalize with the endosomal pathway. Nanoparticle uptake was ~50% inhibited by genistein, an inhibitor of the caveolae-mediated pathway. However, genistein did not inhibit gene expression, and PLL-g-PEG-DNA nanoparticles were not colocalized with caveolin-1 indicating that caveolaemediated endocytosis is not decisive for DNA delivery. Clathrin-mediated endocytosis and macropinocytosis pathways were reduced by 17 and 24%, respectively, in the presence of the respective inhibitors. When cells were transfected in the presence of double and triple inhibitors, transfection efficiencies were increasingly reduced by 40 and 70%, respectively; however, no differences were found between the different uptake mechanisms. These findings suggest that PLL-g-PEG-DNA nanoparticles enter by several pathways and might therefore be an efficient and versatile tool to deliver therapeutic DNA.

INTRODUCTION

Gene therapy has gained a lot of attention for the treatment of chronic diseases, cancer, and genetic disorders (1–4). It is also considered as a valuable alternative for conventional protein therapy since it overcomes inherent problems that are associated with the administration of protein drugs in terms of bioavailability, systemic toxicity, in vivo clearance rate, and manufacturing costs (2, 4, 5). For this reason, safe and efficient delivery systems for therapeutic DNA are developed. Polycationic substances such as poly-L-lysine (PLL), poly-L-ornithine, or polyethylenimine (PEI) alone or as graft- and copolymers with different amounts of poly(ethylene glycol) (PEG) have been shown to form complexes with DNA and are widely used as an attractive alternative to viral vectors in gene therapy (2, 6–11). However, not only the DNA-delivery vehicle itself but also cellular uptake, intracellular stability, and bioavailability of the therapeutic DNA within the cell are crucial for clinical relevance. Therefore, uptake mechanisms of DNA-containing vehicles and trafficking pathways within the cells were analyzed (3, 12–14). Unfortunately, (nano-)particle uptake and intracellular pathways seem to depend on the analyzed cell type (15) as well as on the particular size, shape, charge, and chemistry of the delivery vehicle (16, 17). Especially, when certain cell types or specific uptake pathways are targeted, the uptake mecha-

nism(s) and intracellular pathways need to be analyzed carefully (1, 3, 4, 17). Therefore, rational design of PLL-g-PEG-DNA nanoparticles might benefit from the knowledge raised about the uptake pathways involved and the release mechanisms of therapeutic DNA in order to optimize their performance.

The analysis of gene delivery vehicles is still a complex challenge, as endocytosis may involve several pathways (3, 18–21). The first barrier is to overcome the plasma membrane, followed by cytoplasmatic pathways prior to DNA delivery into the nucleus where gene expression takes place. So far, five major uptake mechanisms are distinguished (14, 18, 21, 22) including phagocytosis that is performed only by specialized cells such as macrophages, neutrophils, or monocytes to eliminate debris and pathogens. In this study, four pinocytotic pathways are studied to assess cellular uptake and intracellular pathway(s) of PLL-g-PEG-DNA nanoparticles: (i) macropinocytosis that is stimulated by growth factors or phorbol esters, which cause actin-driven formation of membrane ruffles that fuse with the plasma membrane forming large endocytotic vesicles known as macropinosomes (14, 23). They either fuse with lysosomes or are recycled back to the cell surface. Large particles up to 1–5 µm in diameter can be internalized by macropinocytosis (14, 22). (ii) Clathrin-mediated endocytosis is a process involving specific receptors that recognize and internalize cargo (24). Ligand-bound receptors are internalized via clathrin-coated vesicles involving GTPase activity and are transformed to early endosomes. Receptor—ligand complexes are separated by low pH generated by vacuolar proton ATPases (25). The ligands are transferred into late endosomes for potential degradation in lysosomes, whereas the receptors are recycled and transported back to the plasma membrane (19). (iii) Caveola-mediated endocytosis is characterized by the evolution of caveolae, which
are associated with caveolin-1/caveolin-2 (or caveolin-3 in the muscle) (26). Caveolae derive from subdomains of sphingolipid and cholesterol-rich cell membrane fractions called lipid rafts (27). Caveolae are considered to be static organelles, whose internalization is induced by specific ligands such as simian virus-40 (SV40) or cholera toxin (18, 28, 29). After internalization, those ligands are either transferred into caveosomes having neutral pH that translocate to the endoplasmic reticulum or the Golgi complex (30) or they enter the endosomal pathway (31). (iv) This pathway is described as clathrin- and caveole-independent, transporting cargo to the GPI-anchored protein-enriched early endosomal compartment (GEEC) and ending in the Golgi complex or in recycling endosomes (18). Glycosylphosphatidylinositol-anchored proteins (GPI-AP) can be internalized via 40–50 nm lipid rafts (18). But regulatory mechanisms for this pathway are still unknown.

A recent report from our group showed evidence that PLL-g-PEG-DNA nanoparticles are a promising tool for effective transfection and delivery of therapeutic DNA as they show long-term stability, a hydrodynamic diameter of 80–90 nm, and high transfection efficiency at a N/P ratio 3.125 of ∼80% (0.01 M phosphate buffered saline, pH 7.4, Sigma). In order to understand the uptake mechanism(s) and intracellular pathway(s) of PLL-g-PEG-DNA nanoparticles, PLL-g-PEG and plasmid DNA were differentially fluorescently labeled, and uptake into COS-7 cells was determined by uptake inhibition analysis. Furthermore, transfected COS-7 cells were analyzed after different time points for colocalization with markers for individual uptake mechanisms, and immunofluorescence staining of cellular compartments was performed. This study may be helpful in the future design of PEGylated PLL-polymers for gene delivery approaches to identify the most efficient uptake and delivery mechanisms.

**MATERIALS AND METHODS**

**Polymer Synthesis.** The polymers were synthesized using poly-L-lysine hydrobromide with a molecular weight of 20 kDa (Sigma), referred to as PLL20 and an N-hydroxysuccinimidyler ester of methoxy-terminated poly(ethylene glycol) (Nektar, USA, MW 5 kDa) as described previously (11, 32). FITC-labeled PLL-g-PEG polymer was synthesized in an analogous manner, using 20% FITC-terminated poly(ethylene glycol) N-hydroxy succinimidyl ester (Nektar, USA, MW 5 kDa) and 80% N-hydroxysuccinimidyler ester of methoxy-terminated poly(ethylene glycol). Grafting (g) was calculated using the absolute number of PEG-moieties per lysine of the PLL backbone and was about 5% for all the polymers. PLL20-g5-g5-g5-g5-g5-g5-FITC or PLL20-g5-g5-g5-g5-g5-g5-FITC was used for all experiments and is referred to as PLL-g-PEG or PLL-g-PEG-FITC, respectively. Reaction rates were determined by 1H NMR (D2O) (δ, ppm, and δ CH2 peaks of lysine at 1–2 ppm, CH2CH2O of PEG at 3.3–3.9 ppm, and the FITC-group at 6.9–7.2 ppm). The polymers were stored in PBS at −20 °C until further use.

**Formulation of PLL-g-PEG-DNA Condensates.** PLL-g-PEG-DNA condensates were formed using a plasmid expressing enhanced green fluorescent protein (pEGFP-N1, Clontech) at a final DNA concentration of 1 μg/150 μL (6.7 μg/mL) in PBS (0.01 M phosphate buffered saline, pH 7.4, Sigma) and a PLL-g-PEG concentration according to the NH23/PPO43− (amino group in PLL/phosphate group in DNA; N/P) charge ratio of 3.125. For a 24-well plate, PLL-g-PEG was provided in 75 μL of PBS, and the DNA dissolved in 75 μL of PBS was added dropwise into the polymer solution. The complexes were equilibrated for 30 min at room temperature (RT) and were always freshly prepared prior to use.

**Plasmid Propagation.** The vector pEGFP-N1 used in this study was transformed into E. coli strain Top10 (Invitrogen) and grown in LB medium supplemented with 30 μg/mL kanamycin. The plasmid DNA was purified using a Maxiprep kit (Qiagen), and the concentration was determined spectrophotometrically at 260 nm.

**DNA Labeling.** Plasmid DNA pEGFP-N1 was covalently labeled using LabelIT nucleic acid labeling kits (Mirus, Cat. No.: MIR3100 (CX-rhodamine) and MIR3700 (Cy5), respectively) according to the manufacturer’s protocol. For colocalization studies, the DNA was labeled with CX-rhodamine (DNA-CX-rh), and for flow cytometry analysis, the plasmid was labeled with Cy5 (DNA-Cy5).

**Cells and Cell Culture.** COS-7 (SV 40 transformed kidney cells of African green monkey, ATCC CRL 1651, ATCC Rocksville, MA) cells were cultured as exponentially growing subconfluent monolayers at 37 °C and 5% CO2. The cell culture was kept in 25 cm2 culture flasks in DMEM (Cat. No.: 21885, low glucose, Glutamax I, GibcoBRL) supplemented with 10% heat inactivated FBS (Cat. No.: F7524, Sigma) and 1% ABAM (Cat. No.: 15240, GibcoBRL). For experiments, cells were used 24 h post seeding.

**Temperature-Dependent Nanoparticle Uptake.** In order to determine energy-dependency of nanoparticle uptake, COS-7 cells were transfected with DNA nanoparticles at either 37 or 4 °C. COS-7 cells were seeded 24 h prior to transfection in 24 well plates (10,000 cells/well). The growth medium then was replaced with 150 μL of PLL-g-PEG-DNA nanoparticles in PBS (per well: 1 μg plasmid pEGFP-N1, N/P ratio = 3.125), and the cells were incubated for 30 min, either at 37 °C or at 4 °C (here, in all the subsequent steps, the solutions were prechilled to 4 °C). Then, 400 μL of 2% serum-containing DMEM was added, and the cells were again incubated at either 37 or 4 °C for an additional 3 h. Finally, the cells were extensively washed 3× with PBS at the corresponding temperatures before DMEM supplemented with 10% FBS at 37 °C was added. After 48 h of incubation at 37 °C for both conditions, the cells were analyzed for GFP expression. To identify the total cell number, Hoechst 33342 nuclear stain (Invitrogen) was used, and ethidium bromide (AppliChem) visualized potential cytotoxicity. Briefly, the cells were incubated for 2 min in PBS containing 4 μg/mL Hoechst 33342 and 10 μg/mL ethidium bromide. After rinsing the cells three times with PBS, the transfection efficiency was analyzed by counting the GFP-expressing cells in 3 nonoverlapping areas of 500 μm2 in relation to the total cell number as determined by the Hoechst nuclear stain. In order to analyze the data, ImageJ 1.38 was used.

In order to visualize nanoparticle uptake at 4 and 37 °C, 8000 COS-7 cells were seeded in 4 well chamber slides (Lab-Tek Chambered #1.0 Borosilicate Cover Glass System, Nunc) and cultured for 16 h in DMEM containing 10% FBS. Complexes were formed by using PLL-g-PEG-FITC and DNA-CX-rh (1 μg/well) at N/P ratio 3.125 and were incubated with the cells for 3.5 h at either 4 °C or 37 °C as mentioned above. After 3× PBS washing, the cells were Hoechst-stained. Finally, cells were fixed with 1% (w/v) paraformaldehyde (PFA, Sigma) in PBS for 30 min at RT and analyzed using a high-resolution TCS-SP5 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). To avoid cross talk, emission signals were collected independently. Image processing was performed using IMARIS software, version 6.0.0 (Bitplane AG, Zurich, Switzerland).

**Colocalization Studies of Nanoparticles Formed by PLL-g-PEG-FITC Polymer and DNA-CX-rh.** PLL-g-PEG-FITC was first confirmed to have transfection efficiency similar to that of nonlabeled PLL-g-PEG. Eight thousand COS-7 cells were seeded in 4 well chamber slides and cultured for 16 h in DMEM containing 10% FBS as indicated above. Complexes were formed by using PLL-g-PEG-FITC and DNA-CX-rh (1 μg/well)
at N/P ratio 3.125 as described above and were applied on the
cells for 30 min and 2, 6, and 24 h. DMEM with 2% FBS was
added 30 min after nanoparticle addition to guarantee cell
viability. After the indicated time points, noninternalized
nanoparticles were removed by rinsing the cells with PBS.

Hoechst 33342 nuclear stain was performed and cells were
rinsed 3× with PBS. Finally, cells were fixed with 1% (w/v)
paraformaldehyde (PFA, Sigma) in PBS for 30 min at RT and
analyzed by CLSM.

Optimization of Endocytosis Inhibitor Concentrations. In
order to determine the most efficient concentrations of several
endocytosis inhibitors without decreasing cell viability, a WST-1
proliferation assay (Roche) was performed in 48 well plates
(TPP, Switzerland). The following inhibitors have been used:

150 µM–10 mM methyl-β-cyclodextrin (Sigma, Cat. No.: C4555),
200 µM genistein (Sigma Cat. No.: 91955), 2 µM–60 µM
cholorpromazine (Sigma, Cat. No.: C8138), and 50 nM–20 µM
wortmannin (Sigma, Cat. No.: 95455). As positive control,
cells were treated with PLL-g-PEG-DNA nanoparticles in the
absence of any inhibitors. Eight thousand COS-7 cells/well
were seeded 24 h prior to the addition of inhibitors. Afterwards,
the cell culture medium was replaced with 270 µL of serum
free DMEM containing the different inhibitors, and the cells
were incubated at 37 °C for 1 h. Then, freshly prepared 30 µL
PLL-g-PEG-DNA nanoparticles in PBS (per well: 0.4 µg
plasmid DNA; N/P ratio = 3.125) were added and incubated
for another 3 h. Finally, the cells were washed 3× with PBS
before they were incubated in 300 µL of growth medium
for 48 h. Subsequently, 30 µL of WST-1 reagent was applied to
each well, and the cells were incubated for another hour at 37
°C and 5% CO2. Absorption at 440 nm was measured using a
microplate reader Infinite M200 (Tecan). Each condition was
performed in quadruple and repeated three times.

DNA Nanoparticle Uptake in the Presence of Endo-
cytosis Inhibitors (FACS Analysis). Twenty-four hours prior
to the experiment, 50,000 COS-7 cells were seeded into a 6
well plate and cultured as describes above. The growth medium
was replaced with 1 mL of serum free medium containing
the different inhibitors using the optimized concentrations. The
cells were incubated for 1 h. Subsequently, the medium was replaced with 500 µL of PBS containing PLL-g-PEG-DNA-Cy5 nano-
particles (per well: 1 µg DNA-Cy5; N/P ratio = 3.125) in
addition to the inhibitors as described above. The cells were
incubated for 30 min, and then an additional 1.5 mL serum free
DMEM medium containing the inhibitors was added. After
a total inhibitor incubation time of 4 h, the cells were rinsed twice
with PBS before they were detached with 500 µL of trypsin-
EDTA (0.25%, GibcoBRL), resuspended, and incubated for 10
min in PBS containing 10 mg of heparin (H9399, Sigma) at
RT to detach cell-surface-bound DNA-containing nanoparticles.
Subsequently, the cells were washed twice with PBS before they
were transferred into tubes (REF 352054, Falcon) for flow
cytometry analysis. The analysis was carried out with a
FACScalibur instrument and CellGate software (BD Bio-
sciences). Dead cells and debris were gated out by forward and
side scatter spectra. The mean fluorescence intensity of 10,000
individual cells was determined. All experiments have been
performed in duplicate and were repeated three times. As
controls, COS-7 cells were transfected with DNA-Cy5-nano-
particles in the absence of inhibitors, and naked DNA-Cy5 was
used.

Transfection Studies with Endocytosis Inhibitors. Eight
thousand COS-7 cells were seeded for 24 h in 24 well plates
and incubated for 1 h with inhibitors dissolved in 200 µL of
serum free DMEM. The optimized inhibitor concentrations
were used as follows: methyl-β-cyclodextrin (5 mM), genistein (200
µM), chlorpromazine (10 µM), and wortmannin (10 µM). Later,
the inhibitor solutions were removed, and 150 µL of freshly
prepared PLL-g-PEG-DNA nanoparticles in PBS (per well: 1
µg pEGFP-N1; N/P ratio = 3.125) containing the same inhibitor
concentrations as those mentioned above were added and
further incubated for 30 min. Then, 400 µL of serum free medium
supplemented with inhibitors was added and the incubation time
extended for an additional 2.5 h. Subsequently, the cells were
washed 3× with PBS before 10% serum-containing medium
was added to further incubate the cells for 48 h. Double
and triple inhibitor transfection experiments were performed in
an analogous manner. In order to determine cell viability of
the multiple inhibitors used, a WST-1 proliferation assay was carried
out in parallel as described above. Finally, the cells were
analyzed for GFP expression by fluorescence microscopy as
mentioned before. As positive control, DNA nanoparticles
without inhibitors were used to transfect the cells.

Nanoparticle Distribution in the Presence of Colocaliza-
tion Markers. Eight thousand COS-7 cells were seeded in 4
well chamber slides (Lab-Tek Chambered #1.0 Borosilicate
Cover glass System, Nunc) and cultured as described before.
After washing the cells with PBS, the condensates, consisting
of 1 µg of plasmid DNA (pEGFP-N1) complexed with PLL-
PEG-FITC at N/P ratio 3.125, were coincubated with either
cholera toxin subunit B (10 µg/mL) labeled with Alexa Fluor
594 (Molecular Probes, AF-cbT) or Texas Red-labeled trang-
ferrin (50 µg/mL; Molecular Probes, TR-tf) for 30 min. DMEM
supplemented with 2% FBS was added to a final volume of
500 µL per chamber slide. Additionally, cells were first
incubated with FITC-labeled condensates for 1.5 h before adding
AF-cbT (10 µg/mL) or TR-tf (50 µg/mL). For both experimental
setups, coincubation of the nanoparticles with the colocalization
markers or when colocalization markers were added after 1.5 h,
the nuclei were Hoechst-stained after 2 h total incubation time.
Then, cells were washed 3× with PBS and fixed in 1% (w/v)
PFA in PBS for 30 min at RT. Cells were washed again with
PBS and analyzed by CLSM as described above.

Immunofluorescence of TFR, Caveolin-1, GM1, and
EEA-1. Eight thousand COS-7 cells were seeded into 4 well
chamber slides and cultured for 16 h prior to incubation with
PLL-g-PEG-DNA-CX-rh (DNA-CX-rh = 1 µg/well) condens-
ates at N/P = 3.125. Cells were stained with antibodies against
transferrin receptor (TFR), caveolin-1, and GM1 after 30 min
and 45 min, and 1 and 2 h. The cultures were stained against
early endosome antigen-1 (EEA-1) after 1, 2, 4, and 6 h. After
the indicated time points, the cell culture medium was removed,
the cells were washed 3× with PBS and fixed in 1% (w/v) PFA
in PBS for 30 min at RT. Cells were washed again with
PBS and analyzed by CLSM as described above.

Statistical Analysis. The mean values were compared by one-
way ANOVA analysis using Origin 7.5 software. Statistical
significance was accepted for $p < 0.05$ after comparing the mean values by Bonferroni test.

RESULTS

PLL-g-PEG-DNA Nanoparticles are Internalized by Endocytosis and Remain Stable within the Cytoplasm. As nonviral gene delivery systems are promising tools for gene therapy approaches, it is important to analyze nanoparticle internalization and trafficking pathways within the cell. This is especially important when comparing to viral gene delivery systems that benefit from their inherent high cell transduction efficiency. Information on particle uptake mechanisms and intracellular pathways will lead to improved design of PLL-g-PEG-DNA nanoparticles for further applications.

As PLL-g-PEG-DNA nanoparticle stability is maintained by ionic interactions between the PLL backbone of positively charged amines in PLL-g-PEG to negatively charged phosphate groups of the DNA, it is crucial to gain insight into nanoparticle uptake kinetics and intracellular stability. Therefore, PLL-g-PEG and DNA were covalently labeled with two different fluorescent labels. An FITC-labeled PEG-moiety was incorporated into PLL-g-PEG-FITC polymer. Scale bar is 10 μm. (B) PLL-g-PEG-DNA (plasmid DNA: pEGFP-N1) nanoparticles were applied on COS-7 cells and incubated for 3.5 h at either 37 or 4 °C. Transfection efficiency was analyzed after 48 h by counting transfected cells in relation to the total number of cells. The experiments indicate mean values ± standard deviation of three independent experiments carried out in triplicate. The values are normalized to particle uptake at 37 °C.

Analysis of PLL-g-PEG-DNA Nanoparticle Uptake into COS-7 Cells. Uptake of PLL-g-PEG-DNA nanoparticles was studied by using different endocytosis inhibitors. Figure 3A lists endocytosis inhibitors that were used to inhibit their respective target pathways. As the effective range of inhibitors is very narrow, cell viability assays were performed 48 h after inhibitor treatment to determine efficient but not toxic inhibitor concentrations (Figure 3C). The inhibitor concentrations used here showed more than 85% cell viability compared to untreated COS-7 cells. To quantify nanoparticle uptake into COS-7 cells, FACS analysis was carried out (Figure 3B). PLL-g-PEG-DNA-Cy5 nanoparticles were individually coincubated with different substances, and inhibition of nanoparticle uptake was determined. As controls, fluorescent-labeled nanoparticles and DNA-Cy5 in the absence of inhibitors were used. Genistein, an inhibitor of caveolae-mediated endocytosis, reduced nanoparticle
uptake to 43 ± 5%. Whereas all other inhibitors reduced nanoparticle uptake only a little, wortmannin by 17 ± 3% and chlorpromazine by 3 ± 2%, indicating the involvement of several pathways, 74 ± 2% of the nanoparticles were internalized when methyl-β-cyclodextrin was applied.

As nanoparticle uptake is not indicative for gene expression, transfection experiments in the presence of single inhibitors were performed (Figure 3D). PLL-g-PEG-DNA nanoparticles were coincubated with the respective inhibitors, and GFP expression was analyzed. In the presence of wortmannin and methyl-β-cyclodextrin, transfection efficiency was reduced by 35 ± 18% and 30 ± 19%, respectively, whereas in the presence of genistein or chlorpromazine, transfection efficiency was not reduced significantly. In order to further analyze if multiple pathways are involved in PLL-g-PEG nanoparticle uptake, double and triple inhibitor experiments were performed. Cell viability with double inhibitors was found to be above 90% (Figure 3E), whereas triple inhibitors decreased cell viability by ~20% (Figure 3G). Double inhibitors showed a reduction in transfection efficiency to ~70% (Figure 3F). The transfection efficiency with triple inhibitors was reduced to ~30% in comparison to that of control cells (Figure 3H). Interestingly, no inhibitor combination used was able to completely block nanoparticle uptake and gene expression, indicating that PLL-g-PEG-DNA nanoparticles might use several uptake mechanisms.

Analysis of PLL-g-PEG-DNA Nanoparticle Pathways within COS-7 Cells. To visualize intracellular pathways of the PLL-g-PEG-DNA nanoparticle within COS-7 cells, PLL-g-PEG-DNA nanoparticles were coincubated with known endocytosis ligands cholera toxin B and transferrin. The nontoxic subunit B of cholera toxin was used to study lipid raft-mediated endocytosis as cholera toxin B binds to membrane sphingolipid GM1 to enter a cell (29, 33). Transferrin is internalized by receptor-mediated endocytosis forming clathrin-coated pits and is widely used as a tracker for clathrin-mediated endocytosis. COS-7 cells were incubated for 2 h with PLL-g-PEG-FITC-DNA nanoparticles and endocytosis markers Alexa Fluor-594-labeled cholera toxin B (AF-ctB) and Texas Red-labeled transferrin (TR-tf) were either coincubated with nanoparticles for 2 h or COS-7 cells were preincubated for 1.5 h with nanoparticles prior to the addition of cholera toxin B for 30 min (Figure 4). When COS-7 cells were preincubated for 1.5 h with the nanoparticles prior to the addition of cholera toxin B or transferrin (Figure 4A and C), cholera toxin B was found attached to the plasma membrane, whereas transferrin was already internalized and localized close to the nucleus. PLL-g-PEG-FITC-DNA nanoparticles were internalized, but only few particles colocalized with the endocytosis markers as indicated by a few yellow particles. When COS-7 cells were coincubated with PLL-g-PEG-FITC-DNA nanoparticles and endocytosis markers for 2 h (Figure 4B and D), cholera toxin B was found in the nucleus not colocalized with PLL-g-PEG-FITC-DNA nanoparticles. Also transferrin and PLL-g-PEG-FITC-DNA nanoparticles were not colocalized within COS-7 cells.

To support the findings obtained with colocalization studies, immunofluorescence stainings with endocytosis markers GM1,
Figure 3. Inhibition of PLL-g-PEG-DNA nanoparticle uptake. (A) Endocytosis inhibitors and their targets used. (B) FACS analysis of COS-7 cells incubated with nanoparticles in the presence of four different endocytosis inhibitors. The graph displays mean fluorescence intensities of one of three independent experiments performed in duplicate. As controls, PLL-g-PEG-DNA-Cy5 without inhibitors, plasmid DNA only, and untreated COS-7 cells were used (not shown). Cell viability as determined by the WST-1 proliferation assay performed in the presence of single (C), double (E), and triple (G) endocytosis inhibitors. Transfection efficiencies after coincubation with single (D), double (F), and triple (H) endocytosis inhibitors. In D, F, and H, unlabeled pEGFP-N1 was used for nanoparticle production. The values were normalized to transfection efficiencies in the absence of any inhibitor (control). The values shown here represent mean values ± standard deviation from two to five independent experiments performed in duplicate. * refers to values that are statistically different from the control (p < 0.05).
of these particles. Cargo uptake is usually energy-dependent and leads to the formation of membranous vesicles (3, 4, 14, 18, 22). PLL-g-PEG-DNA nanoparticles were shown to enter COS-7 cells by energy-dependent mechanisms as reduction of the temperature to 4 °C reduced nanoparticle uptake as shown by confocal imaging. Consequently, transfection efficiency was decreased by 75% at 4 °C. The nanoparticles were shown to enter the cytoplasm within the first 2 h of transfection; later, PLL-g-PEG-DNA nanoparticles accumulate in the perinuclear region preceding nuclear uptake. Furthermore, PLL-g-PEG-DNA nanoparticles were found within the cytoplasm at least for 24 h, and no colocalization with endosomal compartments, as indicated by fluorescence staining against early endosome antigen-1, was observed. These experiments indicate that PLL-g-PEG-DNA nanoparticles translocate efficiently to the nucleus and eventually enter the nucleus, e.g., during mitosis, to express the gene of interest. Most studies in the field of particle uptake focused on pathways via caveolin- or clathrin-coated vesicles and cholesterol- and ganglioside GM1-enriched microdomain-mediated endocytosis (14, 34–36). Therefore, our studies were based on such methodology to classify the intracellular fate of PLL-g-PEG-DNA nanoparticles. Inhibition of uptake experiments were performed in the presence of optimized single inhibitor concentrations of wortmannin (macropinocytosis), chlorpromazine (clathrin-mediated), genistein (caveolae-mediated), and methyl-β-cyclodextrin (caveolae-mediated and caveolae- and clathrin-independent pathways) and with double and triple inhibitor combinations. These experiments revealed that only genistein was able to substantially inhibit (∼55%) PLL-g-PEG-DNA nanoparticle uptake into COS-7 cells. However, transfection efficiency performed in the presence of genistein did not show any reduction. Also, colocalization studies with antibodies against caveolin-1 indicate no correlation between caveolin-1 and PLL-g-PEG-DNA nanoparticles. In addition, caveolae-mediated endocytosis was described to be very fast (>20 min), and the vesicles produced are usually smaller (50–80 nm diameter) (22) than the diameter of PLL-g-PEG-DNA nanoparticles. Therefore, all findings suggest that these nanoparticles do not follow caveolae-mediated pathways and that the inhibitory effects of genistein might be the result of genistein side reactions. It is known that genistein acts as an ATPase inhibitor (37) as well as a hormone analogue (38) that might affect nanoparticle uptake in one way or another. Similar findings with respect to genistein activity were shown when COS-7 cells were transfected with cationic polymer pDMAEMA and PEI nanoparticles (21).

Clathrin-mediated pathways might also be an option for PLL-g-PEG-DNA nanoparticle uptake as particles up to 120 nm in diameter can be internalized (22). Here, no correlation between nanoparticle uptake and markers indicative for clathrin-mediated pathways could be demonstrated. Nanoparticle uptake was not inhibited in the presence of chlorpromazine, nor was transfection efficiency in the presence of chlorpromazine impaired. Moreover, no colocalization was found for PLL-g-PEG-DNA nanoparticles with transferrin, transferrin receptors, and EEA-1.

Also macropinocytotic and caveolae- and clathrin-independent uptake mechanisms could not be assigned as primary uptake pathways for PLL-g-PEG-DNA nanoparticles. Particle uptake and transfection could be inhibited in the presence of wortmannin or methyl-β-cyclodextrin by 17 and 24%, respectively, whereas colocalization between chola toxin subunit B and PLL-g-PEG-DNA nanoparticles as well as the membrane ganglioside GM1 with the nanoparticles failed.

The conclusion is that these PLL-g-PEG-DNA nanoparticles do not strictly follow one of the classical endocytotic pathways, although some inhibition of endocytosis can be observed for several pathways, and transfection efficiencies were reduced when combinations of inhibitors were used (Figure 6). These

**DISCUSSION**

Knowledge about uptake mechanisms and intracellular pathways of nonviral gene delivery vehicles is of great interest for the design of high performance delivery systems for therapeutic DNA. As PLL-g-PEG-DNA nanoparticles were previously identified as potent gene delivery vehicles in COS-7 cells that combine small size (80–90 nm), long storage stability, and low cytotoxicity with high transfection rates (11), this study aimed at elucidating the uptake mechanisms and intracellular pathways of these particles. Cargo uptake is usually energy-dependent

![Image](https://example.com/image.png)

**Figure 4.** Colocalization studies with PLL-g-PEG-FITC-DNA nanoparticles and endocytosis markers Alexa Fluor-594-labeled cholera toxin B (AF-ctB) and Texas Red-labeled transferrin (TR-tf). In A and C, COS-7 cells were preincubated with PLL-g-PEG-FITC-DNA nanoparticles for 1.5 h, before cholera toxin B or transferrin was added for 30 min. After a total of 2 h, the cells were analyzed. In B and D, cholera toxin B and transferrin were coapplied with the nanoparticles for an entire 2 h prior to analysis. The cells were analyzed by CLSM. Blue indicates Hoechst-stained nuclei, green shows PLL-g-PEG-FITC, and red displays colocalization markers for cholera toxin B (in A and B) and transferrin (in C and D). Scale bar is 10 μm.
findings might have been due to a general cellular inhibition, although overall cell viability was not impaired. Nanoparticle uptake has been described for pDMAEMA and PEI nanoparticles to follow the clathrin- and caveolae-dependent pathways.
in COS-7 cells, respectively (21). Another study pointed out that COS-7 cells were very efficiently transfected with alginate–chitosan nanoparticles that were internalized predominantly by caveolae-mediated endocytosis (15). Pegylated poly-

l-lysine (C1K30-PEG) compacted DNA transfected into COS-7 cells also did not show colocalization with early endosome antigen-1 (EEA-1) or transferrin receptor (TFR), indicating lysosomal stability and no involvement of clathrin-mediated uptake pathways (3). As C1K30-PEG-DNA condensates displayed a rod-like shape with a length of up to 300 nm, involvement of macropinocytic uptake could clearly be identified (3). However, in our study the PLL-g-PEG-DNA nanoparticles display a spherical shape with homogeneous hydrodynamic diameter of 80–90 nm (11). One explanation for the failure to assign one prominent uptake pathway into COS-7 cells might be that PLL-g-PEG-DNA nanoparticles are formed by noncovalent bonds between the amino groups of poly-l-lysine and phosphate groups of the plasmid DNA. As the structure of such condensates is not known and might also be flexible in watery solution, it might well be that dangling ends of poly-l-lysine extend to the surface of the condensates and serve as cell-penetrating peptide sequences helping to internalize PLL-g-PEG-DNA nanoparticles (Figure 6). Cell-penetrating peptides cross biological membranes in a nondisruptive way without apparent toxicity and can therefore deliver macromolecular cargo into target cells via endocytotic-dependent and -independent ways (14, 17, 39, 40). They form stable complexes with proteins and nucleic acids, and the interaction is either covalent or noncovalent (40, 41). Cell-penetrating peptides may consist of Pep and MPG families that are short amphipathic peptides consisting of three domains: a variable N-terminal hydrophobic motif, responsible for membrane fusion; a hydrophilic lysine-rich domain, which is required for the interactions with DNA, intracellular trafficking of the cargo, and solubility of the peptide vector; and a linker domain separating the two domains (40, 42, 43). Structural and mechanistic investigations have revealed that the flexibility between the two domains, which is maintained by the linker sequence between the fusion and the DNA-binding motifs, is crucial for macromolecule delivery (42–45). Uptake mechanisms of condensates formed between cell penetration peptides and their cargo are usually energy-dependent, independent of the endosomal pathway, and are directly correlated to the size of particles and the nature of cargo (46, 47). Here, PLL-g-PEG-DNA nanoparticles might function as cell-penetrating condensates where surface-exposed lysines in combination with flexible, noncharged PEG moieties are able to deliver plasmid DNA into the cytoplasm (14) and thereby circumvent the endocytotic machinery. In favor of such a hypothesis might be the fact that PLL-g-PEG enters into the cytoplasm of COS-7 cells independently of the presence of plasmid DNA (Figures 1A, a and 2G). This hypothesis might also be an explanation for the fast and efficient delivery of plasmid DNA combined with the low cytotoxicity of PLL-g-PEG-DNA nanoparticles used in this study. In future studies, this feature needs to be explored for the delivery of therapeutic DNA in the context of gene therapy.

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