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RESEARCH PAPER

PEGylation Improves Nanoparticle Formation and Transfection Efficiency of Messenger RNA

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ABSTRACT

Purpose Cationic polymers have been intensively investigated for plasmid-DNA (pDNA), but few studies addressed their use for messenger-RNA (mRNA) delivery. We analyzed two types of polymers, linear polyethylenimine (l-PEI) and poly-N,*N*-dimethylaminoethylmethacrylate P(DMAEMA), to highlight specific requirements for the design of mRNA delivery reagents. The effect of PEGylation was investigated using P(DMAEMA-*co*-OEGMA) copolymer.

Methods The influence of polymer structure on mRNA binding and particle formation was assessed in a side-by-side comparison with pDNA by methods such as agarose-retardation assay and scanning probe microscopy. Transfection studies were performed on bronchial epithelial cells.

Results Binding of cationic polymers inversely correlated with type of nucleic acid. Whereas P(DMAEMA) bound strongly to pDNA, only weak mRNA binding was observed, which was *vice versa* for l-PEI. Both polymers resulted in self-assembled nanoparticles forming pDNA complexes of irregular round shape; mRNA particles were significantly smaller and more distinct. Surprisingly, PEGylation improved mRNA binding and transfection efficiency contrary to observations made with pDNA. Co-transfections with free polymer improved mRNA transfection.

Conclusions Gene delivery requires tailor-made design for each type of nucleic acid. PEGylation influenced mRNA-polymer binding efficiency and transfection and may provide a method of further improving mRNA delivery.

KEY WORDS cationic polymers · gene delivery · mRNA

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INTRODUCTION

Gene therapy holds great promise for the treatment of numerous acquired and inherited diseases. Although encouraging proof-of-concept has been previously demonstrated for a variety of congenital diseases, including inherited severe combined immunodeficiencies such as X-SCID (1) and ADA-SCID (2) or inherited blinding diseases such as Leber's congenital amaurosis (LCA) (3), disadvantageous properties associated with the viral vectors used in these studies, including either insertional mutagenesis (4) or immunogenicity (5), remain to be overcome. As an alternative, nonviral vectors have been suggested in the field of gene therapy and are considered to minimize the risk of genomic integration or may alternatively allow more precise genomic integration by using site-specific recombinases (6–8). Moreover, they are regarded to be less immunogenic because of the lack of protein structures which could be recognized as foreign by the treated organism. However, one of the most serious limitations of

nonviral vectors is their low gene transfer efficiency. Among the reasons which have been identified to be responsible for this shortcoming are the immobility of plasmid DNA (pDNA) in the cytoplasm (9) and its inefficient transport from the cytoplasm into the nucleus due to the barrier function of the nuclear pore which precisely controls nuclear trafficking of macromolecular import and export (10). Indeed, it has been previously demonstrated that diffusion of pDNA is hardly observed in the cytoplasm and largely restricted by the filamentous actin network of the cell, and only a small fraction of less than 0.01–0.1% of pDNA delivered into the cytoplasm of mammalian cells is finally brought into the nucleus, which is the precondition for successful transgene expression and therefore any gene therapy approach (11, 12). Although intensive efforts have been undertaken to overcome this bottle neck, a general concept has not been proposed to improve pDNA mobility in the cytoplasm nor has its nuclear transport been yet convincingly advanced. In agreement with this obvious mechanistic limitation of nonviral gene vectors, it has been previously suggested to alternatively replace pDNA encoding for the therapeutic protein by messenger RNA (mRNA), which is the direct product of pDNA transcription (13). In contrast to pDNA, mRNA exerts its function in the cytoplasm, where it is translated into the corresponding protein at the ribosomes and therefore does not require nuclear transport. Besides this obvious mechanistic advantage, utilization of mRNA further avoids the risk of insertional mutagenesis and therefore excludes genotoxicity which may lead to tumor formation. Although these inherent benefits of mRNA are obvious, its utilization is thus far restricted to therapeutic applications which do not require long-term expression but where only high levels of short-term expression after single delivery or a few multiple applications is sufficient to cure the cellular defect or achieve the desired therapeutic effect. For this reason, as of yet, mRNA delivery has been intensively and almost exclusively investigated for tumor vaccination (14). Most frequently, mRNA encoding either single tumor-associated antigens or the entire cancer cell transcriptome are transfected into isolated patient dendritic cells *ex vivo*, which are subsequently used for vaccination of the patient (15). This concept has been intensively investigated not only in preclinical models but additionally in clinical trials, although no product has yet received marketing authorisation. Despite a report of successful mRNA expression in the human skin after intradermal administration (16), only very few studies investigated *in vivo* mRNA delivery, predominantly after intramuscular injection (17, 18). However, the therapeutic potential of mRNA application for transcript therapy has been demonstrated very recently by using chemically modified mRNA (19). These developments clearly indicate the wide-spread use of mRNA for therapeutic purposes. It is therefore even more surprising that so far only little attention has been paid to the understanding and optimization of mRNA delivery into cells. Indeed, the repertoire of mRNA delivery technologies that have yet been investigated is restricted to only a handful of different approaches. The by far most widely applied delivery technology is electroporation (20). Although electroporation yields high numbers of mRNA transfected cells, it is limited by severe cell damage and may therefore not represent the most beneficial technology. Among additional delivery technologies, the use of cationic lipids is most prominent, and only a few studies addressed other delivery techniques, including cationic peptides (21, 22), carbonate apatite-cationic liposome conjugates (23) and cationic polymers (21). In particular, the latter are hardly investigated for mRNA delivery, although they have been widely exploited with great success for delivery of other nucleic acids, such as pDNA, oligonucleotides and siRNA, and offer the opportunity to be tailored according to the demands of each of the nucleic acids, respectively. Against this background, we analyzed in more detail the influence of polymer structure on mRNA binding and particle formation to further correlate these parameters with transfection efficiency and allow a more comprehensive prediction of optimal polymer design for mRNA delivery in future. Furthermore, the parameters were analyzed in a side-by-side comparison with pDNA to highlight the specific requirements for the design of mRNA delivery reagents.

MATERIALS AND METHODS

Chemicals and Nucleic Acids

Heparan sulfate was obtained from Sigma-Aldrich (Schnelldorf, Germany). The plasmid pCMVLuc (6.2 kb) containing the *Photinus pyralis* luciferase gene under the control of the cytomegalovirus immediate early promotor (CMV) was kindly provided by Prof. E. Wagner (Department of Pharmacy, Ludwig Maximilians University, Munich, Germany).

The cDNA for pEGFPLuc (2.4 kb) was cloned into the pSTI-A120 vector which was generously provided by Prof. U. Sahin (Division of Experimental and Translational Oncology, Department of Internal Medicine III, Johannes Gutenberg University, Germany). The construct containing the pEGFpLuc cDNA was linearized with SapI enzyme. One µg of the linearized construct was used as a template for the *in vitro* transcription reaction using the Ambion T7 ARCA cap kit (Ambion, England). The quality and integrity of the mRNA

were confirmed by running a BioAnalyzer gel according to the manufacturer procedures using the RNA 6000 LabChip® kit. The kit includes an RNA ladder from Ambion to confirm the predicted size of the mRNA.

Cationic Polymers

Linear polyethylenimine (l-PEI) 22 kDa was synthesized according to Orgis *et al.* (24). After lyophilization the polymer was dissolved in double-distilled water, adjusted to pH 7.4 with hydrochloric acid (Merck Darmstadt, Germany) and dialyzed in double-distilled water (MWCO 8–10 kDa). After sterile filtration, the polymer was characterized via ^{1}H NMR, and concentration was adjusted via $CuSO_4$ assay (25). Molecular weights and molecular weight distributions were analyzed using GPC-MALLS and showed an average molecular weight of 20–22 kDa.

The P(DMAEMA-*co*-OEGMA) copolymer and the PDMAEMA homopolymer were synthesized via atom transfer radical polymerization (ATRP) and described as nonviral gene transfer agents elsewhere (26). For these copolymers, we selected the following nomenclature: P(DMAEMA$_n$-*co*-OEGMA(X)$_m$), where X represents the number of ethylene glycol (EG) units of the OEGMA monomer. The indexes *n* and *m* denote the average number of DMAEMA and OEGMA monomer per P(DMAEMA-*co*-OEGMA) copolymer, respectively. In this study a copolymer with X=9, m=10 and *n*=110 was used. A comparable PDMAEMA homopolymer with *n*=120 was synthesized.

Cell Lines

BEAS-2B (human bronchial epithelia cell line) was obtained from the ATCC (American Type Culture Collection, Wesel, Germany) to culture in minimum essential medium (MEM, Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Karlsruhe, Germany). For transfection efficiency studies, the cells were grown to 80% confluency at 37°C in a 5% CO_2 humidified air atmosphere and in the presence of 1% (v/v) penicillin/streptomycin (Gibco-BRL, Karlsruhe, Germany) for the study to investigate the influence of free polymer.

Peptide Synthesis

The N-terminal sequence of the influenza virus hemagglutinin subunit HA-2 (Influenza-peptide (INF) 7) GLFEAIEG FIENGWEGMIDGWYGC has been previously demonstrated to mediate endolysosomal release and was synthesized on a peptide synthesizer (Applied Biosystems 431A) following a standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protocol as described previously (27).

Preparation of Polymer-Plasmid DNA/mRNA Complexes

Stock solutions of P(DMAEMA)-*co*-OEGMA copolymer were prepared in double distilled water. For one well, nucleic acid solutions (pDNA or mRNA) and polymer stock solutions were diluted separately in 25 µl Hepes Buffered Saline (HBS, 150 mM NaCl, 10 mM Hepes, pH 7.4). The nucleic acid solution was added to the polymer solution and mixed gently by pipetting up and down 5–8 times, resulting in different N/P ratios (defined as the ratio between polymeric nitrogen residues and DNA phosphate groups) with a final pDNA and mRNA concentration of 20 µg/ml and 5 µg/ml, respectively. Subsequently, complexes were incubated for 20 min at room temperature prior to further use. Ternary complexes were generated in the same manner, but nucleic acid, INF7 and the polymer were diluted in HBS to 12.5 µl, 12.5 µl and 25 µl, respectively. The nucleic acid solution was pipetted to the INF7 solution and mixed vigorously. After incubation for 10 min at room temperature, the solution was added to the polymer solution and incubated at room temperature for 10 min before cell transfection.

As control for mRNA delivery, Lipofectamine (Lipofectamine™ 2000, Invitrogen, Germany) complexes were prepared according to the manufacturer's instructions using 1 µl Lipofecatmine diluted in HBS with 0.25 µg mRNA per well.

Size and Zeta Potential Measurements

Particle size and zeta potential measurements of the polymer-pDNA or polymer-mRNA complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). Complexes were prepared in HBS at N/P ratio of 10:1, 20:1 and 40:1 as described above. Complexes were incubated for 20 min and diluted 10-fold with 1 mM NaCl solution before measurement. Plasmid DNA and mRNA concentration was fixed at 10 µg/ml and 5 µg/ml after dilution, respectively. Measurements were performed at 25°C using the following settings: 10 to 30 subrun, viscosity (0.89 cP), refractive index (1.333), dielectric constant (78.2). Results are given as mean ± standard deviation of three measurements (*n*=3).

Atom Force Microscopy (AFM)

Polyplexes were prepared at an N/P ratio of 10 for l-PEI and 40 for P(DMAEMA) and P(DMAEMA-*co*-OEGMA) complexes as described above. Plasmid DNA polyplexes were prepared in HBS at a final pDNA concentration of 0.02 µg/µl, whereas mRNA polyplexes were prepared in water at a final

mRNA concentration of 0.01 μg/μl. After incubation of 20 min at room temperature, a drop of the complex solution was placed on freshly cleaved mica and dried using an air gun to avoid drying effects before measurement. The micrographs were all taken in tapping-mode™ using SiO_2 tips with $\nu = 170$ kHz and a spring constant k ~40 N/m (Anfatec, Oelsnitz, Germany) at a Bioscope equipped with a Nanoscope IV controller (Veeco Instruments, Mannheim, Germany).

Agarose Gel Retardation Assay

The extent of mRNA and pDNA condensation by the tested polymers was investigated by electrophoresis on a 1% agarose gel. Briefly, complexes formed at varying N/P ratios in HBS, as described above, were incubated with different amounts of heparan sulfate (HS) at room temperature for 45 min, mixed with 6 x loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and resolved by agarose gel electrophoresis loaded with 400 ng mRNA or 154 ng pDNA per well.

Transfection Experiments and Luciferase Activity Measurements

For transfection, 100,000 cells were seeded 24 h before transfection in each well of a 24-well plate (TPP, Trasadingen, Switzerland). Cells were rinsed with PBS (Gibco-BRL, Karlsruhe, Germany) immediately before transfection. Two hundred μl of fresh serum-free medium were added per well, and subsequently 50 μl of the gene vector complexes prepared, as described above, corresponding to 0.25 μg and 1 μg of mRNA and pDNA per well, respectively, were added. In order to further investigate the influence of free polymer on transfection efficiency, different amounts of free polymer (corresponding to the amount of polymer needed for N/P 10 and N/P 30 named as +10 and +30, respectively) were added to the serum-free medium prior to the addition of the gene vectors complexes at N/P 10. After 4 h of incubation at 37°C in a humidified 5% CO_2-containing atmosphere, the transfection medium was replaced with MEM including 10% FCS supplemented with 0.1% (v/v) penicillin/streptomycin and 0.5% (v/v) gentamycin (Gibco-BRL, Karlsruhe, Germany). Twenty four hours post-transfection, luciferase activity was measured in relative light units (RLU) using a Wallac Victor^2 1420 Multilabel Counter (Perkin Elmer, Boston, USA) or a FLUOstar microplate reader (BMG Labtech, Germany). Results were normalized to total cell protein using the BioRad Protein Assay (BioRad, Munich, Germany) and bovine serum albumin as protein standard.

Statistical Analysis

Results are presented as mean values ± standard deviation. Statistically significant differences were analyzed by a non-paired Student's *t*-test and preceding F-test. Probabilities of $p < 0.01$ (*) were considered as significant.

RESULTS AND DISCUSSION

Complexation of mRNA with Different Cationic Polymers

In a first set of experiments we analyzed the capacity of different subtypes of cationic polymers to complex mRNA by agarose gel retardation assay. We chose linear polyethylenimine (l-PEI) and poly (N,N-dimethylaminoethyl methacrylate) (P(DMAEMA), which both have been previously investigated by us and others to be used successfully for the delivery of pDNA and siRNA (26, 28, 29).

In addition, we investigated a copolymer of oligo (ethylene glycol) methyl ether methacrylate (OEGMA) and N,N-dimethylaminoethyl methacrylate (DMAEMA), which has been previously demonstrated by us to have excellent biocompatibility and transfection efficiency of pDNA (26).

Whereas l-PEI efficiently complexed mRNA at N/P ratios as low as $N/P = 1$ as indicated by the lack of mRNA migration into the agarose gel, mRNA retardation after complexation with P(DMAEMA) was only observed for N/P ratios >2. Interestingly, P(DMAEMA-*co*-OEGMA) copolymers fully retarded mRNA migration at $N/P = 2$, and partial mRNA complexation was observed at $N/P = 1$ as indicated by the reduced mRNA band intensity when compared with mRNA alone (Fig. 1a). These results demonstrate that l-PEI complexed mRNA more efficiently than P(DMAEMA), whereas P(DMAEMA-*co*-OEGMA) copolymers complexed mRNA with intermediate efficiency. Surprisingly, grafting of P(DMAEMA) with PEG side chains increased its capability of mRNA complexation. These results are contrary to observations which have been previously made for this polymer by us and others when PEGylated cationic polymers were used for complexation of pDNA. Commonly, PEGylation resulted in a decrease of pDNA binding. It has been argued that pDNA condensation is compromised in the presence of PEG moieties, which may adversely affect or even prevent interaction between the cationic moieties and pDNA due to an unfavorable entropy change.

To further asses this observation in more detail, we investigated the binding strength between each of the polymers with mRNA and pDNA by heparan sulphate

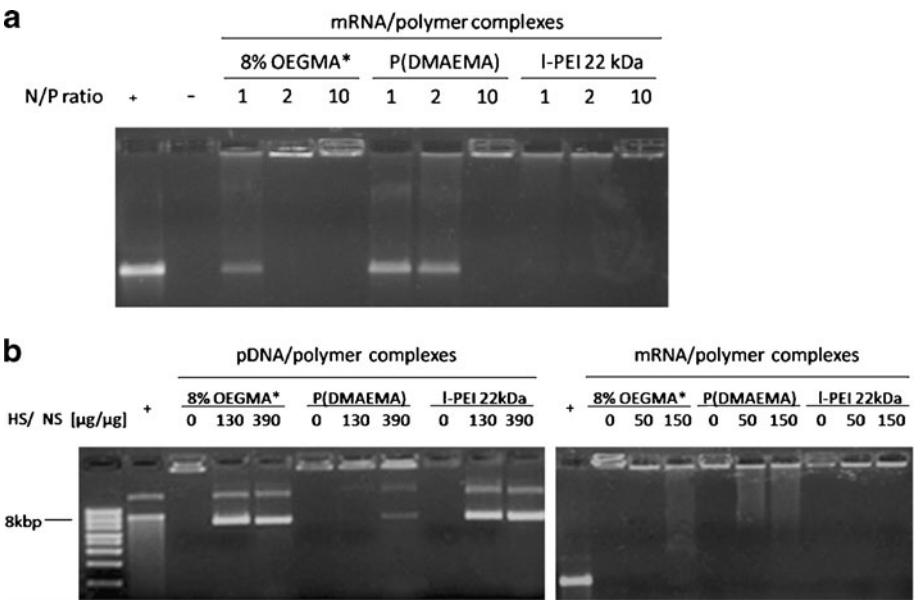

Fig. 1 Agarose gel electrophoresis of mRNA/polymer complexes. (**a**) mRNA condensation studies using different cationic polymers as a function of N/P ratio. (**b**) Binding and condensation properties of different polymers using mRNA or pDNA as nucleic acids. Complexes were incubated with increasing amounts of heparan sulfate (HS) per μg nucleic acid. Uncomplexed nucleic acid was used as a positive control (+) and HBS as negative control (−).

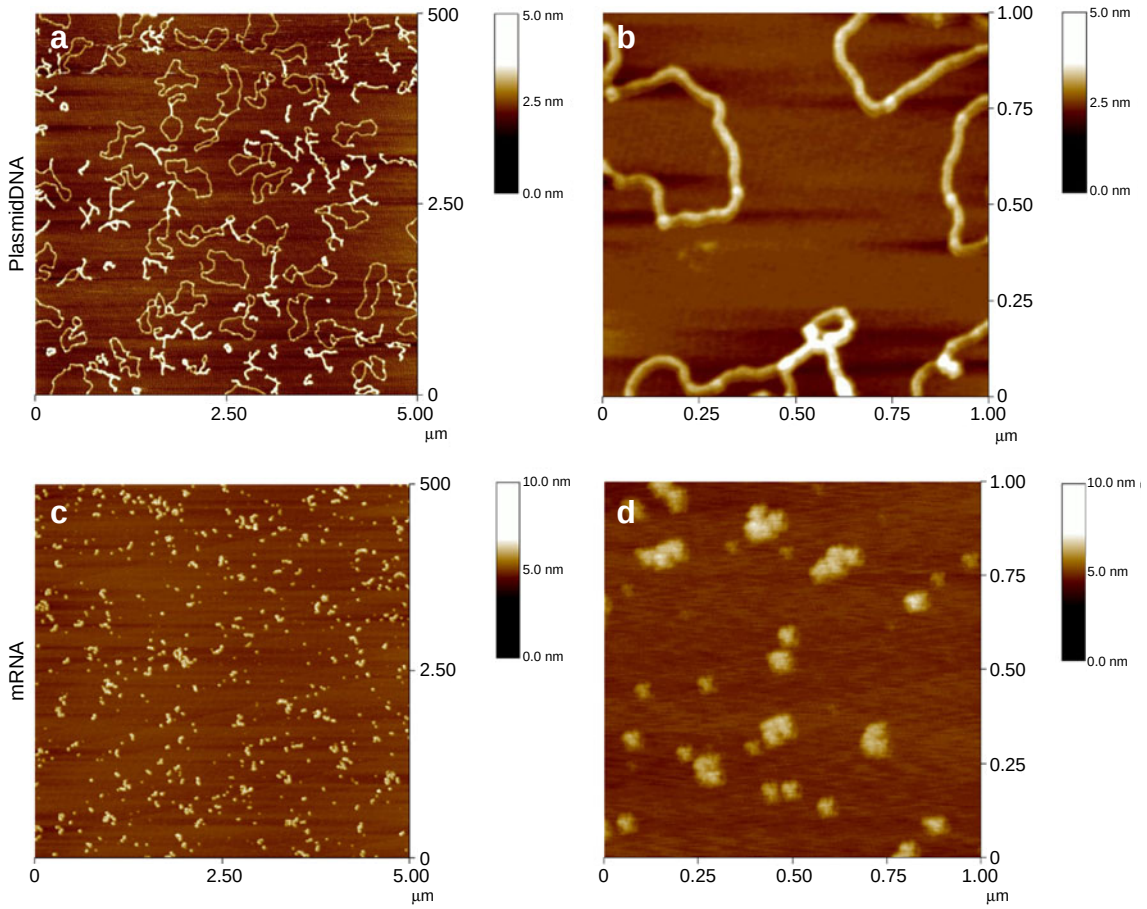

Fig. 2 Atom force microscopy analysis (AFM) of Plasmid DNA (**a**, **b**) and mRNA (**c**, **d**) in free solution. Mica was used as a support.

(HS) competition assay. Heparan sulphate is a negatively charged biopolymer which electrostatically competes with mRNA or pDNA for binding to the positively charged polymers. As has been previously observed, P(DMAEMA) bound to pDNA most strongly, which was indicated by the low amount of released pDNA even at high HS/pDNA ratio, whereas binding was strongly reduced for the P(DMAEMA-*co*-OEGMA) copolymers, and pDNA was more easily released from l-PEI complexes (Fig. 1b). Interestingly, when mRNA was complexed with the same polymers, the order of binding strength was reversed. Whereas mRNA could not be released from the l-PEI complexes even at high HS/mRNA = 150 (w/w) ratio, partial release was observed for P(DMAEMA) polymers at the same HS/mRNA ratio and even at lower HS/mRNA ratio of 50. As observed in the complexation assay from Fig. 1a, binding strength of P(DMAEMA-*co*-OEGMA) copolymers was of intermediate strength as indicated by the partial mRNA release at a HS/mRNA ratio of >150.

These observations illustrate that the structure of the cationic polymer affects binding affinity to mRNA. However, it is quite surprising that PEGylation influenced mRNA

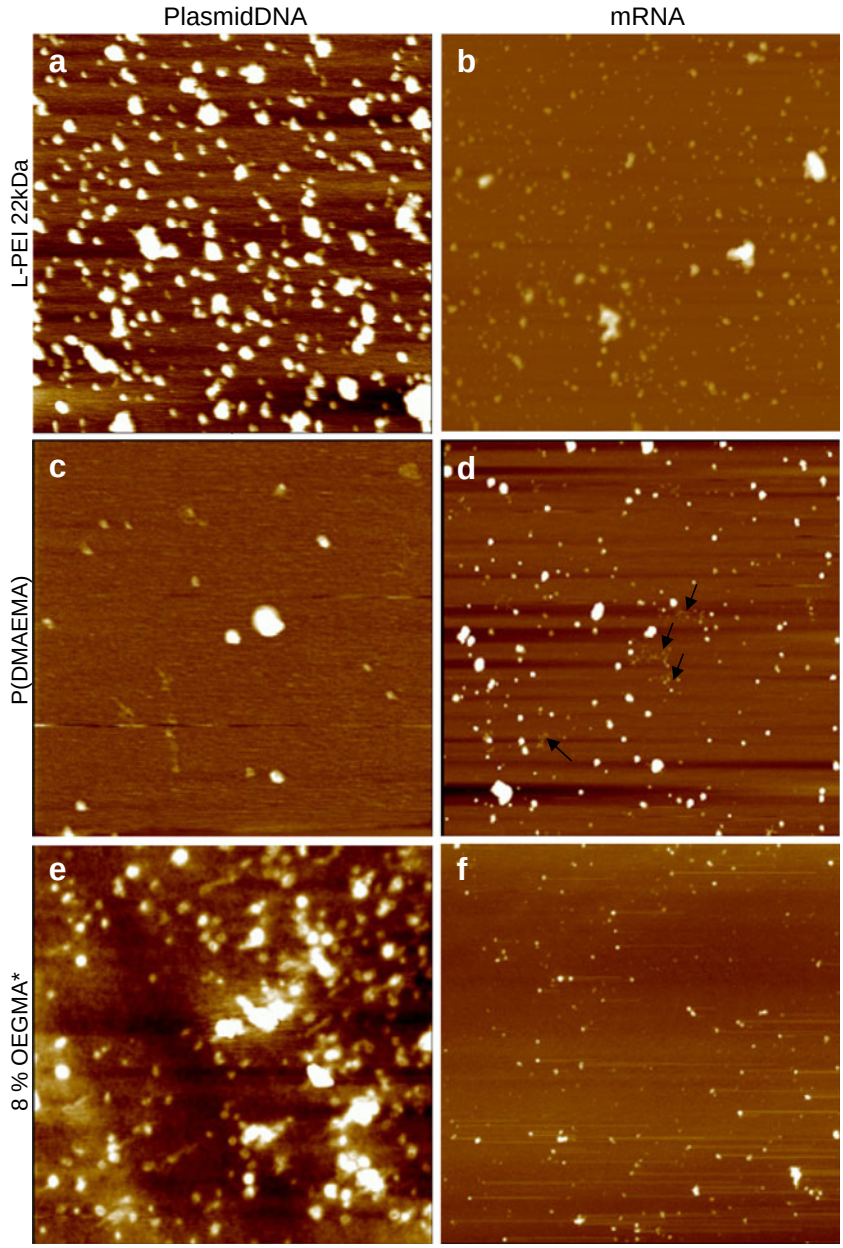

Fig. 3 Atomic force microscopy analysis (AFM) of gene vector complexes. Mica was used as support. Plasmid DNA/polymer and mRNA/polymer complexes were prepared in HBS and water, respectively. N/P ratio of 10 was used for l-PEI (**a**, **b**) and 20 for P(DMAEMA) (**c**, **d**) and P(DMAEMA-co-OEGMA) (**e**, **f**) copolymer (* 9 EG units in the polymer side chain). Images of pDNA/polymer and mRNA/polymer polyplexes (5×5 μm^2). Non-complexed mRNA was marked with *arrow*.

Table I Particle Size Diameter and Height Visualized by Scanning Probe Microscopy. An Area of 5 μm × 5 μm Was Analysed (Except mRNA/l-PEI: 1 μm × 1 μm). Results Are Presented as Means ± Standard Deviation. Plasmid DNA/Polymer and mRNA/Polymer Complexes Were Prepared in HBS and Water, Respectively. N/P Ratio of 10 Was Used for l-PEI and 20 for P(DMAEMA) and P(DMAEMA-co-OEGMA) Copolymer (* 9 EG Units in the Polymer Side Chain)

Particle size diameter (nm) and height (nm) of polyplexes using atomic force microscopy

		l-PEI	P(DMAEMA)	8% OEGMA*
mRNA	diameter (nm)	40 ± 19	40 ± 20	37 ± 15
	height (nm)	3.4 ± 1.6	1.5 ± 0.3	3.8 ± 1.6
pDNA	diameter (nm)	130 ± 72	107 ± 39	107 ± 58
	height (nm)	7.2 ± 2.9	3.8 ± 2.3	3.8 ± 2.6

binding when compared with its parental polymer, because from previous studies with pDNA one may have expected that PEGylation rather would have decreased mRNA affinity. Yet, the apparent effect of PEGylation on binding affinity is probably only indirect. Other physical-chemical parameters, such as particle size, surface charge, mRNA degradation and chain-stiffness, may play an important role here. Some of these aspects are investigated in the following paragraph.

Analysis of Cationic Polymer-mRNA/pDNA Complexes by Atomic Force Microscopy

The experiments from above showed that mRNA as well as pDNA binding to complexation was greatly dependent on the cationic polymer and nucleic acid under consideration. One may further expect that the differences in binding efficiency and strength would additionally affect the size and shape of the resulting particles. In order to address this question in more detail, we analyzed the resulting particles by Atomic Force Microscopy (AFM).

In agreement with previously published reports, naked pDNA appeared as large supercoiled and open circular macromolecules of a diameter of up to ~1 μm and a height of the circular helix of 4–5 nm (Fig. 2a and b). Double-stranded plasmids are quite stiff and can therefore easily be imaged using AFM (30, 31). In contrast, mRNA appeared as rather globular particles with a shape of fluffy clouds and a diameter of ~5–20 nm and a height of 6–10 nm (Fig. 2c and d). These images illustrate that mRNA does not appear as linear stretched macromolecule but adapts a strong secondary and tertiary structure free in solution as shown also previously (32).

Based on these observations it is quite obvious that pDNA and mRNA may behave distinctively different with respect to polymer binding and complexation. AFM analysis confirmed previous findings that each of the cationic polymers resulted in spontaneous self-assembled nanoparticles after mixing with pDNA. The particles appeared to be of irregular round shape with varying size (Fig. 3a, c and e). Whereas P(DMAEMA) and P(DMAEMA-*co*-OEGMA) copolymers condensed pDNA into nanoparticles of comparable average diameter of 107 ±39 nm and 107±58 nm and a height of 4±2 nm, respectively, l-PEI-pDNA complexes were found to be slightly larger in diameter and height (130±72 nm and 7 ±3 nm) (Table I). In contrast to this, complexes formed between each of the polymers and mRNA were significantly smaller in size with comparable diameters of 40±20 nm, 37 ±15 nm and 40±19 nm for P(DMAEMA), P(DMAEMA-*co*-OEGMA) copolymers and l-PEI, respectively (Table I). Whereas P(DMAEMA) condensed mRNA into single particles with predominantly round shape, some aggregation was additionally observed (Fig. 3d). Particles of mRNA P(DMAEMA-*co*-OEGMA) copolymers showed similar shape as observed for P(DMAEMA) but without obvious aggregation. These images clearly indicate that PEGylation favors the formation of monodisperse nanoparticles. P(DMAEMA-*co*-OEGMA) copolymers possibly lead to the formation of poly-ion complex micelles, which are stabilized in aqueous media by a hydrophilic PEG shell (33). This PEG outer-corona probably hampers nanoparticles aggregation. In contrast to the round shape of mRNA particles generated with P(DMAEMA) and P(DMAEMA-*co*-OEGMA) copolymers, l-PEI-mRNA particles predominantly appeared as either individual or clustered small rods (Fig. 3b).

Analysis of the Surface Charge of Cationic Polymer-mRNA/pDNA Complexes

Further, the surface charge of the particles was analyzed by zeta potential measurements. As observed previously, pDNA nanoparticles generated with p(DMAEMA) and l-PEI showed a positive surface charge of ~32 mV and ~38 mV at an N/P ratio above 10, respectively, whereas the surface charge of pDNA nanoparticles generated with P(DMAEMA-*co*-OEGMA) copolymers displayed a significantly lower zeta potential of ~21 mV (Table II). In comparison with pDNA nanoparticles, the surface charge of mRNA nanoparticles was slightly decreased to ~29 mV, ~32 mV and ~19 mV for

Table II Zeta Potential Analysis of Plasmid DNA/Polymer Complexes as a Function of N/P Ratio Tested. Gene Vectors Were Formed in Hepes Buffered Saline (c(pDNA) 100 μg/ml), Incubated for 20 min and Diluted 10-Fold with 1 mM NaCl Before Measurement. Results Represent the Mean ± Standard Deviation of Three Measurements with 10 to 30 Sub-Runs

Zetapotential (mV) of pDNA/polymer complexes			
N/P ratio	l-PEI	P(DMAEMA)	8% OEGMA*
10	38 ± 1	31 ± 1	21 ± 2
20	38 ± 1	34 ± 1	21 ± 1
40	39 ± 2	32 ± 0	21 ± 1

P(DMAEMA), l-PEI and P(DMAEMA-*co*-OEGMA) copolymers, respectively (Table III). The shielding effect of the PEG side-chains was still visible.

Transfection Efficiency of Cationic Polymer-mRNA Nanoparticles in Cell Culture

Next we analyzed the transfection efficiency of each of the mRNA nanoparticles on bronchial epithelial cells. For each of the tested cationic polymers, mRNA expression was dependent on the N/P ratio (Fig. 4a). Whereas for P(DMAEMA) and P(DMAEMA-*co*-OEGMA) copolymers mRNA expression increased with N/P ratio and was highest at N/*P*=40, mRNA expression mediated by l-PEI was highest at N/*P*=10 and progressively declined at higher N/P ratios, most likely due to cytotoxic effects of free polymer. The highest mRNA expression was observed for l-PEI followed by P(DMAEMA-*co*-OEGMA) copolymer and P(DMAEMA). In comparison with Lipofectamine™ 2000, which was used as standard for mRNA transfection, expression mediated by the P(DMAEMA-*co*-OEGMA) copolymer was at the same level, whereas l-PEI mediated 5-fold higher expression levels (Fig. 4b). Interestingly, we observed that PEGylation of P(DMAEMA) increased mRNA transfection 3-fold, whereas in a previous study the same PEGylation reduced pDNA transfection efficiency ~100-fold (26). In our previous study we observed that pDNA nanoparticles generated with the P(DMAEMA-*co*-OEGMA) copolymer were less efficiently released into the cytoplasm. We therefore speculated that PEGylation may have potentially improved endosomal release of the mRNA-P(DMAEMA-*co*-OEGMA) copolymer nanoparticles. To test this hypothesis we formulated P(DMAEMA-*co*-OEGMA) copolymer mRNA and DNA nanoparticles with the well-known endosome-disruptive peptide INF7 which has frequently been used to increase endosomal

Table III Zeta Potential Analysis of mRNA/Polymer Complexes as a Function of N/P Ratio Tested. Gene Vectors were Formed in Hepes Buffered Saline (c(mRNA) 50 μg/ml), Incubated for 20 min and Diluted 10-fold with 1 mM NaCl Before Measurement. Results Represent the Mean ± Standard Deviation of Three Measurements with 10 to 30 Sub-Runs

Zetapotential (mV) of mRNA/polymer complexes			
N/P ratio	l-PEI	P(DMAEMA)	8% OEGMA*
10	32 ± 2	29 ± 2	19 ± 1
20	29 ± 4	28 ± 1	19 ± 3
40	34 ± 2	28 ± 1	19 ± 2

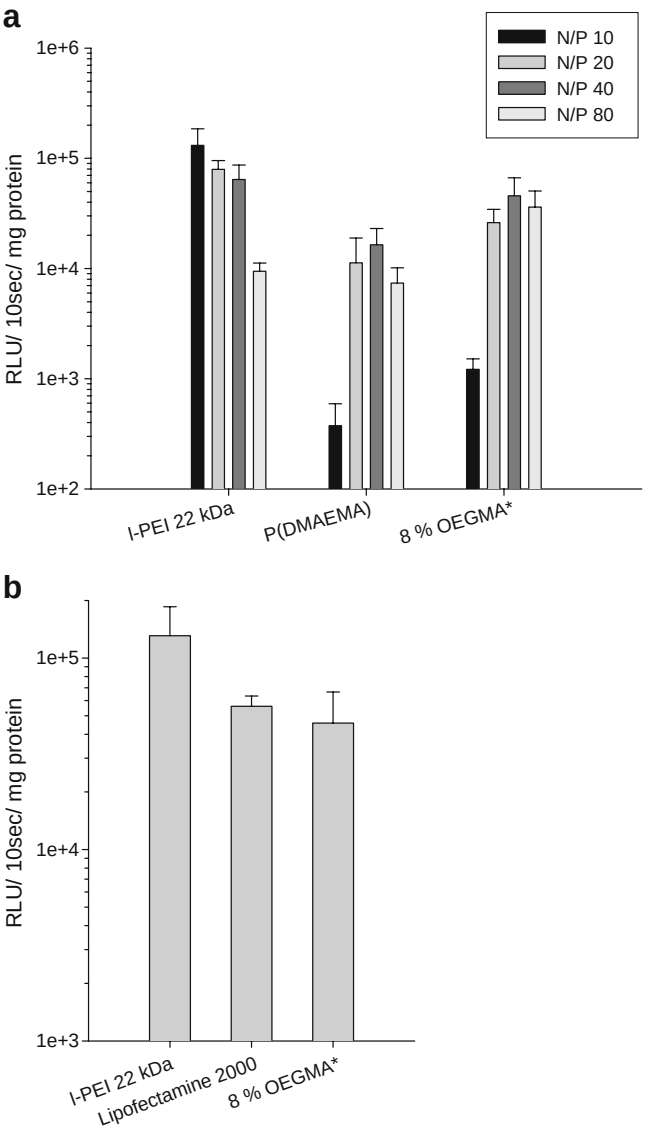

Fig. 4 (**a**) Transfection efficiency of mRNA complexes formed with P(DMAEMA) and PEGylated P(DMAEMA) copolymer at various N/P ratios in BEAS-2B cells. Linear PEI/mRNA complexes were used as positive control. The data are given as the mean ± S.D. ($n=4$). (**b**) Transfection efficiencies of mRNA/P(DMAEMA-*co*-OEGMA) copolymer (8% OEGMA, 9 EG units) gene vector complexes at N/P ratio of 40 and mRNA/l-PEI gene vector complexes at N/P ratio of 10 compared to mRNA/Lipofectamine™ 2000 complexes on BEAS-2B cells. The data are given as the mean ± S.D. ($n=4$).

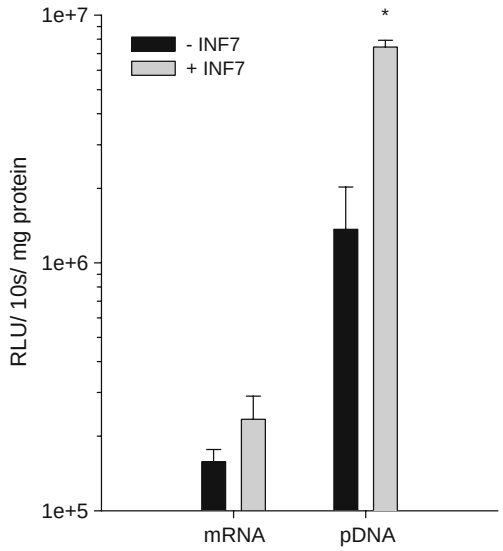

Fig. 5 Effect of lysosomal (INF7) peptide on transfection efficiency of P(DMAEMA-*co*-OEGMA) gene vector complexes on BEAS 2B cells. Plasmid DNA or mRNA polyplexes and ternary complexes were prepared with P(DMAEMA-*co*-OEGMA) copolymer (8% OEGMA and 9 EG units) at N/P ratio of 40 in HBS, respectively. The data are given as mean ± SD ($n = 3$). Statistically significant differences between corresponding samples are denoted with an *asterisk* ($p < 0.01$).

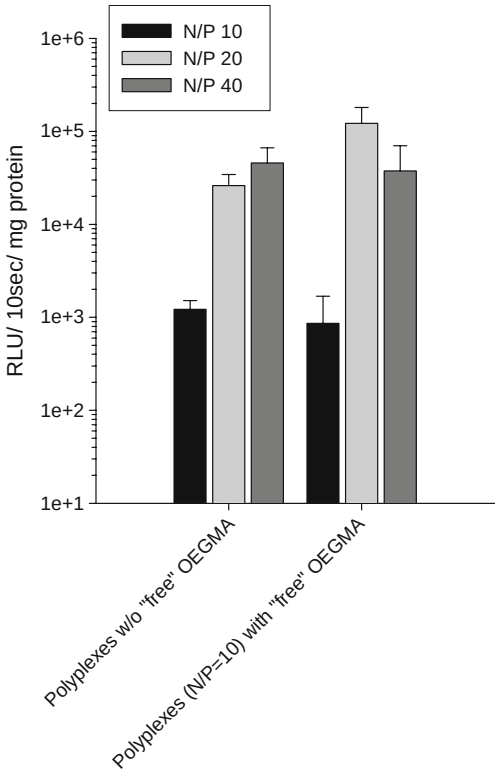

Fig. 6 Transfection efficiency of mRNA/P(DMAEMA-*co*-OEGMA) copolymer (8% OEGMA, 9 PEG units) gene vector complexes at various N/P ratios in the absence (Polyplexes w/o "free" OEGMA) and presence of free polymers (Polyplexes (N/*P* = 10) with "free" OEGMA) on BEAS-2B cells. In the presence of free polymers, N/P 20 and N/P 40 denote Polyplexes at N/P 10 in the presence of +10 and +30 free polymer, respectively. The data are given as the mean ± S.D. ($n = 4$).

release of nonviral gene vector nanoparticles (27). Whereas the INF7 peptide increased mRNA delivery only 1.4-fold, a strong 5.4-fold increase in pDNA delivery was observed (Fig. 5). These results suggest that the copolymers may support endosomal release of the mRNA particles into the cytoplasm. It has recently been demonstrated by Boeckle *et al.* that excess of free polymers, *e.g.* PEI, is present in the transfection solution after formation of polyplexes at high N/P ratios. Moreover, Boeckle *et al.* demonstrated that free PEI was critical for high transfection yields at low DNA concentrations (34). Against this background we performed additional transfection experiments by adding free copolymers to preformed polyplexes at N/*P*=10. As shown in Fig. 6, addition of free copolymer increased expression levels of polyplexes to the same level as observed for polyplexes generated at higher initial N/P ratio. Therefore, these results suggest that free polymer is largely responsible for efficient mRNA delivery. However, more comprehensive studies will be necessary to analyze its underlying mechanism in more detail in future.

CONCLUSION

In conclusion, our results revealed that binding of cationic polymers to high molecular weight nucleic acids such as pDNA and mRNA is largely dependent on structure. Indeed, our results demonstrated that binding behavior may be entirely reversed depending on the nucleic acid under consideration. The most remarkable observation was that PEGylation influenced mRNA binding and improved transfection efficiency. These findings might have not been predicted from previous studies, which were based on pDNA showing that PEGylation rather interfered with nucleic acid binding due to steric effects and therefore may have resulted in reduced transfection rates. Although the underlying mechanism has to be carefully studied in more detail in the future, our results reveal that tailor-made design of cationic polymers has to be considered individually for each type of nucleic acid to identify and generate optimized candidates for efficient delivery.

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