Megalin-targeted Enhanced Transfection Efficiency in Cultured Human HK-2 Renal Tubular Proximal Cells Using Aminoglycoside-carboxyalkyl- polyethylenimine -containing Nanoplexes

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ABSTRACT

Non-viral vectors are of interest as therapeutic gene delivery agents in gene therapy, because they are simple to prepare, easy to modify and have definable safety profiles compared to viral vectors. The potential of gene therapy in the treatment of renal diseases is limited by a lack of effective kidney-targeted gene delivery systems. Aminoglycoside antibiotics gentamicin and neomycin were connected by amide linkages to carboxyl groups on carboxyalkylated-PEI₂₅ (25 kDa PEI) or carboxyalkylated-PEI₁₀ (10 kDa PEI). Aminoglycoside-carboxyalkylated-PEI conjugates were characterized with respect to size, surface charge density, DNA condensation ability, and buffering capacity. Polyplexes prepared by electrostatic interaction between aminoglycoside-carboxyalkylated-PEIs and enhanced green fluorescent protein-expressing (EGFP) plasmid DNA had appropriate nano-scale size (143-173 nm). Their targeting potential was investigated in cultured HK-2 immortalized human cortex/proximal tubule kidney epithelial cells, which expresses megalin, a scavenger receptor that mediates endocytosis of a diverse group of ligands, including aminoglycoside antibiotics. Aminoglycoside-carboxyalkylated-PEIs significantly increased EGFP gene transfection efficiency in HK-2 cells by ~13-fold for aminoglycoside-carboxyalkylated-PEI₂₅ and ~7-fold increase for aminoglycoside-carboxyalkylated-PEI₁₀ relative to the corresponding PEIs without aminoglycosides. The transfection efficiency of polyplexes was dependent on the weight ratio of aminoglycoside-containing ligand in the carrier. In the presence of a range of concentrations of human serum albumin, which competes for megalin binding, aminoglycoside-carboxyalkylated-PEI-mediated transfection was reduced to background levels. These results suggest that aminoglycoside-carboxyalkylated-PEI polyplexes can target megalin-expressing kidney-derived cells *in vitro* resulting in improved transfection efficiency with low cytotoxicity.

Keywords: Gene targeting, Megalin, Polyethylenimine, Aminoglycosides

1. Introduction

Nanoparticle-based gene therapy is a potentially useful method for treating certain diseases for which conventional treatment modalities are not yet available(1). In these systems various carrier systems are used to transfer DNA encoding a specific therapeutic protein into target cells that have or mediate the disease(1). Among various gene delivery carriers, engineered viral vectors transfer genetic materials with high efficiency, but there are considerable difficulties in terms of safety concerns, including immunogenic and toxicological complications, carcinogenesis, inflammatory responses and risk of viral infection when used for therapeutic applications (2). In contrast, non-viral gene therapy vectors have definable toxicity, but low delivery efficiency of the therapeutic nucleic acid into the target cell, and this has proven to be an important obstacle to clinical applications (3).

Chronic kidney disease (CKD) is an increasing human health concern in many parts of the world. It has been reported that 10–15% of the population in developed countries are affected by kidney disease (4, 5). CKD often arises as a complication of diabetes, high blood pressure and inflammatory diseases. Without effective treatment, CKD progresses to renal fibrosis, the main pathological feature of End-Stage Renal Disease (ESRD) (4, 5). Currently, there is no cure for ESRD, and there is interest in developing new treatment strategies that target specific genes responsible for CKD incidence and progression to ESRD. In addition, 3.9 million Americans have a diagnosed kidney disease and as the population ages this number is expected to rise (4). Current therapies, including renal transplantation and dialysis (5), are very expensive

and require long-term administration of drugs that is accompanied by systemic toxicities and side effects (6). If gene therapy could restore or modulate appropriate gene functions, it could assist in the treatment of renal diseases and kidney transplantation. The most challenging issues in successful application of gene therapy for kidney diseases are the development of a reliable gene delivery system and an effective targeting technique. Similarly, modulation of the local immune system through gene therapy could improve patient response to renal grafts. If sufficiently effective, these methods could replace use of transplants and systemic immunosuppressive medications that increase the risk of cancer or infection.

Several experimental techniques have been developed that use nonviral, viral and cellular vectors in renal gene therapy. Adenoviruses appear to be the most efficient viral vector for *in vivo* and *in vitro* transfection, but use of nonviral vectors such as inorganic nanoparticles, cationic phospholipids and polycations have stimulated interest (7, 8). Polycations are effective gene vectors because they possess positive charges that allow them to complex electrostatically with plasmid DNA (pDNA) to form stable polyplexes and for the resulting polyplexes to bind electrostatically to cell surfaces, promote endocytosis and facilitate endosomal escape leading to the rupturing of endocytic vesicles by a proton sponge mechanism. The cationic polymer, polyethylenimine (PEI) has been extensively studied with different molecular weights, different structures (linear or branched) and chemical modifications (6, 9) for *in vitro* transfection activity and subsequently used for gene delivery *in vivo* (7, 10). Relatively high transfection rates (5, 11) with PEI make it an excellent candidate for gene delivery studies, although high molecular weight polycations in polyplexes can result in cytotoxicity.

Recently ligand-decorated nanoparticles have been developed in both academic and industrial laboratories as potential vectors for targeted gene delivery and shown to have promise

in preclinical studies (12), although many complex problems must be addressed before they can be evaluated in clinical trials (13). An important feature for the successful development of targeted nanoparticles is the choice of suitable targeting ligands based on ligand biocompatibility, cell specificity, binding affinity, purity, size, charge and ease of modification (14). Other important factors include production cost, scalability, stability, high intracellular uptake, specificity and efficacy and low cytotoxicity (15).

Gene and drug delivery systems that target various kidney cell types could constitute useful treatment modalities for renal diseases (16). The kidney is a complex, highly differentiated organ composed of a variety of compartments: glomerular, tubular, vascular and interstitial, each with multiple unique cell types, so that any drug or nanocarrier targeting mechanism must be specific for the cell type to be treated (17). Among the various kidney cell types, proximal tubular epithelial cells (PTECs) are of particular interest, because they are the most populous cell type in the kidney, and because they carry out a variety of regulatory and endocrine functions using unique or near-unique transporters. Also, PTECs are associated with a variety of acute and chronic diseases, including acute kidney injury, renal tubular acidosis, phosphate wasting syndromes, Dent's disease, Fanconi's syndrome, cystinuria and other amino acid transport disorders (18). PTECs express large amounts of megalin/cubilin, a near-unique transporter that mediates endocytosis of filtered proteins and a diverse array of other oligocationic compounds that have been filtered by the glomerulus into the urine. Since there is no endothelial layer between the epithelial cells and the tubular fluid, PTECs in the kidney can potentially be targeted through the tubular lumen. The glomerulus guards of the tubular lumen set a limit on the size of drug carriers. Particles with a hydrodynamic diameter below 5-7 nm are quickly passed and excreted, and since most drug carriers are considerably larger (10-200 nm),

glomerular filtration barrier frequently excludes carriers, such as liposomes and antibodies from access to luminal surface of PTECs (19). Some relatively low molecular weight carriers like protein-based, polymeric and folate-conjugated carriers have been reported to be more successful for specific drug targeting of the kidney (20).

Megalin is a 600-kDa transmembrane protein belonging to the LDL-receptor family, which was discovered 30 years ago by Farquhar and Kerjaschki (21). As an endocytic multi-ligand receptor, megalin has been identified as a highly expressed receptor in renal PTECs mediating rescue of ligands, including proteins, biomarkers, vitamins and hormones. In cooperation with the receptor protein cubilin, it also mediates retention of drugs, including aminoglycosides (AGs), and toxins (22). Binding of gentamicin to purified megalin has been demonstrated by the equilibrium dialysis method (23). Several reports have targeted megalin receptor for the specific delivery of drugs (24), siRNA (25, 26) and pDNA (27) and small molecules(14, 24).

In the present study, in order to develop a novel Kidney-targeted drug/gene delivery system, we synthesized aminoglycoside-carboxyalkyl-polyethylenimine -containing nanoplexes and investigated their potential targeting efficiency. we have used pDNA encoding Enhanced Green Fluorescent Protein (EGFP) as a reporter gene and carboxyalkylated cationic PEI polymers with molecular weights of 10 kDa (PEI₁₀) and 25 kDa (PEI₂₅) covalently coupled to two aminoglycoside as new ligands (gentamicin and neomycin) to investigate targeting of megalin-expressing cell lines (HK-2 and MDCK) (28) compared to a non-megalin-expressing control cell line (HepG2) (28, 29) (Figure1). To the best of our knowledge, no other research has reported the use of aminoglycosides as targeting ligands for kidney-derived megalin-expressing

cell lines. Aminoglycoside ligands offer numerous advantages over macromolecules, including well-defined molecular structures, low cost and an extensive history of use in therapy.

2. Materials and methods

2.1. Materials

PEI₁₀ was purchased from Polysciences, Inc. (Warrington, PA, USA). PEI₂₅, 6-bromohexanoic acid, epidermal growth factor (EGF) and bovine pituitary extract (BPE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plasmid EGFP, cell proliferation and reporter gene expression assays were obtained from Promega (Madison, WI, USA) and Invitrogen (Germany), respectively. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), DMEM-F12 and RPMI were purchased from Gibco (Gaithersburg, MD). Penicillin and streptomycin were purchased from Biochrom AG (Berlin, Germany). N-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plasmid pEGFP-N1 was obtained from Prof. Dr. Ernest Wagner, Ludwig Maximilians University of Munich, Munich, Germany. Spectra/Por dialysis membranes were purchased from Spectrum Laboratories, Houston, USA. All other solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest purity available.

2.2. Preparation of ligand derivatives of PEIs

2.2.1. Synthesis of 6-bromohexanoic acid derivatives of PEI

To prepare derivatives with 6-bromohexanoic acid, either PEI₁₀ or PEI₂₅ (500 mg) was dissolved in dimethylformamide (DMF) and chloroform, respectively. To achieve the desired degree of modification, DMF or chloroform solutions containing the proper amounts of 6-bromohexanoic acid based on 10%, 30% and 50% mole percentage were added dropwise to a stirred PEI solution at room temperature over a period of 3 hours. The reactions were allowed to proceed at room temperature for 48 h. The chloroform was removed using a rotary evaporator for modified PEI₂₅. The crude products were dialyzed through dialysis membranes with 8,000 cut-off and 10,000 Da cut-off for modified PEI₁₀ and modified PEI₂₅, respectively. The dialysis was performed once against 0.25M NaCl and then twice against water to remove unreacted alkylating agent, then the retentate was lyophilized. The degree of substitution of primary amines on carboxyalkyl-PEIs (10 or 25 kDa) was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay of unsubstituted free amines remaining.

2.2.2. Aminoglycoside coupling to carboxyalkylated PEIs

The primary amine groups lost through carboxyalkylation of either PEI₁₀ or PEI₂₅ were extensively replaced by free amine groups on gentamicin or neomycin after amide bond linkage between a carboxylate end group of a grafted alkyl chain and an amino group on the aminoglycoside. Carboxyalkyl-PEIs (10 or 25 kDa) were covalently coupled to either gentamicin or neomycin using HOBt and EDC as coupling agents. Briefly, the calculated amounts of the aminoglycosides based on 2.5, 5 and 10% weight percent grafting were dissolved in distilled water containing HOBt. Carboxyalkyl-PEIs (10 or 25 kDa) were activated by EDC through 45 minutes stirring at room temperature. The aminoglycoside and HOBt solution was added dropwise to a gently stirred aqueous solution containing EDC and carboxyalkyl-PEIs (10

or 25 kDa). The reactions were allowed to proceed at room temperature for 48 h. The final products were dialyzed three times against distilled water using Spectra/Por dialysis membrane (6000-10000 Da cut-off, Spectrum Laboratories, Houston, USA) to remove unreacted materials and then lyophilized. Amide bond formation was confirmed by Fourier transform infrared spectroscopy (FTIR).

2.3. Characterization

2.3.1. FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used as a characterization technique to confirm the chemical modification of PEI derivatives. IR spectra were recorded in the range from 4000 to 500 cm^{-1} . All samples were mixed with KBr and pressed into a disc for IR study.

2. 3.2. Nuclear magnetic resonance (NMR) spectroscopy

Chemical conjugation of aminoglycosides (gentamicin/neomycin) to carboxyalkyl-PEIs (10 or 25 kDa) was confirmed by NMR spectroscopy. ^1H NMR spectra of purified products were recorded using a Bruker 400 MHz instrument (Billerica, MA, USA).

2.3.3 TNBS assay

The amount of primary amines on carboxyalkyl-PEIs (10 or 25 kDa) (6-bromohexanoic acid derivatives) was determined by 2,4,6-trinitrobenzensulfonic acid (TNBS) using the standard method (30). The degree of grafting was measured by the differences in the amounts of primary amines on unmodified PEI₁₀ or PEI₂₅ kDa compared to carboxyalkyl-PEIs (10 or 25 kDa) and gentamicin/neomycin conjugated to carboxyalkyl-PEIs (10 or 25 kDa).

2.3.4. Buffering capacity measurement

Each PEI derivative (0.4 mg) was dissolved in 1 ml of double-distilled water adjusted to an approximate pH value of 12 with 1M NaOH. Aliquots (5 µl) of 1M HCl were added sequentially until the pH was reduced to 2.5. After each addition of acid, the pH of the solution was measured with a pH meter (Mettler Toledo, Greifensee, Switzerland). The slope of the plot of pH versus amount of HCl indicates the intrinsic buffering capacity of the modified PEIs.

2.3.5. Ethidium bromide exclusion assay

DNA condensation ability of PEI derivatives was evaluated in polyplexes prepared with EGFP pDNA using the ethidium bromide (EtBr) exclusion assay. Interaction of EtBr within DNA base pairs was determined by fluorescent spectroscopy (excitation: 510 nm, and emission: 590 nm) measured by a Jasco FP-6200 spectrofluorimeter (Tokyo, Japan). The fluorescence intensity of 1 ml of EtBr solution (400 ng/ml) in HEPES-buffered glucose containing 20 mM HEPES, 5% glucose, pH 7.4 mixed with an equal volume of pDNA (5 µg/ml) in the same buffer was set at 100% and the fluorescent intensity of the EtBr solution in the presence of the same amount of free plasmid corresponds to 0% condensation. The percent decrease of EtBr fluorescence intensity was measured in triplicate following addition of PEI or a derivative in increments. Condensation of DNA was estimated as reduced ability to bind EtBr.

2.3.6. Particle size and zeta potential measurements

The mean hydrodynamic particle size and surface charge for vector/pDNA polyplexes were determined using Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV), respectively using Malvern Nano AS instrument and DTS software (Malvern Instruments, UK)

in a salt-free buffer (HEPES-buffered glucose containing 20 mM HEPES, 5% glucose, pH 7.4). Various amounts of cationic polymers were diluted in 125 μ L of buffer and mixed with an equal volume of the same buffer containing DNA. The measurement was carried out in automatic mode and the results were presented as mean $\pm$ S.D. Each mean represents the average value of 30 measurements.

2.3.7. Atomic force microscopy measurement

The size and morphology of nanoplexes at C/P=2 (carrier:plasmid weight ratios; concentration of PEI derivative in mg/ml (2)/concentration of pDNA in μ g/ml (1). Samples were prepared at 0.1 mg/ml and were further characterized using atomic force microscopy (JPK NanoWizard II instrument, Berlin, Germany). Samples (20 μ l) containing PEI₂₅C₅₀G₁₀ and PEI₂₅C₅₀N₁₀ polyplexes were diluted four-fold and deposited onto the center of a freshly split untreated mica disk, then dried at room temperature before imaging. The images were recorded in air at room temperature and a scan speed of 1 Hz, and the phase image and topology were used to determine the morphology and particle size.

2.5. In vitro studies

2.5.1. Cell culture:

HK-2 adult male human cortex/proximal tubule kidney epithelial cells HK-2 immortalized with human papillomavirus 16 (31) (ATCC CCL-131, Manassas, VA, USA); MDCK, Madin-Darby canine kidney epithelial cells (ATCC CCL-3) (32), which are reported to express a cell surface antigen of the kidney distal tubule (33); HepG2, human liver carcinoma (ATCC HB-8065) cells (34) were cultured at 37°C in a humidified 5% CO₂ atmosphere in DMEM-F12 supplemented with 10% FBS, 60 μ g/ml penicillin and 100 μ g/ml streptomycin for

72 h (medium refreshed after 48 h), 24 h, and 24 h in RPMI supplemented with 10% FBS, 60
µg/ml penicillin and 100 µg/ml streptomycin for HK-2, MDCK and HepG2, respectively.

2.5.2. Cytotoxicity assay

The cytotoxic effects of the polyplexes on HK-2, MDCK and HepG2 cells were
evaluated using the MTT colorimetric assay. All cell lines were grown in 96-well plates at an
initial density of 1×10^4 cells per well in 100 ml DMEM-F12 or RPMI media with 10% FBS,
cultured 24 h, Polyplexes prepared at different carrier:plasmid weight ratios (C/P) (C/P ratios of
2, 4 and 6) were used for cytotoxicity experiments. Twenty microliters of polyplexes (equivalent
of 200 ng plasmid) were added into each well. After 4 h of incubation at 37°C, the medium was
replaced with 100 µL fresh medium with serum (10% FBS) and incubated for additional 24 h.
MTT (20 µL of 5 mg/ml in phosphate buffered saline) was added to each well, plates were
incubated at 37°C for 2h, the media were removed and 100 µL DMSO was added to dissolve
formazan crystals. Absorbance was measured at 570 nm (reference wavelength 630 nm) using a
microplate reader (infinite NanoQuant M200, Tecan (Zurich, Switzerland). Treated cells were
compared to control cells ($\text{Cell viability} = A_{\text{treated}} / A_{\text{control}} \times 100$) and expressed as the mean $\pm$ SD of
triplicates.

2.5.3. Demonstration of cell surface megalin

HK-2 and HEPG2 cells were investigated for the presence of megalin receptor on their
surfaces using immunofluorescence confocal microscopy. HK-2 and HepG2 cells were seeded
for 24 h onto poly-L-Lysine (MW 70,000-12000, 0.01%, Sigma, St. Louis, MO, USA) coated

coverslips. Then, cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS), fixed with paraformaldehyde (% in DPBS, 10 min.) and permeabilized with Triton X-100 (0.05%). As primary antibody, rabbit polyclonal anti-human megalin (H245) (Santa Cruz Biotechnology, Dallas, TX, USA) was used at a dilution of 1/100 in PBS with 1% BSA and cells were incubated for three hours at room temperature. Cell-bound antibody was detected with secondary antibody at a dilution of 1/200, polyclonal goat anti-rabbit immunoglobulin conjugated to Alexa-568 (Molecular Probes, Eugene, OR, USA). Cover slips were mounted in Prolong Gold Antifade Mounting (Thermo Fisher Scientific, Waltham, MA, USA). Imaging was performed on an Olympus (Tokyo, Japan) BX61 upright microscope equipped with a FV1000 confocal scan head. PlanApo 60x/1.42 NA or 20x/0.8 NA oil objectives were used to acquire fluorescence (543 nm laser, emission filter 560-660 nm) and differential interference contrast images.

2.5.4. Quantitative measurement of transfection efficiency

Megalín-expressing and non-expressing cells were transfected with a plasmid coding for the reporter gene product EGFP to compare the transfection efficiencies of different aminoglycoside-PEI derivatives prepared in this study (35). The transfection efficiencies of PEIs and aminoglycoside derivatives were evaluated quantitatively using flow cytometry and qualitatively using fluorescent live cell imaging.

For quantitative measurement of transfection efficiency, all cell lines (HK-2, MDCK and HepG2) were seeded at a density of 8×10^4 cells/well in 24-well plates and cultured for 24 h. Cells were treated in serum-free medium with polyplex preparations containing 3 μ g pEGFP DNA at the same C/P ratios [(w/w)] used for cytotoxicity experiments. After 4 h of incubation at 37°C, the medium was removed, replaced with 400 μ L of fresh serum-supplemented medium.

Subsequently, the incubation continued for additional 48 h under normal culture conditions. Cell transfection was quantitatively determined using FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a xenon-ion excitation lamp (488 nm). Aliquot of transformed cells was collected after 48 h of transfection, centrifuged, and then washed twice with Phosphate-Buffered Saline (PBS). The cells were subsequently resuspended in buffered saline solution and analyzed in FACSCalibur flow cytometer. All analyses were performed at a low flow rate ($20 \mu\text{Lmin}^{-1}$) with pressure setting of 0.7 bars. Green fluorescence from GFP (515 nm-545 nm emission filter) measured in the FL1 channel was used to evaluate the transfection efficiency. Data analysis was conducted using CELL Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

2.5.5. Qualitative measurement of transfection efficiency

For qualitative measurement of transfection efficiency, EGFP plasmid DNA was used to prepare polyplexes at different C/P ratios calculated to provide 0.4 μg plasmid DNA per well. Cultured cells (8×10^4 cells/well) were treated with polyplexes for 4 h at 37°C. After replacement of the medium with serum-supplemented fresh DMEM-F12 medium and incubation for 48 h at 37°C, the EGFP expression in transfected cells was studied qualitatively using fluorescent analyzers Zeiss Axio Vert.A1 (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.5.6. Inhibition of transfection by albumin

Inhibition of transfection of pEGFP DNA polyplexes prepared with either PEI-gentamicin or PEI-neomycin by added human serum albumin to the cells (0-500 mg/ml serum-free DMEM-F12 medium) was measured in HK-2 cells using the method described above for

quantitative measurement of transfection efficiency. After incubation for 1 h at 37°C, cells were rinsed three times with ice-cold PBS and EGFP expression measured quantitatively by flow cytometry. The transfection efficiency of both gentamycin- and neomycin-conjugated PEI₂₅ in HK-2 cells were determined when the pre-incubated cells were treated with different concentrations of human albumin for 4 hours, washed three times with PBS and then were transfected using the method described for quantitative measurement of transfection efficiency.

2.5.7 In vivo gene delivery

Male Balb/c mice (6 weeks old, 22.2 g) were purchased from Pasteur Institute (Tehran, Iran) and acclimatized before performing accomplished in accordance with the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences based on the national guidelines from Ministry of Health and Medical Education of Iran, adopted from the 86/609/EEC Directives of European Community. pEFGP plasmid (60 µg/mouse) was complexed with PEI₂₅C₅₀G₁₀ that showed optimal transfection activity in in vitro experiments and with PEI₂₅C₅₀ as control at C/P ratio = 4 in a volume of 200 µl in normal saline. The complexes were incubated for 30 min at room temperature and injected into Balb/c mice through the tail vein. After 36 h post injection, animals were sacrificed by cervical dislocation and several tissues were imaged ex vivo utilizing Ivis Imaging System (Xenogen, Alameda, CA; 100 series) (36, 37).

2.6. Statistical analysis

All statistical analyses were performed using JMP software (version 8) (SAS Institute, Cary, NC, USA). The data were presented as means $\pm$ standard deviation (s.d.), and analyzed by analysis of variance (ANOVA) or student's t-test. P values of ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Synthesis of modified PEIs

Gene delivery to the kidney is a promising treatment modality for the intractable renal disease. Recently, systemic side effects of the kidney drugs and resistance to therapy are the two major obstacles in managing renal system failure. However, selective introduction of therapeutic genes into the kidney may overcome these issues. Ex vivo gene therapy by transfecting the graft during transplantation with genes involved in suppressing immune responses may also provide treatment opportunities to overcome acute kidney rejection for which there is no effective treatment available (38). Recent advances in gene delivery systems have opened the door to possible cures of chronic kidney disease (CKD) which is a major global health concern. Without effective treatment, CKD leads to renal fibrosis and requirement of dialysis and kidney transplantation (19). Important factors like the gene type, the gene vector that carries the gene into the cells and the route of administration affect the therapeutic efficacy. Malek et al. (39) showed that PEI-siRNA complexes exposed lower renal targeting compared to naked siRNA. Although PEI-siRNA complex showed a significantly higher protection of siRNA, but naked siRNA taken up in the kidney was mostly degraded. It is confirmed that positively charged PEI

355 formed non-covalent complexes with siRNA, not only protecting siRNA from nuclease breaking
356 down but facilitating endocytic uptake and following endosomal escape after administration (40).

357 Although the glomerular filtration of compounds may be similar to the extravasation of
358 macromolecules in leaky tumor vessels, the subsequent uptake of compounds in the PTECs is a
359 highly active process. Different receptors and transporters are located in PTECs that can be
360 utilized for the active targeting. However, these transporters will not be suitable targets for
361 renal-specific uptake of the delivered compound, because they are involved in the uptake of
362 small endogenous molecules and drugs (19). Furthermore, unwanted excretion of the
363 intracellularly accumulated substrates from the cells into either the urine or the renal interstitium,
364 by the same transporter or *via* a different transporter expressed in the tubular cell membrane is a
365 major disadvantage for using these transporters. In addition, they are not expressed only in
366 kidneys. Therefore, drug delivery into the PTECs through receptor-mediated endocytosis is an
367 attractive approach. PTECs contain different internalizing receptors facilitating the
368 internalization of small molecules and macromolecules like proteins that are filtered into urine
369 (19). Targeting of drugs to the luminal side of the tubular cells through carriers that attach to
370 these receptors is a suitable and applicable strategy for uptake of drug molecules. It has been
371 shown that uptake of substrate from the tubular lumen side of PTECs is often mediated by two
372 receptors, megalin and cubilin, which are responsible for the receptor-mediated endocytic uptake
373 of various substances from the glomerular ultrafiltrate. Megalin, in cooperation with the receptor
374 protein cubilin, mediates the rescue of ligands, including proteins like albumin, biomarkers,
375 vitamins, hormones, and aminoglycosides. Aminoglycosides are taken up in the proximal tubules
376 (S1 and S2 segments) by a receptor-mediated mechanism as demonstrated by electron
377 microscopic localization of aminoglycosides in coated pits and endocytic compartments (41). We

378 synthesized aminoglycoside-carboxyalkylated-PEIs which significantly increased EGFP gene
379 transfection efficiency into HK-2 cells by ~13 folds for aminoglycoside-carboxyalkylated-PEI25
380 and ~7 folds for aminoglycoside-carboxyalkylated-PEI10 compared to the corresponding PEIs
381 without aminoglycosides. The transfection efficiency of polyplexes was highly dependent on the
382 weight ratio of aminoglycoside-containing ligand in the carrier, which was inhibited in the
383 presence of a range of concentrations of human serum, which competes for megalin binding,
384 reducing the aminoglycoside-carboxyalkylated-PEI-mediated transfection to background levels.

385 According to our results, we were able to demonstrate specific renal delivery of PEI25/EGFP
386 nanoparticles into kidney PTECs in vitro and in animal model. Previous reports have shown that
387 siRNA conjugated with specific antibodies were successfully delivered into podocytes (42).
388 Nanoparticles with defined size ($\sim 75 \pm 25$ nm) could target the mesangium (43); and cationic
389 cyclodextrin-containing polymer based siRNA nanoparticles were able to accumulate and
390 disassemble in the glomerular basement membrane (44). Furthermore, subcutaneous injection of
391 cholesterol-conjugated siRNA led to accumulation of siRNA predominantly in the kidney cortex
392 and glomeruli (45). The suggested role of aminoglycoside-carboxyalkylated-PEI25/EGFP
393 conjugates (with a size about 143 nm) for the delivery of nucleic acids to the PTECs raises the
394 question of how the PEI25-Gen/EGFP nanocomplexes are filtered. The rapid clearance of these
395 nucleic acid nanoparticles is confusing since they exceed range of molecular weight cut-off
396 (MWCO) for glomerular filtration (26). One possibility is that the PEI25-Gen/EGFP complex
397 disassembles at the GBM and subsequently reassembles. As suggested by the work of
398 Zuckerman et al. and Naeye et al., for either cationic cyclodextrin-containing polymer based
399 siRNA nanoparticles or siRNA-loaded dextran nanogel, it may be that a fraction of PEI25-
400 Gen/EGFP nanoparticles comprising very small nanoparticles could pass through the endothelial

layer and then become disassembled at the glomerular membrane (GBM) (44, 46). After filtration into the urinary space, the PEI25-Gen conjugate may re-associate with the EGFP plasmid facilitating uptake of siRNA into the PTEC in accordance with the observations reported for cationic cyclodextrin-containing polymer based siRNA nanoparticles (44). It has been reported previously that most cationic polymer-based nucleic acid delivery systems are rapidly eliminated from circulation in mice, monkeys and humans (47-49). Zuckerman et al. hypothesized that paradoxical renal clearance of polycation-nucleic acid nanoparticles results from their attachment to and disassembly by components of the renal filtration barrier. They introduced three key properties of such nanoparticles (diameters between 10 and 100 nm, positive zeta potentials, and electrostatically driven self-assembly) making them susceptible to this mechanism of clearance. The renal filtration barrier located within the glomerulus of the nephron, consists of three layers that must be traversed to enter the urinary space. These three layers include the glomerular endothelium with pores in the range of 80 to 100 nm, the glomerular basement membrane (GBM), a 300 to 350 nm-thick basal lamina rich in heparan sulfate and charged proteoglycans (pore size of 3 nm) which filters small molecules by charge and size, and the podocyte filtration slits lies behind the GBM that are around 32 nm wide (50). The glomerular filtration apparatus, taken in its entirety, possesses an effective size cutoff of 10 nm, and is responsible for facilitating the rapid clearance of small molecules and free nucleic acids. It was indeed demonstrated that gold NPs up to 130 nm could extravasate *via* glomerular endothelium. Then, NPs are ended up in the GBM that contains high number of negatively charged proteoglycans such as heparin sulfate which might compete with the nucleic acids in the NPs (51). As recently published by Naeye et al., PEGylated dextran nanogels also revealed a prolonged accumulation of siRNA in kidney and a significant delayed transit of the siRNA

toward the bladder. It is therefore plausible to conclude that the sub-150 nm fraction of the Dex-NGs developed in their study could also be disassembled through the recently described mechanism. Again in accordance with our data, Boletta et al. tested a panel of reagents including a monocationic lipid, DOTAP, a polycationic lipid, Transfectam, and three different forms of the PEI. Kidneys injected with PEI₂₅/DNA complex yielded activity level significantly higher than the other transfection condition. Dose-dependent luciferase expression was shown for 10, 50, 100 µg of PEI₂₅/DNA complexes. They confirmed that applying β-galactosidase (β-Gal) as receptor, transgene expression was localized almost exclusively in PETCs (52).

An alternative model is that the PEI₂₅-Gen/EGFP complex is disassembled partially at the GBM, suggesting that the size of the partially disassembled complex is within the range of molecular weight cut-off (MWCO) for glomerular filtration. However, it has been reported that 200 to 300 nm carbon nanotubes which are much larger than the MWCO, can be cleared through glomerular filtration. This cannot be explained by conventional theory (53) and suggests that other mechanisms may be involved to enable even larger complexes to pass through the GBM. Benigni et al. reported that perfusion of the kidney with PEI₂₅/DNA complexes had acceptable gene transfer activity for delivering of CTLA4Ig gene with high toxicity which resulted in large areas of hypoperfusion characterized by injured glomeruli and tubuli, and capillary thrombosis. According to their results, the toxicity could be reduced at lower concentrations of the PEI₂₅/DNA complexes although at lower concentrations, the transfection efficiency was unacceptably low. They reported that the observed toxic effect of PEI 25kDa prevents possible future application of this polymer in clinical transplantation thus, it seems that unmodified PEI 25kDa cannot be an ideal gene transfer vector for the kidney (54). One of the best advantages of PEI 25kDa is its ability in facilitating the endosomal disruption, and consequently faster release

of the siRNA into the cytoplasm. This demolition ability is believed to be dependent on protonation of PEI leading to swelling and disruption of the endosomal compartment avoiding increased acidity and protecting siRNA digestion (40). Previous studies have targeted kidney *via* active targeting of therapeutics to proximal tubular cells. Dolman et al. successfully targeted PTECs *via* Imatinib-ULS-lysozyme conjugate which was mediated by megalin receptor (55).

In the present study, we designed a kidney-specific pDNA nanocomplex which had demonstrated specific PTECs targeting in the kidney. It has been reported that therapy of some kidney diseases would benefit from water-soluble drug delivery systems that specifically target proximal tubular cells (20). In this study to reduce the cytotoxic effects characteristic of high molecular weight branched PEIs (either 10 or 25 kDa) we initially modified PEI₁₀ and PEI₂₅ by carboxyalkylation of primary amine groups at the molar ratios calculated to give 10%, 30% and 50% primary amine substitutions (Figure 2). Alkylation with a range of percentages (10%, 30% and 50%) of 6-bromohexanoic acid was selected to serve as linker, because six carbons (hexanoate) was found to be the optimal alkyl chain length in the previous work. These modifications also provided terminal functional carboxylate groups, to which aminoglycosides could be attached by amide bond formation in the next step. The TNBS assay was used to measure (i) the extent of PEI primary amine group substitution by carboxyalkyl moieties; and (ii) the number of additional primary amine groups added when oligoamine antibiotics were coupled to carboxylate groups. Coupling of antibiotics to carboxyalkyl-PEIs was carried out with amounts calculated to derivatize 10%, 30% and 50% of the primary amines of PEIs. The TNBS assay was used to determine the extent of alkylation of PEIs by 6-bromohexanoic acid measured by decrease in primary amines. The results indicated that the degree of substitution obtained on carboxyl groups at the ends of carboxyalkyl chains was 7.5%, 22.5% and 39.9% mol% for PEI₁₀

substitution and 8.3%, 26.5% and 47.3% for PEI₂₅ substitution at 10%, 30% and 50% theoretical substitution, respectively, consistent with yields in the range of 75-90%. The reproducibility of the chemical synthesis procedures was demonstrated by carrying out several independent preparations.

3.2. Characterization of modified PEIs

3.2.1. FTIR spectroscopy

Conjugation of gentamicin to PEI was confirmed using FTIR spectroscopy (Figure 3). The amide carbonyl stretch absorbance peak at 1640 cm⁻¹ confirmed the presence of amide bonds in PEI derivatives. Peaks at 2860 and 2920 cm⁻¹ corresponded to methylene groups of carboxyalkyl chains, while signal at 1737 cm⁻¹ was the characteristic peak for carbonyl group of terminal carboxylic acid.

3.2.2. NMR spectroscopy

Chemical structures of final products were confirmed by ¹H-NMR spectra. Spectra of PEI₂₅C₅₀G₁₀ and PEI₂₅C₅₀ recorded in D₂O are presented in Figure 4A-B. CH₂ protons of PEI were observed in the range of 2.5-3 ppm. CH₂ protons in the spectrum of unmodified PEI appear at 2.5 and 2.6 ppm, but alkylation of PEI with hexanoate adds additional peaks downfield (Figure 4A). The signals for protons present in gentamicin moiety were clearly appeared in the range of 2.9-3.8 ppm and 5.3 (56). In the ¹HNMR spectrum of the PEI-carboxyalkyl derivative in which 10% of the carboxylate was conjugated with gentamicin, peaks a and b were assigned to (OH-CH₂-CO-Alkyl chain) and (O-CH-O-Gen), respectively (Figure 4B). The percentage of grafting

of gentamicin onto PEI₂₅ was calculated to be approximately 9.7 % by integrating the ratio of areas under peaks at 1.5 ppm (OH-CH₂-CO-Alkyl chain) and 5.3 ppm (O-CH-O-Gen).

3.2.3. Buffering capacity of modified PEIs

The proton sponge effect is widely believed to be the main mechanism of endosomal escape in polycation-mediated gene delivery. Polymers with higher buffering capacity in the endosomal pH range of pH 4.5-7.5 gave better transfection efficiency (57). The buffering capacity of PEIs and their derivatives was evaluated by titration of 1 mg/ml solutions with 1M HCl over a pH range of 2-12. Because PEI₂₅C₅₀ and PEI₁₀C₅₀ were the most efficient carriers among aminoglycoside-PEI conjugates, their buffering capacity is presented in Figure 5A-D. In general, increased amounts of carboxyalkylation of either PEI₁₀ or PEI₂₅ resulted in increased buffering capacity in the endosomal pH range (Figure 5B, 4C). Buffering capacity was also increased as the percentage of gentamicin/neomycin grafting increased (Figure 5A). For carriers based on both PEI₁₀ and PEI₂₅ kDa, gentamicin grafting produced higher buffering capacity compared to neomycin conjugates (Figure 5B and 5D).

3.2.4. DNA condensing activity of modified PEIs

Ethidium bromide (EtBr) exclusion was used to estimate the ability of polyplexes to condense DNA. When EtBr intercalates into DNA, its fluorescence is strongly enhanced. When polycation vectors effectively condense DNA, intercalation by EtBr is prevented, which results in decreased fluorescence intensity relative to uncondensed DNA. EtBr exclusion assay data (Figure 6) showed that all PEI derivatives prepared in this study were able to efficiently condense plasmid DNA. For the aminoglycoside-PEI₁₀ conjugates, condensation was complete

at a $C/P = 1$. Significantly greater condensing activity ($P < 0.001$) was observed with unconjugated PEI₂₅ relative to the aminoglycoside-PEI₂₅ conjugated vectors at $C/P = 1.5$, but there were no significant differences between condensing activity of unmodified PEI₂₅ kDa and their corresponding conjugates at C/P ratios greater than 1.5.

3.2.5. Particle size and zeta potential measurements on modified PEIs

If renal-specific drug and gene targeting could be achieved, it would allow improved management of renal diseases. Given the important role in kidney disease played by proximal tubular epithelial cells (PTECs), they represent an attractive target for polymeric drug delivery systems (19, 20). In the present study known ligand-receptor relationships of PTECs were used to design conjugates of the oligocationic antibiotics gentamicin and neomycin to branched PEI₁₀ and PEI₂₅, which were predicted to facilitate endocytosis in megalin-expressing PTECs. An efficient gene delivery vector needs to have an optimal size and charge density. Charge density is needed to condense nucleic acid and protect it from extracellular degradation (58). Nano-sized particles are needed for efficient cell uptake. The size and zeta potential measurements for polyplexes prepared with pEGFP plasmid DNA and gentamicin/neomycin conjugated carboxyalkylated PEIs are presented in Table 1. Use of aminoglycoside-carboxyalkylated PEIs resulted in smaller nanoplexes compared to unmodified PEIs. All resulting nanoplexes ($C/P = 2$) had an average size of about 143-163 nm with gentamicin-PEI₂₅; 153-157 nm with neomycin-PEI₂₅; 166-170 nm with gentamicin-PEI₁₀; and 150 nm with neomycin-PEI₁₀ (Table 1), indicated that addition of either aminoglycosidic ligands to carboxyalkylated PEIs reduced the particle size from 230 to 143 nm for PEI₁₀ and from 205 to 166 nm for PEI₂₅. It was previously shown that alkylated PEIs coupled with oligoamines were able to form nanoparticles with

smaller sizes (56-97 nm) (30), presumably because the aminoglycoside added to the net positive charge and primary amine density. The zeta potentials of polyplexes were in the range of 18.6±0.95 to 29.76±1.82mV for conjugates based on PEI₂₅ and 14.3±4.0 to 19.3±1.7mV for those based on PEI₁₀ (Table 1). Grafting the largest amount of gentamicin studied to carboxyalkylated-PEI₂₅ and carboxyalkylated-PEI₁₀ increased the surface zeta potential from 26.2 to 29.4 mV and from 19.3 to 27.7 mV in PEI₂₅C₅₀G₁₀ and PEI₁₀C₅₀G₁₀ respectively. As expected, coupling aminoglycosides to carboxyalkylated PEIs increased zeta potentials to values closer to or even higher than those with unmodified PEIs. The increased positive charge should increase the electrostatic association of resulting polyplexes with the negatively charged membranes of cells in the initial step of the transfection process (59).

3.2.6. Atomic force microscopy (AFM) size measurement of modified PEIs

AFM analysis was used to further characterize the particle size and morphology of PEI₂₅C₅₀G₁₀ and PEI₁₀C₅₀G₁₀ nanoplexes (Figure 7). The images revealed that the complexes were spherical and nano-sized at C/P = 2. The estimated average diameters of PEI₂₅C₅₀G₁₀ nanoparticles and PEI₁₀C₅₀G₁₀ nanoparticles were 143 and 160 nm, respectively as shown in Figure 7A and 7B. AFM size estimates were in general agreement with the particle sizes obtained by DLS analysis.

3.3. Cell surface megalin expression

PTECs are associated with a variety of acute and chronic diseases, including acute kidney injury, renal tubular acidosis, phosphate wasting syndromes, Dent's disease, Fanconi's syndrome,

cystinuria and other amino acid transport disorders (18). As an endocytic multi-ligand receptor, megalin has been identified as a highly expressed receptor in renal PTECs mediating rescue of ligands, including proteins, biomarkers, vitamins and hormones. In cooperation with the receptor protein cubilin, it also mediates retention of drugs, including aminoglycosides (AGs), and toxins (22). Binding of gentamicin to purified megalin has been demonstrated by the equilibrium dialysis method (23). Several reports have targeted megalin receptor for the specific delivery of drugs, siRNA and pDNA (24-27, 39) and small molecules (14, 24). Expression of megalin on the surface of HK-2 cells was visualized using an anti-megalin polyclonal rabbit anti-human megalin immunoglobulin and a polyclonal goat anti-rabbit immunoglobulin secondary antibody conjugated to Alexa-568, a fluorescent dye with an orange/red emission color. Confocal microscopy confirmed the presence of megalin on the surface of HK-2 cells, while no red fluorescence was observed in the control HepG2 cells (Figure 8).

3.4. Cell toxicity evaluation of modified PEIs

The cytotoxicities of polyplexes prepared with plasmid pEGFP DNA and gentamicin- or neomycin-conjugated carboxyalkylated PEIs were compared at different C/P ratios (2, 4 and 6) with that of polyplexes formed with unmodified branched PEI₁₀ and PEI₂₅ in cultures of both megalin-positive cell lines (HK-2) and megalin-negative cells (HepG2) (Figure 9). Cytotoxicity was estimated under the same conditions as the transfection assay measured as reduced viability after 24 hours in culture measured using a commercial MTT colorimetric assay. Polyplexes prepared with unmodified branched PEI₁₀ and PEI₂₅ at C/P = 6 exhibited the highest toxicities, with cell viabilities decreased to 64.4 and 47.4 %, respectively in both megalin-positive and megalin-negative cell lines. The cell viability of polyplexes prepared with gentamicin- or

neomycin-conjugated PEIs with 50% carboxyalkylation was in the range of 80-98 % at C/P = 2, which was significantly ($P < 0.01$, t -test), greater than the viability observed with polyplexes prepared with unmodified PEIs. However, there was no significant difference between the toxicities of polyplexes prepared with gentamicin-conjugated carboxyalkylated PEIs and those prepared with neomycin-conjugated carboxyalkylated PEIs ($P < 0.35$, ANOVA) (Figure 9).

3.5. Transfection efficiency of modified PEIs in megalin-expressing cells

3.5.1. Flow cytometry analysis of megalin-expressing cells

Megalin has been used in several studies to target delivery of SiRNA to cultured mammalian cells (19, 20, 25-27, 55). The transfection efficiencies for the reporter gene *EGFP* were compared by flow cytometry with polyplexes prepared from aminoglycoside-conjugated carboxyalkylated-PEI₂₅ and carboxyalkylated-PEI₁₀ at various C/P ratios in megalin-expressing cell lines HK-2 and MDCK (MDCK results are shown in Supplementary Materials) and a megalin non-expressing control cell line HepG2 (Figure 10). The observed transfection efficiencies were compared to transfection efficiencies measured with the corresponding polyplexes prepared from unmodified PEI₂₅ and PEI₁₀. In megalin-expressing cell lines, carboxyalkylated-PEIs conjugated to gentamicin and neomycin exhibited higher transfection efficiencies at C/P > 2 than in the control megalin non-expressing cell line ($P \leq 0.01$, t -test). Among all vectors synthesized with PEI₂₅, PEI₂₅C₅₀G₁₀ and PEI₂₅C₅₀N₁₀ yielded polyplexes with the highest transfection efficiency at C/P = 4 ($P \leq 0.01$, t -test). Increasing aminoglycoside grafting increased gene transfection efficiency at different substitution values (2.5, 5 and 10%) for both the PEI₂₅ and PEI₁₀ series ($P \leq 0.01$, t -test). Comparison of gene transfection levels

between formulations bearing different aminoglycoside percentage showed that An ~13-fold (PEI₂₅C₅₀G₁₀ and PEI₂₅C₅₀N₁₀) compared to those with the lowest level of aminoglycoside engraftment (PEI₂₅C₅₀G_{2.5} and PEI₂₅C₅₀N_{2.5}) at C/P = 2 ($P \leq 0.0001$, *t*-test). A similar 7-fold increase in gene transfection was observed for polyplexes prepared in the PEI₁₀C₅₀ series at the higher level of aminoglycoside engraftment for transfection at C/P = 6 ($P \leq 0.01$, *t*-test). Similar results were obtained comparing the megalin-expressing cell line MDCK with HepG2 as negative control.

3.5.2. Qualitative Assessment of EGFP transfection by fluorescence imaging

EGFP transfection levels were also qualitatively assessed by fluorescent microscopy imaging for HK-2 cells transfected at different C/P ratios with polyplexes prepared with each vector. Photomicrographs of cell cultures treated with polyplexes having the highest transfection efficiency are shown in Figure 11 (A and B), and compared with HK-2 cultures treated with polyplexes prepared from underivatized PEI₁₀ and PEI₂₅. The highest EGFP transfection levels were observed in HK-2 cells treated with polyplexes prepared with PEI₂₅C₅₀G₁₀ (Figure 11).

3.6. Evaluation of the megalin-specificity of aminoglycoside antibiotics conjugated to carboxyalkylated PEIs

Published studies have indicated that in NK-2 cells albumin competes for megalin when nucleic acid transfection systems that target megalin are used (60). Other studies reported that binding of radiolabeled gentamicin to megalin was significantly decreased in the presence of ethylenediaminetetraacetic acid (EDTA) (a chelating agent for divalent ions including Ca²⁺) and

of receptor-associated protein. With an approximately 70% decrease in the presence of both (28). Cytochalasin D (an endocytosis inhibitor) (61) and maleate (a megalin-shedding agent) also inhibit megalin uptake of gentamicin. Megalin has also been shown to be involved in renal handling of aminoglycosides (62).

Based on these reports studies were undertaken to determine if albumin inhibits transfection of HK-2 cells with polyplexes prepared with EGFP-expressing plasmid DNA plus gentamicin or neomycin conjugated to carboxyalkylated-PEIs. The studies were conducted with a range of concentrations of human serum albumin (0 to 20 mg/mL) and with polyplexes prepared from the carriers with the highest transfection efficiency (PEI₂₅C₅₀G₁₀ and PEI₂₅C₅₀N₁₀). Human serum albumin at the lowest concentration tested caused reduction in percent cell transfected to about half with both gentamicin- and neomycin-based polyplexes (Figure 12). Higher albumin concentrations caused greater reductions in percent cells transfected until at 10 mg/ml and 20 mg/ml transfection was reduced to background levels (Figure 12A). Qualitative assessment of EGFP transfection by fluorescence imaging was also used to demonstrate of megalin-dependence of transfection with polyplexes prepared from aminoglycoside antibiotics conjugated to carboxyalkylated PEIs by completely inhibition of fluorescence with 20/mg/ml added human serum albumin (Figure 12B). The transfection efficiency of both gentamycin- and neomycin-conjugated PEI in HK-2 cells when the pre-incubated cells were treated with different concentrations of human albumin, was reduced after 4 hour incubation with albumin. A complete inhibition of fluorescence with 20 mg/ml of added human serum albumin was observed (Figure 12C).

3.7. *In vivo* transfection efficiency

The transfection efficiency of polyplexes prepared with either PEI₂₅C₅₀ or PEI₂₅C₅₀G₁₀ as a gentamicin derivative, was further compared in vivo in groups of twelve Balb/c mice. PEI₂₅C₅₀G₁₀ was selected for in vivo experiments because it exhibited the highest transfection efficiency at C/P=4 with acceptable cytotoxicity after either 4 or 24 h incubation with cells. The complexes were administered intravenously in Balb/c mice and EGFP activities in kidneys, lungs, spleen and heart were determined 36 h post injection. Significant EGFP gene expression was observed following the administration of PEI₂₅C₅₀G₁₀/pDNA polyplex which resulted in much higher EGFP gene expression compared to polyplex prepared with PEI₂₅ in kidneys (Fig. 13). No EGFP gene expression was observed in heart and spleen. A lower EGFP gene expression was also detected in lung and liver for the PEI conjugated to gentamicin which could be attributed to lower expression of megalin in lung and PEI clearance pathway in liver.

4. *Conclusion*

Megalín functions as one of the most important receptors on proximal tubule epithelial cells in the kidney. Megalín mediates endocytosis of a numerous ligands, such as albumin, low molecular weight proteins and oligocations, including aminoglycoside antibiotics. In this study we prepared a series of novel aminoglycoside-conjugated carboxyalkylated-PEI polycationic derivatives and tested them as non-viral megalín-targeting gene carriers. Polyplexes prepared with gentamicin-conjugated carboxyalkylated-PEIs and neomycin-conjugated carboxyalkylated-PEIs exhibited significantly higher transfection efficiency with the megalín-expressing kidney-derived cell lines HK-2 and MDCK, than with the control megalín non-expressing, non-kidney-

derived cell line HepG2, as well as exhibiting significantly higher transfection efficiency with megalin-expressing cell lines than polyplexes prepared with underivatized polycations PEI₂₅ and PEI₁₀. Specificity for megalin was demonstrated by showing that free albumin added to the medium was an effective inhibitor of the transfection process with polyplexes prepared with gentamicin-conjugated carboxyalkylated-PEI₂₅ and neomycin-conjugated carboxyalkylated-PEI₂₅. Gene transfection efficiency increased with increasing the ligand substitution values. In general, gentamicin derivatives were better transfection reagents than the corresponding neomycin derivatives, suggesting that gentamicin derivatives would be more useful in future studies aimed at developing renal gene targeting therapeutics for clinical applications.

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Figures and table CAPTIONS:

Table 1. Size and zeta potential data for polyplexes prepared with EGFP plasmid DNA and PEI₂₅, PEI₁₀ and gentamicin and neomycin derivatives of carboxyalkylated PEIs.

Figure 1. Schematic illustration of the present research.

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Fig. 5. Titration curves of A) Water and a range of carboxyalkylation levels (10%, 30% and 50%) modification of PEI₂₅; B) PEI₂₅ unmodified, and carboxyalkylated PEI₂₅ with 5% and 10% gentamicin. C) Water and a range of carboxyalkylation levels (10%, 30% and 50%) modification of PEI₁₀, D) PEI₁₀ unmodified, and and carboxyalkylated PEI₁₀ with 5% and 10% gentamicin. The pH was initially adjusted to 12 using 1M NaOH, then pH changes were measured after adding five microliter aliquots of 1M HCl.

Figure 6. Ethidium bromide assay results for A) gentamicin-PEI conjugates of PEI₂₅C₅₀, B) neomycin-PEI conjugates of PEI₂₅C₅₀, C) gentamicin-PEI conjugates of PEI₁₀C₅₀, D) neomycin-PEI conjugates of PEI₁₀C₅₀. Condensation of EGFP plasmid DNA aminoglycoside-PEI derivatives was measured as decreased fluorescence of ethidium bromide dye intercalated in DNA.

Figure 7. Atomic force microscopy (AFM) micrographs of polyplexes formed with EGFP plasmid DNA and (A) PEI₂₅C₅₀G₁₀ polyplexes or (B) PEI₁₀C₅₀G₁₀ at C/P = 2.

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Figure 9. Comparison of the cytotoxicity of polyplexes prepared from underivatized PEI₂₅ and PEI₁₀ with polyplexes prepared from aminoglycosides conjugated to carboxyalkylated-PEIs in megalin-positive cells (HK-2) and in megalin-negative cells (HepG2). A: Gentamicin conjugated to carboxyalkylated-PEI₂₅ (Aa, Ac); gentamicin conjugated to carboxyalkylated-PEI₁₀ (Ab, Ad). B: Neomycin conjugated to carboxyalkylated-PEI₂₅ (Ba, Bc); neomycin conjugated to carboxyalkylated-PEI₁₀ (Bb, Bd). Polyplexes were prepared with plasmid DNA encoding EGFP at the indicated C/P ratios. Cells were treated with polyplexes for four hours under the same conditions used in transfection assays, and then the medium changed to fresh medium without polyplexes. Cell viability was assessed at 24h using the MTT assay. Polyplexes with significantly lower cytotoxicity than polyplexes prepared the underivatized PEI₂₅ and PEI₁₀ controls are indicated by * for P<0.05 or ** for P<0.01 and *** for P<0.001 (t-test).

Figure 10. Transfection activity of polyplexes prepared with EGFP-expressing plasmid DNA and gentamicin or neomycin conjugated to carboxyalkylated-PEI₂₅ and carboxyalkylated-PEI₁₀ (please see supplementary) compared to that of polyplexes prepared with underivatized PEI₂₅ and PEI₁₀. Transfection activity of polyplexes prepared with a) gentamicin-conjugated carboxyalkylated-PEI₂₅ in HK-2 cells; b) neomycin-conjugated carboxyalkylated-PEI₂₅ in HK-2 cells; c) gentamicin-conjugated carboxyalkylated-PEI₂₅ in HepG2 cells; and d) neomycin-conjugated carboxyalkylated-PEI₂₅ in HepG2 cells. HK2 was used as a megalin-expressing (positive control) cell line, and HepG2 was used as a megalin non-expressing (negative control) cell line. Please see supplementary for seeing transfection activity of polyplexes prepared with EGFP-expressing plasmid DNA and gentamicin or neomycin conjugated to carboxyalkylated-PEI₁₀ compared to that of polyplexes prepared with underivatized PEI₁₀. Numbers of EGFP-expressing cells are represented as mean $\pm$ SD of triplicate measurements. *for $P < 0.05$ or ** for $P < 0.01$ and *** for $P < 0.001$ (t-test).

Figure 11. Fluorescent imaging of EGFP expression in viable HK-2 megalin-expressing cells transfected with polyplexes prepared with EGFP-expressing plasmid DNA and each vector used in the study. Photomicrographs are presented showing green fluorescence expression in HK-2 cells 48 h after transfection with polyplexes prepared from EGFP-expressing plasmid DNA and different polyplexes in different C/Ps.

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assessment of EGFP transfection by fluorescence imaging used to demonstrate the inhibitory effect of albumin, an important megalin-binding ligand, on gene transfection in HK-2 cells by polyplexes prepared with the two most effective vectors prepared in the study, PEI₂₅C₅₀G₁₀ and PEI₁₀C₅₀N₁₀. C) Gene transfection efficiency of polyplexes prepared with gentamicin-carboxyalkylated-PEIs and with neomycin-carboxyalkylated-PEI in pre-incubated HK-2 cells with a range of human serum albumin concentrations. Cells were treated with different concentrations of human albumin for 4 hours, then washed three times with PBS and transfected using the method described for quantitative measurement of transfection efficiency.

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PEI ₂₅ and derivatives	Zeta potential (mv ± SD)	Size (nm ± SD)	PEI ₁₀ and derivatives	Zeta potential (mv ± SD)	Size (nm ± SD)
Gentamicin Derivatives					
PEI ₂₅ C ₅₀ G _{2.5}	23.8±1.8	163.9±6.7	PEI ₁₀ C ₅₀ G _{2.5}	15.2±1.0	170.3±5.3
PEI ₂₅ C ₅₀ G ₅	26.9±1.3	156.9±6.6	PEI ₁₀ C ₅₀ G ₅	18.9±1.8	166.6±11.8
PEI ₂₅ C ₅₀ G ₁₀	29.4±1.3	143.6±8.4	PEI ₁₀ C ₅₀ G ₁₀	19.3±2.1	170.9±13.4
Neomycin Derivatives					
PEI ₂₅ C ₅₀ N _{2.5}	19.1±2.4	157.1±12.4	PEI ₁₀ C ₅₀ N _{2.5}	14.3±1.7	173.8±3.2
PEI ₂₅ C ₅₀ N ₅	22.0±1.3	158.3±7.7	PEI ₁₀ C ₅₀ N ₅	18.1±3.1	171.9±4.1
PEI ₂₅ C ₅₀ N ₁₀	27.6±1.0	153.2±4.2	PEI ₁₀ C ₅₀ N ₁₀	19.3±4.0	163.2±5.5
Underivatized					
PEI ₂₅	26.2±1.3	200.3±27.3	PEI ₁₀	27.7±1.6	205.0±9.1

1- Megalin Mediated Endocytosis; 2- Proton Sponge Effect; 3- Endosomal Escape EGFP Gene Expression

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