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Different Strategies for Formation of PEGylated EGF-Conjugated PEI/DNA Complexes for Targeted Gene Delivery

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With the aim of generating gene delivery systems for tumor targeting, we have synthesized a conjugate consisting of polyethylenimine (PEI) covalently modified with epidermal growth factor (EGF) peptides. Transfection efficiency of the conjugate was evaluated and compared to native PEI in three tumor cell lines: KB epidermoid carcinoma cells, CMT-93 rectum carcinoma cells, and Renca-EGFR renal carcinoma cells. Depending on the tumor cell line, incorporation of EGF resulted in an up to 300-fold increased transfection efficiency. This ligand-mediated enhancement and competition with free EGF strongly suggested uptake of the complexes through the EGF receptor-mediated endocytosis pathway. Shielded particles being crucial for systemic gene delivery, we studied the effect of covalent surface modification of EGF−PEI/DNA complexes with a poly(ethylene glycol) (PEG) derivative. An alternative way for the formation of PEGylated EGF-containing complexes was also evaluated where EGF was projected away from PEI/DNA core complexes through a PEG linker. Both strategies led to shielded particles still able to efficiently transfect tumor cells in a receptor-dependent fashion. These PEGylated EGF-containing complexes were 10- to 100-fold more efficient than PEGylated complexes without EGF.

INTRODUCTION

Cancer gene therapy by systemic gene delivery would be a most attractive way for treatment of cancer. Approaching the tumor via blood circulation should allow several advantages over the local intratumoral injection such as reaching multiple distant metastases. Unfortunately, the required tumor-targeted gene delivery system does not exist yet. An ideal gene delivery system has to fulfill several criteria to access tumor tissue: (1) ligands able to mediate cell specific recognition and internalization into target cells must be incorporated; (2) the gene delivery system must be inert against nonspecific interactions with the biological environment such as blood components and nontarget cells.

Synthetic gene delivery systems have recently attracted much attention particularly those based on polyethylenimine (PEI) polymers. Native PEI polymers of various molecular weights and topological isomers have been shown to be efficient for transfection of eukaryotic cells (*1*−*4*), and receptor-mediated endocytosis was achieved by chemical modification of PEI backbone with ligands such as sugars (*5*−*8*), peptides (*9*), and proteins (*10*). PEI polymers have also been used for gene delivery in vivo (*1*, *11*−*17*) and particularly for systemic delivery to the lung (*18*, *19*). However, unspecific interactions of the DNA complexes with blood components and nontarget cells strongly reduce the applicability of complexes. Recently, our group reported tumor-targeted gene delivery based on transferrin−PEI conjugate (*20*, *21*). Surface modification of transferrin-containing complexes with hydrophilic poly(ethylene glycol) (PEG) polymers strongly reduced interactions with blood components and prolonged circulation in blood after systemic delivery. Moreover, PEGylated transferrin-containing complexes mediated gene expression in distant tumor and showed reduced tropism for the lung compared to nonmodified PEI/DNA complexes.

Epithelial growth factor (EGF) receptor is an attractive therapeutic target for tumor targeting because it is overexpressed in a high percentage of human carcinomas including glioblastoma and lung, liver, breast, head, neck, and bladder cancers (*22*). The natural EGF ligand is a 53-residue polypeptide that binds specifically and with high affinity (*23*) to the EGF receptor. The binding of EGF triggers the dimerization of the receptor and the clustering of EGF−EGF receptor into coated pits that are internalized.

With the aim of specific gene delivery to EGF receptor-bearing tumor cells we synthesized a EGF−PEI conjugate and tested its ability to deliver DNA in three carcinoma cell lines. Keeping in mind future systemic gene delivery, the effect of PEGylation of preformed EGF-containing complexes was studied. We also describe an alternative novel way to generate PEGylated ligand-containing complexes where ligands are projected away from core complexes through a PEG linker.

EXPERIMENTAL PROCEDURES

Chemicals. Branched polyethylenimine with an average molecular weight of 25 kDa (PEI 25K) was obtained from Aldrich (Vienna, Austria). For complex preparation, PEI 25K was diluted with water, neutralized with HCl solution, and used as a 0.43 mg/mL working solution (10 mM of amine functions, assuming a MW of 43 Da for the repeating unit). Linear polyethylenimine

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(PEI 22K), kindly provided by Jean-Paul Behr (Faculté de Pharmacie, Illkirch, France) and commercially available from Euromedex (Exgen 500, Euromedex, Souffelweyersheim, France), was also used as a neutral 10 mM working solution. Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from Fluka (Buchs, Switzerland); chloroquine, 1,4-dithio-L-threitol (DTT), bovine serum albumin (BSA) from Sigma (St. Louis, MO); α-vinyl sulfone-ω-*N*-hydroxy succinimide ester poly-(ethylene glycol) (VS-PEG-NHS, MW 3400), α-maleimide-ω-*N*-hydroxysuccinimide ester poly(ethylene glycol) (MAL-PEG-NHS, MW 3400) from Shearwater Polymers (Huntsville, AL); mouse epidermal growth factor (mEGF) from Serotec (Oxford, England); cell culture media, antibiotics, foetal calf serum (FCS), Geneticin (G418-sulfate) from Life Technologies (Gaithersburg, MD). Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) described in Plank et al. (*24*) was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany).

Synthesis of Thiol-Functionalized PEI 25K. PEI 25K was diluted in water, neutralized with 32% aqueous hydrochloric and subjected to gel filtration as described previously (*10*). To 50 mg of PEI 25K (1.16 mmol of amine functions, quantified by ninhydrine assay at 570 nm (*25*)) in 758 μL was added 394 μL of a 20 mM ethanolic solution of SPDP (2.5 mg, 7.9 μmol). The reaction was carried out for 1 h at room temperature. Low molecular weight products were removed by gel filtration on a Sephadex G25 superfine (Pharmacia, Uppsala, Sweden; 300 × 10 mm column) in 0.25 M NaCl, 20 mM Hepes, pH 7.3. After dialysis and concentration under vacuum, a 720 μL solution containing 0.97 mmol of PEI monomer (41.5 mg) with 4.2 μmol of pyridyldithiopropionate linker (PDP; quantified spectrophotometrically at 343 nm by release of thiopyridone after reduction of an aliquot with excess DTT (*26*)) was obtained (i.e., ca. 2.5 PDP groups per PEI molecule). Half of this solution (20.7 mg PEI, 0.48 mmol of amine functions) was mixed under argon with an excess of DTT (73 μmol) and was left to react for 1 h at room temperature. For removal of the low molecular weight products an additional gel filtration on a Sephadex G25 superfine (0.25 M NaCl, 20 mM Hepes pH 7.3; 100 × 10 mm) was performed yielding 1.5 mL of thiol-functionalized PEI 25K that contained 242 μmol of PEI monomer (10.4 mg) modified with 1.25 μmol of thiol functions (quantified by Ellman assay at 412 nm (*27*)); i.e., ca. three thiol groups per PEI molecule.

Synthesis of PDP- and Thiol-Functionalized mEGF. A solution of 4 mg mEGF (0.66 μmol) in 1 mL of 16 mM Hepes buffer pH 7.9 was mixed with 3.1 mg of SPDP (10 μmol) in 0.5 mL of ethanol. The reaction and the purification steps (see below) were carried out in the presence of ethanol (20−50%) to avoid precipitation of the PDP-functionalized product. The reaction was left to react for 3 h at room temperature. The functionalized peptide was purified by either dialysis (MWCO 1000; Spectra Por, Spectrum, Houston, TX) against 50% aqueous ethanol or gel filtration on a Sephadex G10 (Pharmacia, Uppsala, Sweden; 100 × 10 mm column) in 20 mM Hepes pH 7.3/20% ethanol.

The purified conjugate contained about 0.9 PDP group per mEGF peptide (±10%, depending on the preparation).

Thiol-functionalized mEGF (mEGF-SH) was obtained by reduction of an aliquot of mEGF-PDP (at a concentration of about 50 μM) with DTT (1 mM) for 1 h under argon. Low molecular weight products were removed by gel filtration on a Sephadex G25M PD-10 column (Pharmacia, Uppsala, Sweden) in 20 mM Hepes pH 7.3/30% ethanol. Quantification of thiol functions with Ellman assay indicated that the conjugate contained about one thiol group per mEGF peptide (±15%).

Synthesis of mEGF-PEI 25K Conjugate. Two solutions of PDP-functionalized mEGF were pooled, and 2 mL of the resulting solution that contained 4.2 mg EGF (0.69 μmol) was modified with 0.56 μmol of pyridyldithiopropionate linker was mixed under argon with 7.5 mg of thiol-functionalized PEI 25K (174 μmol of amine functions, 0.9 μmol thiol functions) in 1.08 mL of 0.25 M NaCl, 20 mM Hepes, pH 7.9. After 1 day at room temperature, the reaction mixture was loaded on a cation-exchange column (Macro-Prep High S, BioRad, München, Germany; 100 × 10 mm column) and was fractionated with a salt gradient from 0.5 to 3.0 M sodium chloride with a constant content of 20 mM Hepes, pH 7.3. The major amount of conjugate eluted between 2.7 and 3.0 M salt and was pooled. After dialysis against HBS (MWCO 6000−8000; Gibco BRL, Gaithersburg, MD) and concentration under vacuum, 6.5 mL of mEGF−PEI solution was obtained that contained 148 μmol PEI of monomer (6.4 mg) modified with 0.32 μmol of mEGF peptide (2.0 mg); i.e., approximately 1.3 mEGF peptides per PEI molecule. Before use, EGF conjugate samples were diluted to 10 mM PEI monomer.

Formation of PEI/DNA, mEGF−PEI/DNA, and PEGylated mEGF−PEI/DNA Complexes. DNA complexes used for the transfection of KB and Renca-EGFR cells were prepared as described by Boussif et al. (*1*) with some modifications. Briefly, 4 μg of pCMVLuc plasmid (12 nmol of phosphate) and 7.2 μL of a 10 mM PEI or EGF−PEI (72 nmol of amine functions) were each diluted in 100 μL of the indicated buffer: HBS (Hepes buffered saline: 145 mM NaCl/20 mM Hepes pH 7.3), HBS 1/2 (HBS twice diluted with water) or 5 mM Hepes pH 7.3, and mixed together. After 15 min incubation an aliquot of the complex solution was added to the cells (100 μL/well for example corresponding to 2 μg DNA/well).

Complexes that were subsequently PEGylated were prepared in advance. Fifteen minutes after mixing DNA and polymer solutions, 0.6 μL of a 6 mM VS-PEG-NHS (or MAL-PEG-NHS) stock solution in DMSO (3.6 nmol) was added and left to react overnight.

For titration of different N/P ratios on CMT-93 cells mEGF−PEI/DNA or PEI/DNA complexes were prepared by mixing 5 μg of pCMVLuc plasmid, prediluted in 250 μL of HBS, with different amounts of mEGF−PEI 25K or PEI 25K prediluted in 250 μL of HBS (2.25 μg of PEI corresponding for example at N/P 3.6).

Sequential Addition of Bifunctional PEG and mEGF−SH to PEI/DNA Complexes. pCMVLuc plasmid (20 μg, 60 nmol of phosphate) and PEI (360 nmol of amine) were each diluted in 0.5 mL of the indicated buffer previously saturated with argon. The two solutions were gathered and mixed with argon stream. The mixture was kept under argon until the end of the protocol. After 15 min, 3 μL of a 6 mM VS-PEG-NHS (or MAL-PEG-NHS) stock solution in DMSO (18 nmol) were added. Two hours later, various amounts of EGF-SH (between 7.2 and 144 pmol; as indicated in the Figure legend) were added to 200 μL aliquots of the complex solution and left to react overnight.

Cells and Cell Culture. KB human epidermoid carcinoma cells (ATCC CCL-17) and CMT-93 murine rectum carcinoma cells (ATCC CCL-223) were cultured in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/mL streptomycin. Renca mouse renal carcinoma cells stably cotransfected with the plasmids pLTR-EGFR and pSV2neo (*28*)

(kindly provided by Winfried Wels, Georg-Speyer-Haus, Frankfurt am Main, Germany), named Renca-EGFR, were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 mg/mL Geneticin. All cell lines were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Transfection. One day before transfection, KB and Renca-EGFR cells were seeded at 4×10^4 cells per well in 24-well dishes. Prior to transfection, medium was removed and replaced with 1 mL (or as indicated in Figure legend) of fresh culture medium complemented with 10% FCS. Transfection complexes were added to the cells, and after 4 h incubation, transfection medium was removed and replaced by 1 mL fresh culture medium.

Murine CMT-93 cells were seeded at 5×10^5 cells per 25 cm^2 tissue culture flask 1 day before transfection. Prior to transfection, medium was removed and replaced by 2 mL of fresh culture medium complemented with 10% FCS. Transfection complexes (in 0.5 mL of HBS) were added to the cells. After 4 h incubation, transfection medium was removed and replaced by fresh culture medium.

The cells were grown for additional 20 h and then washed in PBS and lysed (0.25 M Tris·HCl pH 7.4, 0.1% Triton X-100). Luciferase activity and protein concentration were determined from aliquot of the lysate using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) and Bio-Rad protein assay (Bio-Rad, Hercules, CA), respectively. Values are given as light units integrated over 10 s per mg of protein.

RESULTS

Synthesis of mEGF−PEI 25K Conjugate. mEGF was covalently coupled to the PEI backbone through a disulfide bridge as described in Experimental Procedures. Murine EGF can bind both the murine and human EGF receptor (*29*) and was chosen because it lacks lysine residues in its primary sequence and can react with SPDP-activated ester only through the N-terminus. It has been shown that the derivatization of this free amino group had little effect on EGF receptor binding compared to native EGF (*30*). Modification of this amino group with the heterobifunctional cross-linker SPDP (*26*) rendered the peptide poorly soluble, and ethanol had to be present in the reaction and purification steps. The reaction was carried out with an excess of SPDP (15 molar equiv excess) and led to a mEGF−PDP conjugate containing almost one linker per peptide. PEI with an average molecular weight of 25 kDa was also modified with SPDP and then treated with an excess of DTT. A thiol-functionalized PEI conjugate that contained approximately three thiol groups per PEI molecule was obtained. Reaction between thiol-functionalized PEI and mEGF−PDP followed by purification by cation-exchange chromatography resulted in a mEGF−PEI conjugate that contained 1.3 mEGF peptides per PEI molecule.

EGF Receptor-Mediated Gene Transfer in Various Carcinoma Cell Lines. Transfection efficiency of mEGF−PEI 25K-containing DNA complexes was compared with EGF-free PEI 25K/DNA complexes in three carcinoma cell lines (Figure 1). Formulation of complexes in three different Hepes buffers containing different concentrations of NaCl (Hepes: 0 mM, HBS 1/2: 75 mM and HBS: 150 mM) at a PEI-nitrogen to DNA-phosphate ratio of 6 (N/P = 6) resulted in complexes of various sizes (Table 1) which were tested in transfection experiments with Renca-EGFR cells (Figure 1A) and KB cells (Figure

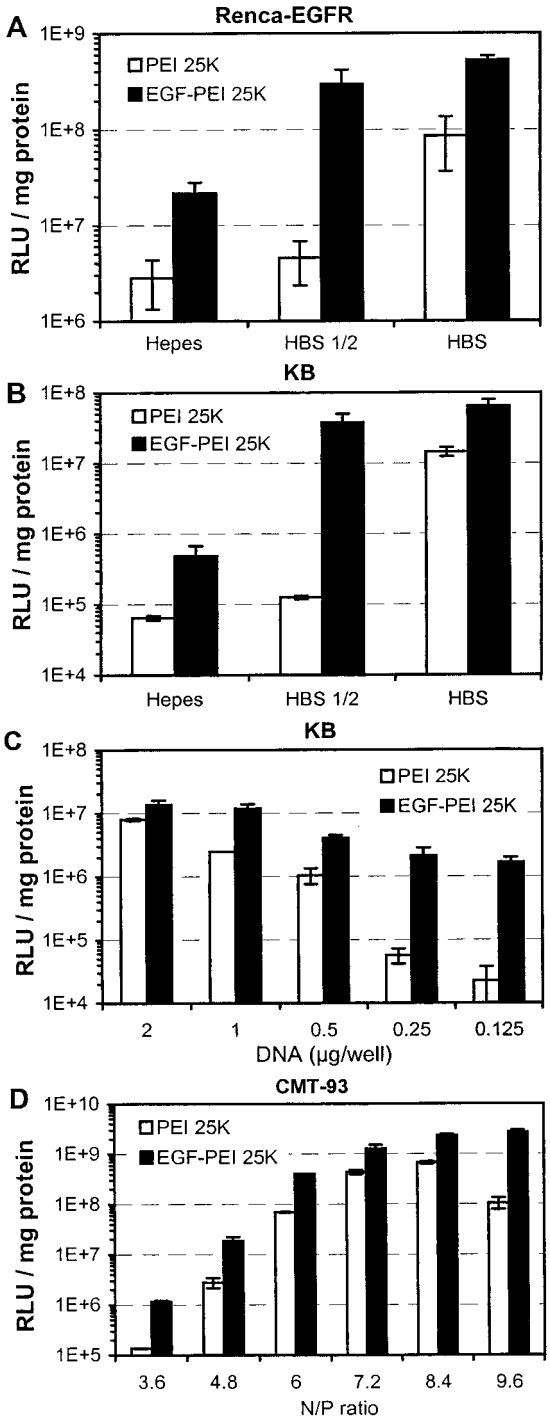

Figure 1. EGF-mediated gene transfer to Renca-EGFR mouse renal, KB human epidermoid and CMT-93 murine rectum carcinoma cells. Renca-EGFR (A) and KB cells (B) were transfected with pCMVLuc plasmid (2 μg/well) complexed with PEI 25K or mEGF−PEI 25K/PEI 25K (1:1) at a PEI (nitrogen)/DNA (phosphate) ratio of 6 (N/P = 6) in either 5 mM Hepes pH 7.3, HBS 1/2 or HBS. After 4 h, transfection medium was replaced with fresh culture medium and cells were grown for additional 20 h. Luciferase activity was measured as described in Experimental Procedures. Values are the mean ± SD of two independent experiments made in duplicate. (C) KB cells were transfected with various amounts of pCMVLuc plasmid (2 to 0.125 μg per well) complexed with PEI 25K or mEGF−PEI 25K/PEI 25K (1:1) at N/P = 6 in HBS. Luciferase activity is the mean ± SD of two independent experiments made in duplicate. (**D**) CMT-93 cells were transfected with pCMVLuc plasmid (5μg/flask) complexed with various amounts of PEI 25K or EGF−PEI 25K (at indicated N/P ratios) in HBS. Luciferase activity is shown as mean ± SD of duplicate.

Table 1. Biophysical Characteristics of DNA Complexes and Their PEGylated Derivatives[a]

	size (nm)			
	Hepes	HBS 1/2	HBS	zeta (mV) Hepes
PEI 25K	57 ± 14	116 ± 36	478 ± 111	+20.0 ± 1.7
PEI 25K + MAL-PEG-NHS	47 ± 11	96 ± 30	446 ± 118	−0.7 ± 1.1
PEI 25K + MAL-PEG-NHS + EGF-SH	49 ± 15	81 ± 23	403 ± 118	−0.6 ± 1.4
EGF-PEI 25K/PEI 25K	53 ± 12	447 ± 82	590 ± 132	+21.5 ± 2.3
EGF-PEI 25K/PEI 25K + MAL-PEG-NHS	48 ± 14	553 ± 128	> 1 μm	−0.6 ± 1.0
PEI 25K/PEI 22K	43 ± 11	~ 1 μm	~ 1 μm	+28.0 ± 2.5
PEI 25K/PEI 22K + MAL-PEG-NHS	42 ± 10	> 1 μm	> 1 μm	−0.1 ± 1.4

[a] Complexes were prepared as described in Experimental Procedures in 1 mL total volume of the indicated buffer containing 20 μg DNA (for zeta potential measurement of PEI 25K/PEI 22K/DNA complexes DNA concentration was 50 μg/mL). Size and zeta potential of non-PEGylated and PEGylated complexes were measured 20 min and 18 h after their formation respectively, using a Zetasizer 3000HS (Malvern Instruments, Worcestershire, England). Three measurements were carried out per sample.

1B). EGF-containing DNA complexes showed a 5- to 300-fold increase in gene delivery efficiency compared to unmodified PEI/DNA complexes. This ligand-mediated enhancement was found for all formulations. Large complexes formulated in physiological salt concentration (HBS) were more efficient than small complexes prepared in salt-free medium (5 mM Hepes pH 7.3), similar as observed previously with other cell lines using transferrin-modified PEI/DNA complexes (*31*). Chemical ligation of mEGF to the PEI 25K backbone did not change the biophysical properties (size, zeta potential) of resulting complexes generated either in Hepes or HBS buffer (Table 1). This strongly suggests the expected biological involvement of EGF in the increase of gene expression. In contrast, in HBS 1/2, the difference of size between both type of complexes might also contribute to the improved gene expression.

Comparative transfection was also investigated with sub-micromolar amounts of DNA on KB cells (Figure 1C). Luciferase expression of PEI complexes generated in HBS dropped rapidly with the decrease of DNA amount while expression of EGF−PEI complexes remained relatively high.

Luciferase gene expression was examined as a function of the PEI-nitrogen to DNA-phosphate ratio used to generate the complexes. Gene expression in CMT-93 murine rectum carcinoma cells was strongly dependent on the N/P ratio (Figure 1D). Increasing the N/P ratio from 3.6 to 8.4 increased the efficiency of both EGF-containing and EGF-free DNA complexes by more than three orders of magnitude. There was a 3- to 30-fold ligand-mediated enhancement at all N/P ratios tested. With ligand-free complexes a drop in transfection efficacy was found at high N/P ratio correlating with some toxicity.

Small particles generated in salt-free medium led to low gene expression in comparison with large particles generated in HBS (Figures 1A and 1B). We tested whether the addition of an endosomolytic compound during the transfection could enhance the efficiency of small complexes. Therefore, the lysomotropic drug chloroquine was added during transfection in cell culture medium of Renca-EGFR cells at a concentration of 100 and 200 μM (Figure 2A). Addition of chloroquine resulted in a 100-fold increase of gene expression with both EGF-containing DNA complexes and EGF-free DNA complexes while the ligand-mediated enhancement remained approximately constant.

To test whether the EGF receptor was involved in EGF−PEI-mediated gene transfer, a competition assay was performed using small complexes formulated in salt-free medium. Addition of exogenous mEGF in cell culture medium during transfection of Renca-EGFR did not interfere with PEI/DNA receptor-independent gene transfer (Figure 2B). However, competition of EGF-containing DNA complexes with free mEGF (100 molar equiv excess) decreased gene transfer efficacy down to nearly the values found for the receptor-independent transfection. On the other hand, addition of BSA, a protein that does not interact with EGFR, did not significantly affected gene transfer efficiency of both type of complexes. Taken together, competitive and comparative transfection ex-

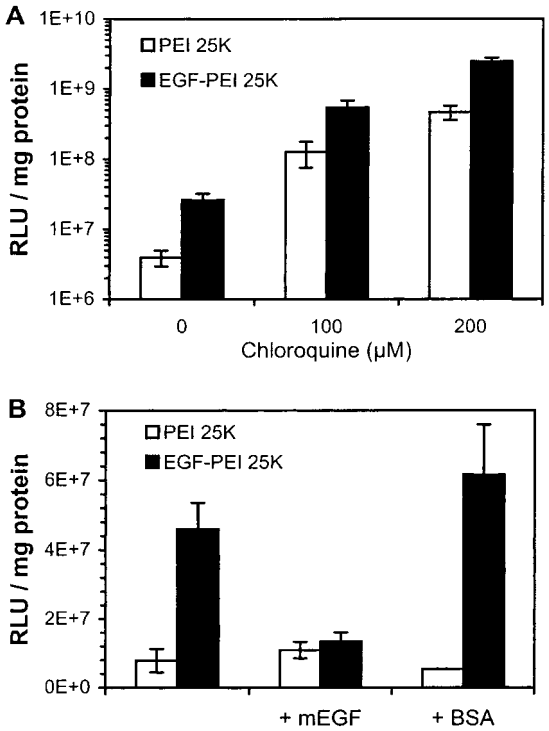

Figure 2. (A) Chloroquine strongly enhances transfection efficiency of small particles generated in salt free medium on Renca-EGFR cells. Renca-EGFR were transfected with pCMVLuc plasmid (2 μg/well) complexed with PEI 25K or mEGF−PEI 25K/PEI 25K (1:1) at N/P = 6 in Hepes. Transfection was carried out in the presence of 0, 100, or 200 μM chloroquine. After 4 h, transfection medium was replaced with fresh culture medium and cells were grown for additional 20 h. Luciferase activity values are the mean ± SD of two independent experiments made in duplicate. (B) Competitive inhibition of EGF receptor-mediated gene transfer on Renca-EGFR cells. Renca-EGFR cells incubated in 0.5 mL of fresh culture medium containing 200 μM chloroquine were transfected with pCMVLuc plasmid (1 μg/well) complexed with PEI 25K or mEGF−PEI 25K/PEI 25K (1:1) at N/P = 6 in Hepes. For the inhibition and the control experiments transfection was carried out in the presence of free mEGF (12 μg/well) and BSA (12 μg/well), respectively. After 4 h, transfection medium was replaced with fresh medium and cells were grown for additional 20 h. Luciferase activity is shown as mean ± SD of duplicate.

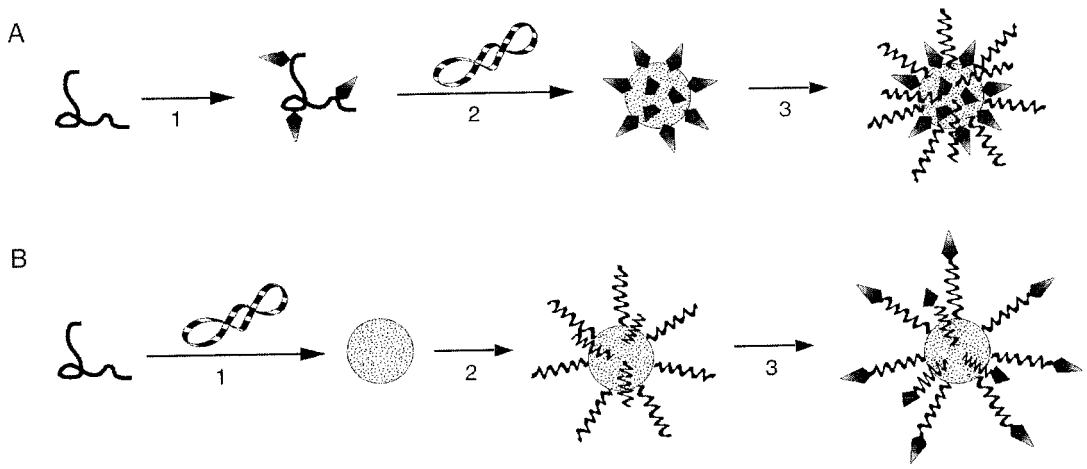

Figure 3. Schematic presentation of alternatives for the formation of PEGylated ligand-containing DNA complexes. Strategy A: A ligand is covalently conjugated to a polycation (step 1). Condensation of DNA with this conjugate leads to ligand-containing DNA complexes (step 2) which are subsequently modified with a PEG derivative reacting with free amino groups of the polycation (step 3). Strategy B: DNA is condensed with a polycation (step 1), and the resulting complexes are modified by a heterobifunctional PEG which first reacts with amino groups of the polycation (step 2). Subsequently ligands are incorporated into the complexes by covalent conjugation with the distal end of the PEG (step 3). The latter strategy should allow a better accessibility of the grafted ligand for its receptor.

periments strongly suggested involvement of EGF receptor in gene delivery with EGF−PEI-based systems.

Transfection Efficiency of PEGylated EGF-Containing DNA Complexes. Targeted transfection particles for systemic gene delivery should have uncharged surface to avoid nonspecific electrostatic interaction with blood components and nontarget cells. On the other hand, DNA is usually efficiently condensed using an excess of polymer, giving rise to cationic particles. One way to overcome this dilemma is to covalently modify the surface of ligand-containing DNA complexes with an electroneutral polymer such PEG (*20*) (Figure 3, strategy A). Coating the DNA particle with PEG shields its cationic surface and prevents interaction with macromolecules by steric hindrance. On the other hand PEG potentially might also shield small ligands bound to the polymer and thus preventing to some extent the binding to the receptor on the target cell. As described in strategy B (Figure 3), incorporation of the ligand at the distal end of PEGylated PEI/DNA complexes should allow better accessibility of the ligand for its receptor and thus should increase cellular internalization of DNA complexes.

These two strategies using EGF as ligand were evaluated with Renca-EGFR cells (Renca mouse renal carcinoma cells stably transfected with pLTR-EGFR plasmid carrying human EGF receptor cDNA (*28*)) and KB cells (human epidermoid carcinoma cells that endogenously express human EGF receptor). Transfection of Renca-EGFR was performed in the presence of chloroquine with small complexes formulated in salt free medium. It was previously observed that addition of chloroquine on Renca-EGFR cells increases considerably PEI-mediated gene transfection (Figure 2A), in contrast to KB cells where no beneficial effect is observed. PEGylation of PEI/DNA complexes dramatically decreased the zeta potential (which reflects the surface charge) close to the neutrality (Table 1) and was accompanied by a reduction of gene expression (Figure 4A). Addition of increasing amounts of thiol-functionalized mEGF to PEGylated complexes without changing their biophysical properties (size, zeta potential) increased gene transfection by two-orders of magnitude. In contrast, addition of the functionalized ligand to PEI/DNA complexes without reactive PEG linker had no effect. This finding strongly suggested that EGF binding at the distal end of the PEG allows the delivery of PEGylated complexes in a ligand-dependent manner. We compared this strategy with the one previously developed in our group (*20*) (Figure 3, strategy A). As previously observed, EGF−PEI conjugate-containing DNA complexes were more efficient than those prepared with native PEI 25K. PEGylation of EGF-containing DNA complexes decreased the zeta potential close to the neutrality (Table 1) and also gene expression. This PEGylated complexes were still about 5-fold more effective than EGF-free PEI/DNA complexes but about one order less efficient than PEGylated complexes obtained according to strategy B (Figure 3).

The two strategies were also used for the delivery of PEGylated EGF-containing DNA complexes in KB cells in the absence of chloroquine (Figure 4B). Subsequent addition of PEG and thiol-functionalized EGF to PEI/DNA complexes generated in HBS 1/2 increased their efficiency by more than one order of magnitude. Complexes resulting from the PEGylation of EGF−PEI conjugate-containing DNA complexes showed approximately the same level of gene expression.

Transfection with Different PEI Polymers Complexes and Their PEGylated Derivatives. Among all available PEI, which differ by their average molecular weight and topology, linear PEI 22K had shown the most promising properties to deliver DNA (*4, 18*). We were interested to know whether the combination of PEI 22K with mEGF−PEI 25K could enhance the ligand-mediated gene delivery in our in vitro cell models. KB cells were transfected with various amount of DNA complexed with PEI 25K, EGF−PEI 25K/PEI 25K (1:1), PEI 25K/PEI 22K (1:1), or EGF−PEI 25K/PEI 22K (1:1) in HBS 1/2 at N/P = 6 (Figure 5A). Incorporation of PEI 22K in the preparation of ligand and ligand-free PEI 25K/DNA complexes strongly increased gene expression especially at low DNA amounts. At 60 ng of DNA, PEI 22K-containing complexes remained efficient (10^7−10^8 RLU/mg) whereas PEI 22K-free complexes showed only low activity. Interestingly, a ligand-dependent enhancement was observed with PEI 22K-containing complexes on KB cells but also on Renca-EGFR cells independently of the medium in which the complexes were prepared (Figure 5B).

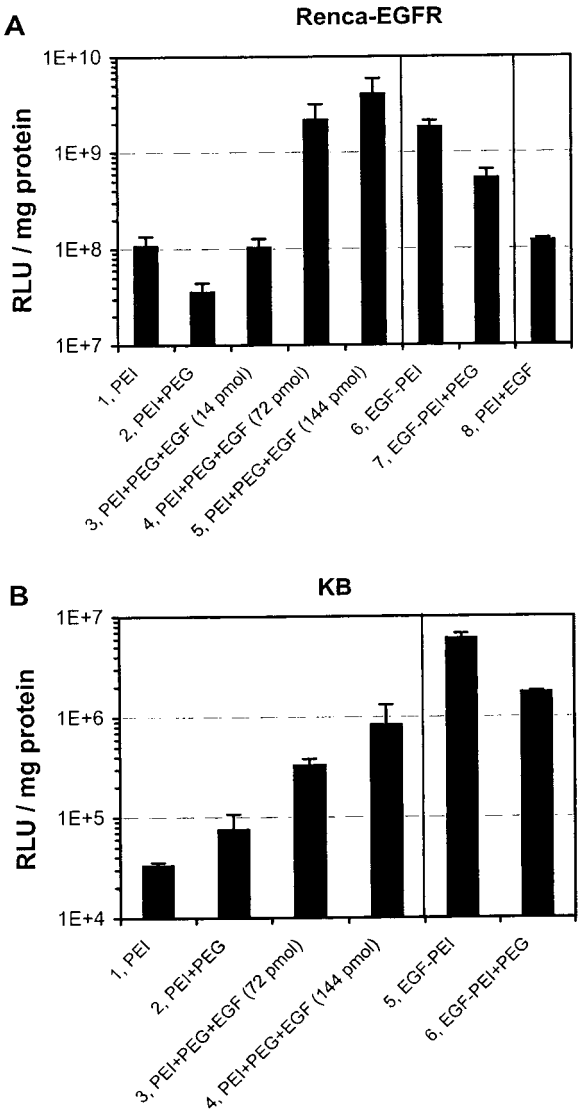

Figure 4. Transfection efficiency of PEGylated EGF-containing DNA complexes on Renca-EGFR and KB cells. (A) Renca-EGFR cells incubated in fresh culture medium containing 200 μM chloroquine were transfected with the following complexes generated as described in Experimental Procedures in Hepes buffer at a N/P ratio of 6 (2 μg DNA/well). Column 1: PEI 25K/DNA; 2: PEI 25K/DNA modified with VS−PEG-NHS; 3, 4, and 5: as in 2 and subsequently modified with 14, 72, and 144 pmol of thiol-functionalized mEGF, respectively; 6: mEGF−PEI 25K/PEI 25K (1:1)/DNA; 7: mEGF−PEI 25K/PEI 25K (1:1)/DNA modified with VS−PEG-NHS; 8: as in 1 in the presence of 144 pmol mEGF in culture medium during the transfection. Luciferase activity values are the mean ± SD of two independent experiments made in duplicate. (B) KB cells incubated in fresh culture medium were transfected with the following complexes prepared in HBS 1/2 at a N/P ratio of 6 (2 μg DNA/well). Column 1: PEI 25K/DNA; 2: PEI 25K/DNA modified with MAL-PEG-NHS; 3 and 4: as in 2 and subsequently modified with 72 pmol and 144 pmol of thiol-functionalized mEGF, respectively; 5: EGF−PEI 25K/PEI 25K (1:1)/DNA; 6: EGF−PEI 25K/PEI 25K (1:1)/DNA modified with MAL-PEG-NHS. Luciferase activity is shown as mean ± SD of duplicate.

Transfection efficiency of PEGylated EGF-containing PEI 25K/PEI 22K complexes was studied in KB cells (Figure 6). As observed previously with PEI 22K-free complexes (Figure 4B), subsequent addition of PEG and thiol-functionalized EGF to PEI/DNA complexes strongly increased transfection efficiency to a level comparable to the one obtained by PEGylation of EGF-containing DNA complexes.

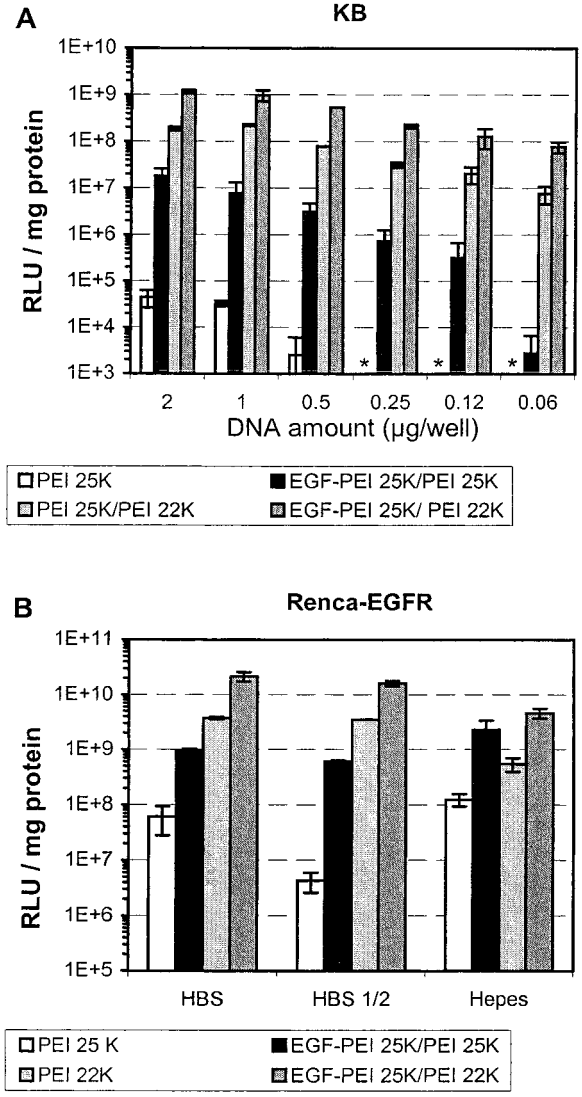

Figure 5. Effect of different PEI polymers (linear PEI 22K versus branched PEI 25K) on transfection efficiency. Complexes were generated from a mixture of mEGF−PEI 25K and different PEIs and tested on KB and Renca-EGFR cells. (A) KB cells were transfected with various amounts of pCMVLuc plasmid (2 to 0.06 μg per well) complexed with PEI 25K, mEGF−PEI 25K/PEI 25K (1:1), PEI 25K/PEI 22K (1:1) or mEGF−PEI 25K/PEI 22K (1:1) at N/P=6 in HBS 1/2. Luciferase activity is shown as mean ± s.d. of duplicate (* is $<10^3$ RLU/mg of protein). (B) Renca-EGFR cells were transfected with pCMVLuc plasmid (2 μg/well) complexed with PEI 25K, mEGF−PEI 25K/PEI 25K (1:1), PEI 22K or mEGF−PEI 25K/PEI 22K (1:1) at N/P = 6 in either Hepes buffer (in the presence of 200 μM chloroquine in cell culture medium during the transfection), HBS 1/2 or HBS. Luciferase activity is shown as mean ± SD of duplicate.

DISCUSSION

Our aim is to develop synthetic gene delivery systems that can be administered by intravenous application. One important aspect for the construction of such systems is the use of a convenient ligand able to mediate cell specific recognition and internalization into target cells. EGF receptor is overexpressed in many human tumors of epithelial origin and may therefore serve as a useful cell surface target (*22*). Gene delivery into EGF receptor-expressing cells was previously demonstrated using polylysine conjugates where receptor ligand (EGF or anti-EGF receptor antibody) and polylysine were covalently coupled (*32, 33*) or bound through a biotin/streptavidin linkage (*34*−*36*). Also other synthetic systems were

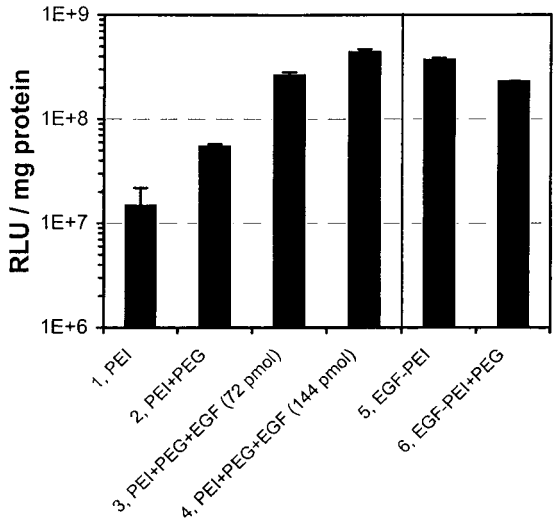

Figure 6. Transfection efficiency of PEGylated EGF-containing PEI 25K/PEI 22K complexes on KB cells. Complexes were prepared as described in Figure 4B with PEI 25K/PEI 22K (1:1) instead of PEI 25K (Columns 1−4) and EGF−PEI 25K/PEI 22K instead of EGF−PEI 25K/PEI 25K (Columns 5 and 6). Column 1: PEI 25K/PEI 22K/DNA; 2: PEI 25K/PEI 22K/DNA modified with MAL-PEG-NHS; 3 and 4: as in 2 and subsequently modified with 72 pmol and 144 pmol of thiol-functionalized mEGF respectively; 5: EGF−PEI 25K/PEI 22K/DNA; 6: EGF−PEI 25K/PEI 22K/DNA modified with MAL-PEG-NHS. Luciferase activity is shown as mean ± SD of duplicate.

described including chimeric TGF-alpha fusion protein (*37*) and EGF-containing liposomes (*38, 39*). In these cases gene delivery in EGF receptor-expressing cells was effective only in the presence of an endosomolytic agent such as chloroquine or inactivated adenovirus. Chloroquine would be difficult to apply in vivo at the required dose due to its toxicity. The use of inactivated adenovirus could lead to undesired immune problems inherent of the virus.

Recently some new polycations such as PEI (*1*) and polyamidoamine dendrimers (*40, 41*) have been described as particularly efficient gene delivery vectors without the need for additional endosomolytic agents. These cationic polymers share with chloroquine a strong buffering capacity that promotes escape of the complexes out of the endosomal compartment (*1, 42*). We combined the powerful gene delivery activity of PEI with the specificity of EGF-receptor binding by generating an EGF−PEI conjugate able to deliver DNA into tumor cells with considerably higher efficiency compared to the native PEI complexes. When EGF was incorporated in polyplexes, 10-fold lower amounts of DNA complexes were required for similar efficiency. High transfection ability with low dose of DNA is essential for in vivo application. This ligand-mediated enhancement and competition experiments performed with free EGF strongly suggest that EGF-containing DNA complexes are taken up specifically by the EGF-receptor mediated endocytosis pathway. This is also supported by recent data from our group by FACS analysis (Ogris et al., submitted to *AAPS Pharm Sci.*) where enhanced uptake of EGF-containing complexes was demonstrated.

For in vivo administration, small DNA particles are required for escape out of normal blood vessels to reach surrounding tissues such as hepatocytes (*43*) and much effort is applied in this direction (*44−46*). Preparation of EGF-polyplexes in salt-free buffer generates small particles that, however, are relatively inefficient. These complexes are not limited by the EGF receptor uptake process, but by ineffective endosomal release, as suggested by the large increase of transfection efficiency in the presence of chloroquine. This finding is in good agreement with previous observations using transferrin-PEI particles (*31*). For tumor-targeted gene delivery the DNA particle size is less problematic because of the leakiness of the tumor vasculature. Indeed accumulation of large DNA complexes (approximately 500 nm) was observed recently in our group (*20, 21*). Therefore, we evaluated also EGF−PEI complexes of medium-large size. Interestingly linear PEI 22K but also in mixture with PEI 25K (1:1) was more efficient to deliver DNA than the branched PEI 25K alone, particularly at low DNA doses. Indeed, gene expression obtained with 60 ng of DNA complexed with EGF−PEI 25K/PEI 22K was higher (10^8 RLU/mg protein) even compared to much higher amounts (2 μg) of EGF−PEI 25K/PEI 25K complexes (Figure 5).

Gene delivery systems for intravenous application must be inert against nonspecific interactions with biological environment such as nontarget cells and blood components in order to maintain their physical integrity and to avoid rapid elimination via activation of the immune system. Covalent surface modification of preformed polyplexes with PEG has been shown to strongly reduce nonspecific interactions with erythrocytes and plasma proteins (*20*) and it also reduces complement system activation (*47*). Incorporation of PEG in polyplexes was also achieved using block copolymer as condensing agent such polylysine−polysaccharide (*48*), polylysine−PEG (*49−54*), and PEI−PEG (*55, 56*). Major drawback of this latter strategy is the crowding effect of the neutral polymer that can hinder proper DNA condensation in dense particles (*49, 55*).

We evaluated two post-PEGylation strategies to generate shielded EGF-containing complexes, where EGF was either coupled to PEI (Figure 3A) or to the distal end of a PEG spacer arm (Figure 3B). In the latter strategy the ligand is incorporated at the final step of complex preparation. Optimal amounts of ligand giving best transfection efficiencies can be rapidly found. The later strategy should also allow a better accessibility of the ligand as suggested by studies on liposomes. Indeed, it was shown that incorporation of PEG in immunoliposomes can substantially weaken antigen/antibody interaction (*57, 58*). In contrast, coupling the ligand to the distal end of the PEG produced stabilized liposomes still allowing efficient binding to the target receptor (*59, 60*). We showed that both types of shielded EGF-containing complexes were effective to deliver DNA. Efficiencies were approximately 10- to 100-fold higher compared to EGF-free PEGylated complexes. In contrast to the published liposome work, the beneficial effect of grafting the ligand at the distal end of PEG compared to coupling to PEI and PEGylation was less clear with polyplexes. With small complexes this strategy (Figure 3B) resulted in 8-fold higher gene expression than with strategy illustrated in Figure 3A, but with large complexes efficacies were nearly similar. This strongly suggests that the PEG covalently coupled to the surface of EGF-containing PEI/DNA complexes only weakly interferes with the binding to the EGF-receptor. This is quite surprising knowing that a brush of PEG (MW 5000) extending out of the surface of liposomes has been described to have a thickness of 10 nm (*61*). Nevertheless, this is in line with a recent finding by Ogris et al. (submitted to *AAPS Pharm Sci.*) suggesting that PEGylation does not reduce internalization of EGF-containing complexes.

In summary we have generated EGF-containing polyplexes able to efficiently transfect cells in a receptor-dependent fashion. PEGylated EGF-containing complexes maintained high efficiency and are interesting candidates for in vivo evaluation in tumor models.

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