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An outer shell of positively charged poly(ethyleneimine) strongly increases the transfection efficiency of calcium phosphate/DNA nanoparticles

Viktoriya Sokolova · Sebastian Neumann · Anna Kovtun · Svitlana Chernousova · Rolf Heumann · Matthias Epple

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Abstract Nanoparticles with an inner core of calcium phosphate, followed by layers of DNA and calcium phosphate and an outer layer of poly(ethyleneimine) (PEI) were prepared, characterized, and tested on different cell lines (HeLa, T24, and NIH3T3). A considerable increase in transfection efficiency was found for such nanoparticles, compared to the commercial reagent Polyfect® (a cationic dendrimer). The DNA is incorporated into the nanoparticles and protected from the attack by enzymes (nucleases) inside the cytoplasm of cells. The outer layer of PEI leads to electrosteric colloidal stabilization and gives a positive charge to the nanoparticle, which is helpful for the penetration through the negatively charged cell membrane.

Introduction

In molecular biology as well as in gene therapy, DNA has to be introduced into living cells in order to express proteins of interest. This approach is called transfection. DNA alone cannot penetrate the cell membrane due to its negative charge; therefore, suitable carriers are needed. These can be viruses [1, 2] or chemically based nanoparticles like liposomes or polymer/DNA aggregates [3–5], and inorganic nanoparticles [6]. There are also physical and chemical transfection techniques as alternatives to the viral transfection like electroporation [7], microinjection [8], and the gene gun [9]. DNA-loaded calcium phosphate (CaP) nanoparticles present an interesting option [10] because CaP is highly biocompatible due to its presence in mammalian hard tissue [11]. In the last years, precisely defined nanoparticulate CaP delivery systems were developed by several groups [4, 12–18] including ours [19, 20].

We have demonstrated that the transfection efficiency of CaP/DNA nanoparticles could be considerably increased if the DNA was incorporated into the nanoparticle, i.e., protected by another layer of CaP. An outer second layer of DNA ensured colloidal electrosteric stabilization, leading to so-called triple-shell nanoparticles (structure CaP/DNA/CaP/DNA from core to outside) with a strong negative charge [19]. The transfection efficiency of triple-shell nanoparticles was comparable to that of the commercially available transfection agent Polyfect®, which is a cationic dendrimer [19]. The same procedure worked for gene silencing with siRNA [21]. Recently, we showed that the good biocompatibility of CaP/DNA nanoparticles can be explained by low disturbance of the intracellular calcium level during transfection [22]. Polycationic polymers like poly(ethyleneimine) (PEI) form aggregates with DNA, which can be used for non-viral gene delivery [23, 24]. The positive charge of the resulting aggregates is beneficial for penetrating the cell membrane [25]. Several cell lines take up PEI/DNA complexes by endocytosis and the complexes enter the endolysosomal compartment. PEI shows a "proton sponge effect" because every third atom of PEI is a protonable amino nitrogen, leading to a considerable buffer capacity over almost the entire pH range [23]. The high transfection efficiency of PEI can be explained by its buffering capacity of the endosome. This buffering triggers a massive proton accumulation leading to passive chloride influx resulting in osmotic swelling and finally endosome disruption

V. Sokolova · A. Kovtun · S. Chernousova · M. Epple (✉)
Institute of Inorganic Chemistry and Center for Nanointegration Duisburg-Essen (CeNIDE), University of Duisburg-Essen, Universitaetsstr. 5-7, 45117 Essen, Germany
e-mail: matthias.epple@uni-due.de

S. Neumann · R. Heumann (✉)
Department of Biochemistry, Molecular Neurobiochemistry, University of Bochum, Universitaetsstr. 150, 44780 Bochum, Germany
e-mail: rolf.heumann@rub.de

[23, 26]. Furthermore, PEI promotes nuclear localization with and without DNA [27]. The successful transfection by all other non-viral transfection techniques except for electroporation depends on the cell cycle. In contrast to this, PEI can be used to transfect cells independently from their cell cycle state [28]. PEI with low molecular weight showed a low cytotoxicity in cellular studies [29], but PEI with high molecular weight led to higher cytotoxicity [30]. This was ascribed to huge clusters of PEI on the cell membrane, which induced necrotic cell death [31].

Recently, Shkilnyy et al. [32] showed that PEI can be used as template for the synthesis of stable spherical CaP/polymer hybrid particles. However, these particles were not functionalized with DNA or used in any cellular context. Here, we show how the outer layer of DNA in the triple-shell nanoparticles was substituted by PEI, leading to a higher transfection rate than the negatively charged triple-shell particles with DNA on the outside. The transfection efficiency was tested on three different cell lines (HeLa, T24, and NIH3T3) by two different reporter systems, and the cell viability was measured.

Materials and methods

Plasmid DNA was purified from *Escherichia coli* using the Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany). The pcDNA3-EGFP plasmid encoded the enhanced green fluorescing protein (EGFP), and the pcDNA3.1(+)-firefly-luciferase plasmid encoded firefly-luciferase. Branched poly(ethylenimine) (PEI) with a molecular weight of 25,000 g mol^{-1} from Aldrich was used.

DNA-coated CaP nanoparticles (single-shell; CaP/DNA) were prepared as described in reference [19] by a rapid precipitation method using 0.5 mL of $Ca(NO_3)_2$ solution (6.25 mM), 0.5 mL of $(NH_4)_2HPO_4$ solution (3.74 mM), and 0.2 mL of pcDNA3-EGFP (1 mg mL^{-1}). CaP/DNA/CaP/PEI triple-shell nanoparticles were prepared by adding a layer of CaP on the surface of the single-shell nanoparticles. 1.2 mL of the CaP/DNA nanoparticle dispersion were taken with an Eppendorf pipette and rapidly mixed with 0.5 mL of calcium nitrate (6.25 mM) and then 0.5 mL of diammonium hydrogen phosphate (3.74 mM), followed by the addition of either 0.1, 0.5, or 1.0 mL of PEI (2 mg mL^{-1}), giving three different dispersions. These three dispersions had concentrations of 87, 74, and 63 μg DNA mL^{-1}, and 87, 370 and 625 μg PEI mL^{-1}, respectively.

DNA/PEI particles without a CaP core were prepared as control: 0.2 mL of pcDNA3-EGFP solution (1 mg mL^{-1}) were diluted in 1 mL of water and then mixed with 0.1, 0.5, and 1.0 mL of PEI (2 mg mL^{-1}), respectively.

The nanoparticles functionalized with pcDNA3.1(+)-firefly-luciferase were prepared in the same way, except that the concentration of DNA was 1.2 mg mL^{-1}, therefore, the used amount of DNA was 0.16 mL per 1 mL of CaP dispersion. We used either 0.1 or 0.5 mL of PEI (2 mg mL^{-1}) per 1 mL of CaP dispersion. These dispersions had concentrations of 87 or 74 μg DNA mL^{-1}, and 87 or 370 μg PEI mL^{-1}, respectively.

For the transfection experiments, the following cell types were used: HeLa (human transformed cervix epithelial cells), T24 (a human bladder carcinoma cell line), and NIH3T3 (a mouse embryonic fibroblast cell line). All cells were obtained from the American Type Culture Collection (ATCC). They were cultured either in DMEM (HeLa) or in RPMI 1640 (T24 and NIH3T3), supplemented with 10% fetal calf serum (FCS) at 37 °C and 5% CO_2 (T24 and NIH3T3) or 10% CO_2 (HeLa) in humidified atmosphere and subcultivated according to standard cell culture protocols. Twelve hours prior to transfection, the cells were trypsinized and seeded in cell culture dishes with 5×10^4 cells per 24-well dish (density 10^4 cells cm^{-2}).

The transfection with the dendrimer-based commercial agent Polyfect® (Qiagen, Hilden, Germany) was carried out according to the manufacturer's recommendation: 2 μg DNA were dissolved in 100 μL medium without FCS to which 22 μL Polyfect®-solution was added. After mixing, the dispersion was incubated for 5 min, and then 600 μL cell culture medium were added. The culture medium was replaced by 1.5 mL fresh medium and the transfection mixture was added. The transfection mixture remained on the cells for 7 h and was then replaced by fresh cell culture medium. The standard transfection method with CaP was carried out as follows: 4 μL aqueous solution containing 4 μg of DNA was mixed with 10 μL of 2.5 M $CaCl_2$ solution. The dispersion was incubated for 5 min. The volume of the dispersion was adjusted to 100 μL with water and 100 μL HEPES buffered saline solution (2HBS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES, pH 7.05 ± 0.01) were added. Cell culture medium (RPMI 1640 with FCS) was added up to 1 mL. From the cell culture, the culture medium was removed, and the transfection mixture was added. After 7 h of incubation, the transfection mixture was replaced by fresh cell culture medium. The transfection with custom-made nanoparticles was carried out as follows: 40 μL of the particle dispersion were thoroughly mixed with 1 mL cell culture medium. From the cell culture, the cell culture medium was removed and 0.5 mL of the mixture was added. After 7 h, the transfection mixture was replaced by fresh cell culture medium. The transfection with pcDNA3.1(+)-firefly-luciferase plasmid was carried as follows: 40 μL of the particle dispersion were thoroughly mixed with 0.5 mL cell culture medium and then 0.5 mL of this mixture was added to the cell culture. After 7 h the transfection mixture was exchanged by fresh cell culture medium and incubated for further 48 h.

The efficiency of the expression of EGFP was determined by fluorescence microscopy at a magnification of 200×. The transfection efficiency was computed by the ratio of cells in which EGFP was expressed to the total examined number of cells.

The transfection efficiency with luciferase was detected by counting luminescence signals on a multilabel plate reader (Wallac 1420, Perkin Elmer). 48 h after transfection, the cell culture medium was replaced with 360 µL of the mixture of two buffers (substrate buffer: Tricine 21.5 g L^{-1}, $MgSO_4$ 3.9 g L^{-1}, EDTA 0.06 g L^{-1}, DTT 30.7 g L^{-1}, Coenzyme A 1.25 g L^{-1}, Luciferin 0.78 g L^{-1}, ATP 1.76 g L^{-1}, pH 7.8; and lysis buffer: Triton X-100 3%, glycerol 10%, DTT 2 mM, Na_2HPO_4 25 mM, Tris (HCl) 25 mM, pH 7.8) in PBS in the volume ratio of substrate buffer:lysis buffer:PBS = 1:1:4. The cells were incubated at room temperature for 15 min and then 150 µL of the lysate was transferred into a black 96-well plate. The time for measuring the luciferase activity was 10 s per well and the obtained values were corrected for background values. The obtained activity values in counts per second (cps) were normalized to the activity of cells transfected by Polyfect® taken as 100% ($N = 3$).

The cell viability was probed by applying an MTT-assay 48 h after the transfection. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Taufkirchen, Germany) was dissolved in PBS (5 mg mL^{-1}) and finally diluted to a concentration of 1 mg mL^{-1} in cell culture medium. The cell culture medium of the transfected cells was replaced by 300 µL of the MTT solution and incubated for 1 h at 37 °C under 5% CO_2. The dark blue formazan salt was dissolved in 300 µL DMSO by incubating the cells for further 30 min. Then a 50-µL aliquot was taken for spectrophotometric analysis using an ELISA-Reader (SLT Labinstruments, Salzburg, Austria) at $\lambda = 560$–600 nm. The absorption levels of transfected cells were normalized by those of untransfected cells, thereby indicating the relative level of cell death.

Dynamic light scattering (DLS) of the colloidal dispersions was performed with a Malvern Zetasizer (Nano ZS, 633 nm laser; Smoluchowsky method). Scanning electron microscopy (SEM) was performed with a FEI Quanta 400 ESEM instrument after gold–palladium alloy sputtering.

Results and discussion

We have previously described the transfection by multishell CaP/DNA nanoparticles [19]. In the present study, these particles were modified by replacing the outer layer of DNA by PEI, giving the particles a positive charge. A schematic representation is shown in Fig. 1.

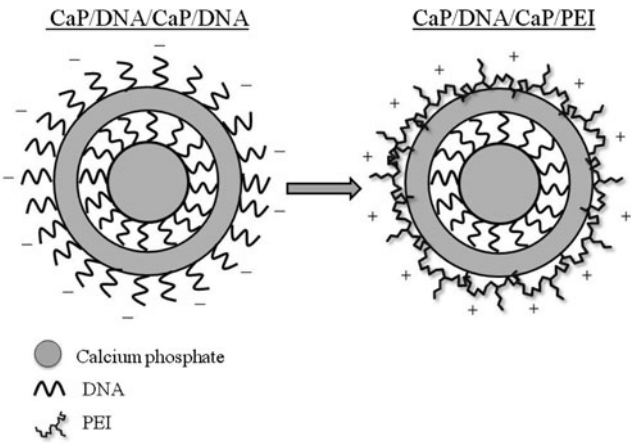

Fig. 1 Schematic representation of two types of nanoparticles: negatively charged CaP/DNA/CaP/PEI and positively charged CaP/DNA/CaP/PEI

Table 1 Dynamic light scattering data of functionalized calcium phosphate (CaP) nanoparticles

Sample	PDI	Particle size (*Z*-average) (nm)	Zeta potential (mV)
CaP/DNA	0.235	35	−32
CaP/DNA/CaP/PEI (0.1 mL)	0.306	285	+36
CaP/DNA/CaP/PEI (0.5 mL)	0.234	150	+37

PDI is the polydispersity index from dynamic light scattering

The results of DLS are shown in Table 1. In the dispersion of CaP/DNA/CaP/PEI, nanoparticles with a size around 285 nm (PEI 0.1 mL) and 150 nm (PEI 0.5 mL) were present; for DNA/PEI aggregates, the particle size was too large to be detected (i.e., in the µm range), and for CaP/DNA nanoparticles, a particle size of 35 nm was found. CaP/DNA nanoparticles without PEI showed a strongly negative charge due to the negatively charged phosphate groups of adsorbed DNA. The particles with an external coating of PEI carried a strongly positive charge due to the positively charged amino groups of PEI molecules (Table 1). The PEI on the surface of nanoparticles protected the particles from aggregation, due to the presence of electrostatic and steric stabilization. Scanning electron microscopy confirmed the spherical morphology of nanoparticles (Fig. 2).

The transfection efficiency of the nanoparticles was assessed on HeLa cells by the expression of either EGFP or firefly-luciferase. As controls, the commercial transfection reagent Polyfect® and the standard CaP precipitation method were used.

Transfection studies with DNA/PEI alone resulted in very low levels of gene expression (Table 2). However, the

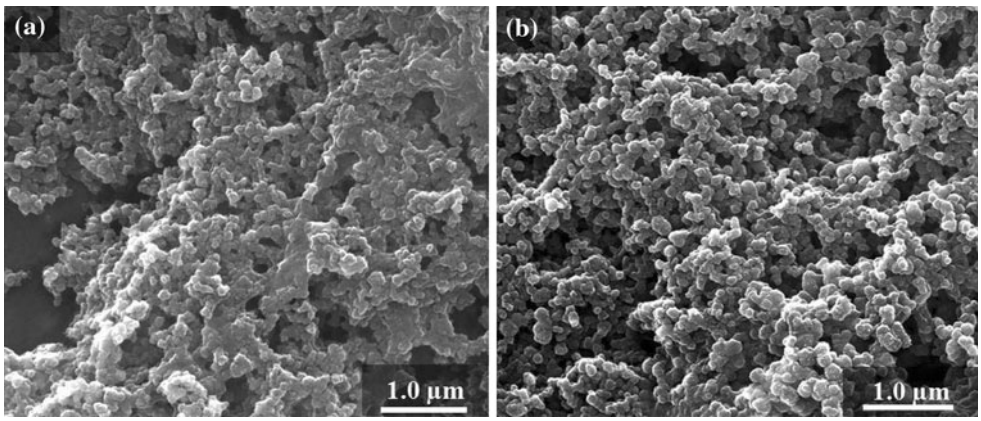

Fig. 2 Scanning electron micrographs of CaP/DNA/PEI nanoparticles. **a** CaP/DNA/CaP/PEI with 0.1 mL of PEI. **b** CaP/DNA/CaP/PEI with 0.5 mL of PEI. Note that the particle size in SEM is smaller than that obtained by dynamic light scattering because by DLS, the hydrodynamic radius is measured

Table 2 Results of transfection experiments for HeLa with two types of reporter systems, EGFP and luciferase expression

Method	Reporter gene expression		Cell viability (%)
	EGFP transfection efficiency (%)	Luciferase activity normalized to Polyfect® (%)	
Polyfect®	30 ± 2	100 ± 2	83 ± 2
Standard calcium phosphate method	5 ± 1	1.1 ± 0.9	96 ± 3
CaP/DNA/CaP/PEI (0.1 mL)	11 ± 3	233 ± 150	101 ± 4
DNA/PEI (0.1 mL)	5 ± 1	67 ± 38	92 ± 6
CaP/DNA/CaP/PEI (0.5 mL)	44 ± 9	306 ± 237	84 ± 5
DNA/PEI (0.5 mL)	14 ± 4	170 ± 19	78 ± 4
CaP/DNA/CaP/PEI (1.0 mL)	48 ± 13	–	43 ± 3
DNA/PEI (1.0 mL)	16 ± 5	–	47 ± 6

All values are given as average ± standard deviation

transfection efficiency increased with the content of PEI in the nanoparticles from 11 to 48% of transfection efficiency for EGFP (Polyfect®: 30%) and from 233 up to 306% of luciferase activity for the luciferase reporter system (Polyfect® set to 100%). The difference in the efficiencies between EGFP expression and luciferase expression can be explained as follows: in the case of the EGFP reporter system, we detected the number of transfected cells directly by fluorescence by the expressed EGFP protein. However, in the case of firefly-luciferase, we determined the transfection efficiency indirectly by monitoring the catalytic activity of the enzyme measuring the luminescence. Therefore, the absolute efficiency can be hardly compared, but the correlation of data obtained from these independent methods is clear. We observed the same tendencies in both systems: the increase of PEI content led to the increase of the transfection efficiency, but simultaneously to increased cytotoxicity.

CaP/DNA/CaP/PEI nanoparticles showed a significant increase in efficiency compared to other transfection reagents with either EGFP or luciferase plasmids. In the case of the EGFP expression, the transfection efficiency increased by a factor of 2–4.5 compared to the standard CaP precipitation method and by a factor of 1.5 compared to Polyfect®. In the case of the luciferase reporter system, the activity increased 20 times compared to the standard CaP precipitation method and 2–3 times compared to Polyfect®. The transfection efficiencies are shown in Fig. 3.

In general, an increase of the amount of PEI resulted in higher transfection efficiencies with both reporter systems. However, it should be kept in mind that very high concentrations of PEI are cytotoxic [29–31], and that cytotoxicity is a possible reason of concern for novel gene delivery systems. An MTT test showed that the cell viability decreased with increasing PEI concentration (Table 2). The optimal concentration of PEI for HeLa cells was 0.5 mL. The cell viability in this case was 84%, which is comparable with that of Polyfect® (83%). Only 43% of HeLa cells survived at a PEI concentration of 1.0 mL.

To show the efficiency of custom-made nanoparticles for different cell types, we tested pcDNA3-EGFP-loaded nanoparticles on two other cell lines, i.e., T24 and NIH3T3. The optimal concentration of PEI for these cell lines was 0.1 mL, in contrast to 0.5 mL of PEI for HeLa cells. Apparently, T24 and NIH3T3 cells are more sensitive to PEI; therefore, its concentration must be lower. For T24 cells, the CaP nanoparticles were twice as effective (22%) as Polyfect® (10%), for NIH3T3 cells, both agents were comparable (9–10%) (Table 3). The standard CaP precipitation method gave about 5% for all cell lines, which was comparable to the DNA/PEI aggregates (i.e., without CaP).

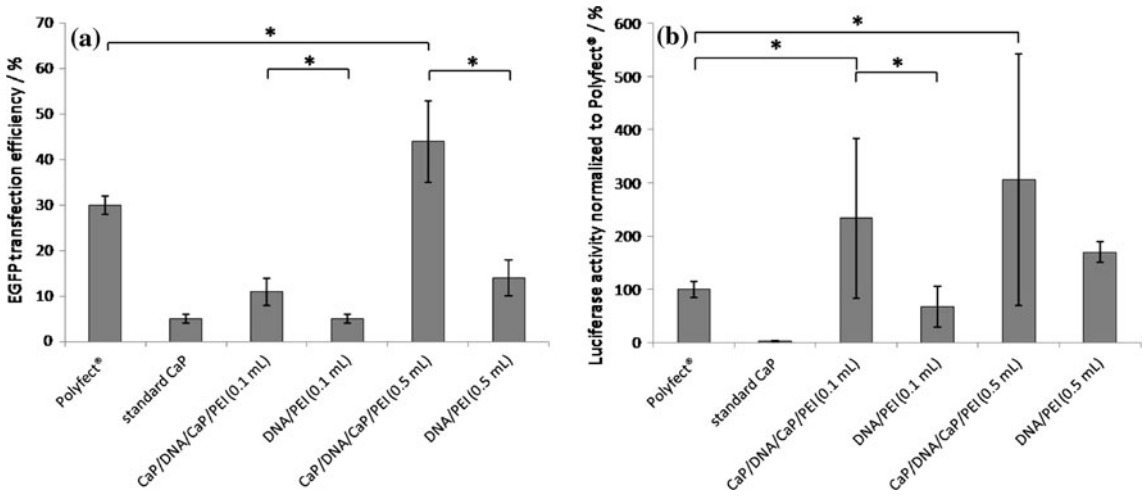

Fig. 3 Comparison of the transfection efficiency (**a**) and luciferase activity (**b**) of HeLa cells by different methods and two reporter systems (EGFP and luciferase). The luciferase activity of the standard calcium phosphate method, CaP/DNA/CaP/PEI, and DNA/PEI was normalized to activity obtained by Polyfect® (100%). The significance was estimated by student's *t*-test: * $p < 0.05$

Table 3 Results of the transfection experiments and the MTT cell viability test with T24 and NIH3T3 cells

Cell line	Method	EGFP transfection efficiency (%)	Cell viability (%)
T24	Polyfect®	10 ± 2	75 ± 10
	Standard calcium phosphate method	4 ± 1	85 ± 2
	CaP/DNA	5 ± 2	100 ± 10
	CaP/DNA/CaP/DNA	6 ± 3	105 ± 7
	CaP/DNA/CaP/PEI (0.1 mL)	22 ± 3	90 ± 2
	DNA/PEI (0.1 mL)	4 ± 1	84 ± 4
	CaP/DNA/CaP/PEI (0.5 mL)	–	0
	DNA/PEI (0.5 mL)	–	0
NIH3T3	Polyfect®	10 ± 5	42 ± 3
	Standard calcium phosphate method	4 ± 2	65 ± 6
	CaP/DNA/CaP/PEI (0.1 mL)	9 ± 4	96 ± 5
	DNA/PEI (0.1 mL)	2 ± 1	84 ± 5
	CaP/DNA/CaP/PEI (0.5 mL)	12 ± 3	24 ± 4
	DNA/PEI (0.5 mL)	5 ± 1	30 ± 5

The transfection efficiency is given as average ± standard deviation. For T24 cells with 0.5 mL PEI, all cells had died

Note that Polyfect® showed equal or even higher cytotoxicity than the nanoparticles with highest PEI content.

Figure 4 shows the cell morphology and EGFP fluorescence after transfection of three cell lines (HeLa, T24, and NIH3T3) with Polyfect® and CaP/DNA/CaP/PEI at the optimal concentration of PEI for every cell line. For T24 and NIH3T3, the best transfection efficiency was found at 0.1 mL of PEI, but at higher concentrations, the cell viability decreased (see Table 3).

Conclusions

Multi-shell nanoparticles with PEI (i.e., CaP core–DNA shell–CaP shell–PEI shell), showed a considerable increase in the transfection efficiency, compared to the nanoparticles without PEI. We have found two optimal concentrations of PEI depending on the cell type (0.5 mL for HeLa cells and 0.1 mL for T24 and NIH3T3 cells) for which the transfection efficiency was comparable to or even higher than Polyfect®.

DNA/PEI aggregates were used for cell transfection and also imaged during their pathway within cells [33]. In CaP/DNA/CaP/PEI dispersions, the nanoparticles are present as well as some free DNA/PEI aggregates, which may also contribute to the transfection efficiency. However, the fact that the DNA/PEI aggregates alone have a much lower efficiency than the nanoparticles demonstrates that the high transfection efficiency is due to the nanoparticulate system. The outermost layer of PEI on the CaP gave the necessary

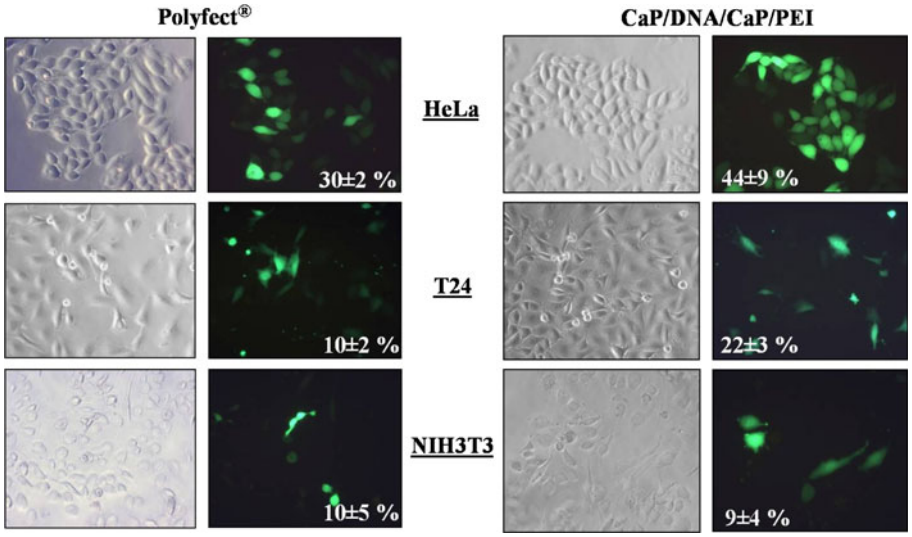

Fig. 4 Transmission light microscopy and EGFP fluorescence microscopy (magnification ×200 in all cases) of HeLa, T24, and NIH3T3 cells transfected with the commercial transfection agent Polyfect®, and with CaP/DNA/CAP/PEI nanoparticles (0.5 mL PEI for HeLa cells and 0.1 mL PEI for T24 and NIH3T3 cells). Transfected cells appeared green as a whole due to EGFP fluorescence. The increase in transfection efficiency from Polyfect® to CaP/DNA/CAP/PEI nanoparticles by HeLa and T24 cells is obvious

positive charge of nanoparticle surface, which is helpful for the penetration of nanoparticles through the negatively charged cell membrane. However, a compromise between high transfection efficiency and minimal cytotoxicity must be found for PEI as functionalizing agent.

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