Efficient megalin targeted delivery to renal proximal tubular cells mediated by modified-polymyxin B-polyethylenimine based nano-gene-carriers

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Non-viral vectors have attracted great interest, as they are simple to prepare, easy to modify and relatively safe, compared to viral vectors. Kidney-targeted gene delivery systems depict a promising technology to improve drug efficacy in renal diseases treatments. In order to develop a novel kidney-targeted gene delivery system, we synthesized polyamine-PEI conjugates using polymyxin B as ligand and investigated their potential targeting efficiency. After grafting either PEI25 kDa or PEI10 kDa with polymyxin B through amidation reaction, the modified-polymyxin-PEI/DNA nanoparticles were produced via electrostatic attraction between the cationic polymers and EGFP plasmid. The properties of modified polymers including size, surface charge density, DNA condensation ability, buffering capacity and cytotoxicity were evaluated. Results revealed that the average size of modified-polymyxin-PEI25 kDa was about 143–180 nm and modified-polymyxin-PEI10 kDa 115–194 nm. The zeta potentials were in the range of 16.4 ± 1.87 to 23.43 ± 1.25 mV and 11.3 ± 1.4 to 19.3 ± 2.1 mV for conjugates based on PEI25 and PEI10 respectively. The AFM images revealed that the complexes were spherical and nano-sized at C/P = 4. The buffering capacity of both PEI 10 and 25 kDa increased as the percentage of polymyxin B grafting increased. In vitro study demonstrated that modified-polymyxin-PEI conjugates could remarkably improve the gene transfection efficiency to kidney cells. The transfection efficiency of the polyplexes was dependent on the weight ratio of ligand in the formulation (~12 and 8 fold increase for PEI25 and PEI10 kDa, respectively) and was significantly higher than that of unmodified PEI/DNA nanoparticles. These results suggest that modified-polymyxin-PEI/DNA nanoparticles can effectively target megalin-expressing kidney cells and show improved transfection efficiency and low cytotoxicity in In vitro and in vivo studies. Animal studies confirmed the in vivo study. Thus, these conjugates can be considered as a safe and efficient non-viral therapeutic therapy vector for kidney diseases.

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1. Introduction

Extracellular and intracellular delivery of therapeutic nucleic acid molecules is the biggest hindrance for efficient gene therapy. The aim of the delivery system is to improve the biodistribution and cellular recognition [1]. Cationic liposomes and cationic polymers are two typical classes of non-viral vectors. Compared with viral vectors, non-viral ones are considered promising vehicles for gene therapy because of their low toxicity, biocompatibility, and controllability [2,3], although their low efficacy limits their application as a mature gene delivery system. As many barriers prevent gene materials from reaching their intended target and functioning within cells [4], safe and effective delivery remains an important challenge for the clinical development of non-viral vectors [5,6]. Polycations are effective gene vectors because they possess positive charges that allow them to form complexes electrostatically with plasmid DNA (pDNA) to form stable polyplexes, bind electrostatically to cell surfaces, promote endocytosis and facilitate endosomal escape leading to rupturing of endocytic vesicles by a proton sponge mechanism [7–9]. The cationic polymer, polyethylenimine (PEI) has been extensively studied with different molecular weights, different structures (linear or branched) and chemical modifications for in vitro transfection activity and subsequently used for gene delivery in vivo [10,11]. Its relatively high transfection rates [12] make PEI an excellent
candidate for gene delivery studies. The high molecular weight of the polycations in polyplexes can result in cytotoxicity. Recently, a great enthusiasm for the development of ligand-decorated nanoparticles has been generated by both academia and industry [13]. Despite enormous potential of ligand-decorated nanoparticles in targeted drug and gene delivery systems, there is still a long way to develop formulations for therapeutic applications in clinical trials. It seems that the reason for the clinical limitations is that the development of targeted nanoparticles is complex and multifaceted [14]. An essential feature for the successful targeted NPs depends on the choice of suitable targeting ligands based on ligand biocompatibility, cell specificity, binding affinity, purity, size, charge and ease of modification [15]. Also production cost, scalability and stability are the other important factors that have to be taken into account in the choice of ligands [16]. The main goal in fabrication of novel delivery agents is to achieve therapeutic systems with improved intracellular uptake, high specific targeting along with improved efficacy and low cytotoxicity [13]. Kidney-targeted gene and drug delivery systems represent a promising technology to improve drug efficacy and safety in renal diseases treatment [17]. When designing drug or nanocarriers to treat kidney diseases, it is necessary to identify what kind of cell is going to be targeted. The kidney is a highly differentiated organ composed of a variety of compartments: glomerular, tubular, vascular and interstitial. Thus, the disease targeting is determined by the vector and the targeted site. Kidney poses unique challenges because of its complexity, its filtration barrier and the high rate of blood flow [18]. Millions of nephrons make up the functional portions of each kidney. Since there is no endothelial layer between the epithelial cells and the tubular fluid, proximal tubular epithelial cells (PTECs) in the kidney can be targeted through the tubular lumen. PTECs have large numbers of internalizing receptors on their luminal membranes, which are able to absorb a diverse array of compounds that have been filtered by the glomerulus into the urine. This propensity prevents glucose and proteins to be absent in the urine of healthy people. The glomerulus guard of the tubular lumen sets a limit on the size of the drug carriers. Particles with a hydrodynamic diameter below 5–7 nm are quickly filtered and excreted, and since most drug carriers are considerably larger (10–200 nm), glomerular filtration barrier frequently excludes drug carriers, for example liposomes and antibodies, from accessing the luminal surface of PTECs [19]. Some relatively low molecular weight carriers like protein-based, polymeric and folate-conjugated carriers were reported to be more successful for specific targeting to kidney [20]. Recently researchers have applied different types of receptors and many ligands to enhance targeted delivery of cargoes. Some famous ligand-receptor pairs having been introduced to target the payloads include folate/folate receptor, RGD peptide/integrin αvβ5, NGR peptide/aminopeptidases N receptor, and epidermal growth factor (EGF)/EGF receptor (EGFR) [21,22]. Megalin is a 600-kDa transmembrane protein belonging to the LDL-receptor family discovered 30 years ago by Farquhar and Kerjaschki [23]. As an endocytic multi-ligand receptor, megalin has been identified as a highly expressed receptor in renal PTECs mediating rescue of filtered ligands, including proteins, biomarkers, vitamins and hormones. In cooperation with the receptor protein, cubilin, it also mediates retention of drug, including polymyxin B, aminoglycosides (AGs), and toxins [24]. Megalin, a member of the low-density lipoprotein receptor gene family, is a multi-ligand receptor expressed in the apical membrane of PTECs that plays a central role in the endocytic process. Earlier studies demonstrated the strong affinity of polymyxin B toward megalin. Evidences provided that based on the used drug concentration, the affinity of polymyxin B is even higher than that of gentamicin [25, 26]. The complete mechanism and roles of megalin-associated adaptor proteins in megalin trafficking are largely unknown [27]. Several reports have targeted megalin receptor for the specific delivery of drugs [28], siRNA [29,30] and pDNA [31] using a variety of ligands including antibodies and antibody fragments, aptamers, peptides, sugars, and small molecules. PTECs express high levels of internalizing receptors at their luminal membrane, which are able to mediate endocytosis of filtered proteins and a broad variety of other oligocationic compounds that have been filtered through the glomerulus into the urine [19,20,32]. Furthermore, targeting of therapeutic agents to the proximal tubular epithelial cells in the kidney can be relatively easily achieved from the tubular lumen, since there is no endothelial layer between the epithelial cells and the tubular fluid [20]. Drug targeting to the proximal tubular cells may offer new tools for the treatment of tubulointerstitial fibrosis by reducing toxicity of drug that exert unwanted side effects and/or by increasing the renal efficacy of antibiotic pharmaceuticals [19,33].

Based on the aforementioned reasons and as a part of our interest in the synthesis of nanomaterials and investigation of their applications in diagnosis and treatment of kidney diseases [30] we applied megalin-targeted nanoparticles of modified-polyoxymyxin-PEI conjugates for the delivery of EGFP plasmid to confirm the renal targeting capability of modified-polyoxymyxin-PEI conjugates. In this study, polymyxin B conjugates of carboxyalkylated PEs were prepared which contain: 1) PEI (either 10 or 25 kDa) as a polycation for nucleic acid condensation, 2) 6-bromohexanoic acid to provide low cytotoxicity, better biocompatibility and prolonged circulation, and 3) polymyxin B, as ligand conjugated via 6-bromohexanoic acid spacer to target megalin-positive cells. These included kidney cell uptake and cytotoxicity assays on MDCK as megalin-positive and HepG2 as megalin-negative cell lines (Fig. 1).

2. Experimental

2.1. Materials

Branched polyethyleneimine with an average molecular weight of approximately 10 kDa (PEI 10 kDa), was purchased from Polysciences, Inc. (Warrington, PA, USA). Branched polyethylenimine (b-PEI; average MW = 25 kDa), 6-bromohexanoic acid, polymyxin-B sulphate (Sigma-Aldrich), polymyxin acylase as enzyme (Sigma), Fmoc-chloride (Fluka, 98%), piperidine (Aldrich, 99%) were used as supplied. 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), Dulbecco’s modified Eagle’s medium (DMEM), 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-diphenyltetrazoliumbromide (MTT) were obtained from Sigma-Aldrich (Munich, Germany). Spectra/Por dialysis membranes were purchased from Spectrum Laboratories, Houston, USA. All other solvents and reagents were purchased from Sigma-Aldrich (Munich, Germany) and were of the highest purity available.

2.2. Preparation of modified-PMX-PEI derivatives

2.2.1. Modification of polymyxin-B sulphate with fluorenylmethylcarbonyl chloride (Fmoc)

Fmoc-polymyxin (Fmoc-PMX) was prepared in aqueous medium. A mixture of fluorenylmethylcarbonyl chloride (200 mg) and 1 M Na2CO3 aqueous (5 cm3) was added to a solution of polymyxin-B sulphate (500 mg in 10 cm3 water) and was stirred at room temperature overnight. Fmoc-PMX was insoluble in water and it was filtered off and washed with water several times to remove unreacted reagents [34,35].

2.2.2. Enzymatic treatment of Fmoc-PMX

In order to remove the terminal alkyln chain of PMX, we used polymyxin acylase according to a previously reported method [34]. Briefly, 50 mg Fmoc-PMX in 16 ml Tris HCl (50 mM) was mixed with 2 ml polymyxin acylase (1 mg per 1 ml PBS pH 8.0) and stirred at 25 °C overnight. After the reaction completion, the insoluble final product was separated by filtration. The unreacted reagents were removed by washing several times with PBS.
2.2.3. Synthesis of carboxyalkylated derivatives of PEI

To prepare derivatives with 6-bromoalylacarboxylic acid, 10 kDa branched PEI (500 mg) was dissolved in dimethylformamide (DMF) and chloroform, respectively. To achieve the desired degree of modification, DMF solutions containing the proper amounts of 6-bromoalylacarboxylic acid based on 10%, 30% and 50% mol percentage were added dropwise to a stirred PEI solution at room temperature over a period of 3 h. The reactions were allowed to proceed at room temperature for 48 h. The chloroform was removed using a rotary evaporator, followed by storage overnight at room temperature under vacuum to remove the residual chloroform. The crude products were dissolved in water and dialyzed against 0.25 M NaCl through dialysis membranes with 8000 and 10,000 Da cut-off for modified PEI25 kDa. Then, the mixtures were dialyzed twice against water to remove unreacted alkylating agent followed by lyophilization.

2.2.4. Polymyxin B coupling to carboxyalkylated PEIs

The primary amine groups lost through carboxyalkylation of either PEI10 or PEI25 kDa were extensively replaced by free amine groups on polymyxin B after amide bond linkage between a carboxylate end group of a grafted alkyl chain and an amino group on the polymyxin B. Carboxyalkyl-PEIs (10 or 25 kDa) were covalently coupled to polymyxin B using HOBt and EDC as coupling agents. Briefly, the calculated amounts of the ligand 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 min stirring at room temperature. The polymyxin B 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 (6000–10,000 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). The degree of substitution of primary amines on PEI was determined by 2,4,6-trinitrobenzensulfonic acid (TNBS) assay. Synthesized conjugates are abbreviated as PmCnPMXz in which m shows the molecular weight of PEI (kDa), n indicates the molar percentage of carboxyalkyl chain and z shows the molar ratio of polymyxin in the formulation.

3. Characterization

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 to disk for IR study.

3.2. Nuclear magnetic resonance (NMR) spectroscopy

Chemical conjugation of polymyxin B to carboxyalkylated PEIs was confirmed by NMR spectroscopy. ¹H NMR spectra of purified products were recorded using Bruker 300 MHz instrument (USA).

3.3. TNBS assay

The amount of primary amines of carboxyalkylated PEI (6-bromohexanoic acid derivatives) was determined by 2, 4, 6-trinitrobenzensulfonic acid (TNBS) using the standard method [36]. The degree of grafting was measured by the differences in the amounts of primary amines on unmodified PEI10 or PEI25 kDa compared to polymyxin B conjugated PEIs.

3.4. Buffering capacity measurement

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

DNA condensation ability of polypelexes was evaluated by 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 HBG complexed with pDNA (5 μg) was set at 100% and the fluorescent intensity of the EtBr solution in the presence of free plasmid corresponds to 0% condensation. The decrease in EtBr fluorescence intensity following addition of PEI or a derivative indicates its interaction with the plasmid. The results were reported as mean ± S.D., n = 3.

3.6. Particle size and zeta potential measurements

The average particle size of vector/pDNA complexes was determined using Dynamic Light Scattering (DLS) on a Malvern Nano ZS instrument (Malvern instrument, UK). Scattered light was detected at 173° angle. The N/P ratio of polypeplex preparation was varied preparing a range of concentrations of conjugates in HBG buffer (HEPES-buffered glucose containing 20 mM HEPES, 5% glucose, pH 7.4), which were each added to an equal of DNA solution containing 5 μg plasmid in the same buffer, mixed by gently pipetting and incubated for 30 min at room temperature. Various amounts of cationic polymers were diluted in 125 μL 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 ± S.D. Each mean represents the average value of 30 measurements.

3.7. Atomic force microscopy measurement

The size and morphology of nanoplexes at C/P = 4 (carriers/pDNA) were also characterized by atomic force microscopy (JPK NanoWizard II instrument, Berlin, Germany). 20 μL of PEI25C50PMX10 stock (0.25 mg/mL), which showed the highest gene transfection efficiency, was deposited onto the center of a freshly split untreated 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.

3.8. In vitro study

3.8.1. Cell culture

MDCK, Madin-Darby canine kidney (ATCC CCL-3); HepG2, human liver carcinoma Hep G2 (ATCC HB-8065) cells were cultured at 37 °C in a humidified 5% CO2 atmosphere in DMEM supplemented with 10% FBS, 100 U/mL penicillin for 72 h, medium refreshed after 48 h and confluence was achieved after 3–4 days.

3.8.2. Cytotoxicity assay

The cytotoxic effects of the polypeplexes on 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 × 104 cells per well in 100 μl DMEM or RPMI media with 10% FBS, cultured 24 h, polyplexes prepared at different carrier:plasmid weight ratios (C/P) (C/P: 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 serum containing (10% FBS) growth medium and incubated for additional 24 h. 20 μL of MTT (5 mg/mL in phosphate buffered saline) was added to each well, plates were incubated at 37 °C for 2 h, the media were removed and 100 μL DMSO was added to dissolve formazan crystals. The absorbance was measured at 570 nm (reference wavelength 630 nm) using a microplate reader (infinite NanoQuant M200, Tecan, treated cells related to that of in control cells (Cell viability = A_treated / A_control × 100) and expressed as the mean ± SD of triplicate.

3.8.3. Transfection experiment

Transfection efficiency of synthesized nanocarriers was evaluated both quantitatively using flow cytometry and qualitatively using fluorescent live cell imaging. For cell transfection, all cell lines (MDCK and HepG2) were seeded at a density of 4 × 104 cells/well in 24-well plates and cultured for 24 h. Cells were treated with polyplexes in serum free medium containing 3 μg pEGFP at the same C/P ratios [(w/w)] used for the toxicity experiments. After 4 h of incubation at 37 °C, the medium was removed, replaced with 400 μL of serum supplemented medium. Subsequently, the incubation continued for additional 48 h similar to the growth condition. Cell transfection was quantitatively determined using FACS-Calibur flow cytometer (BD biosciences, 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 PBS saline solution. The cells subsequently resuspended in buffer saline solution and were analyzed in FACS-Calibur flow cytometer. All analyses were performed at a low flow rate (20 mg/mL) 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. The data analysis was conducted using CELL Quest Pro software (BD Biosciences, USA).

3.8.4. EGFP expression

Megalin-expressing and non-megalin-expressing cells were transfected with plasmid coding for EGFP to determine the transfection efficiency of different modified-polymyxin-PEI derivatives [37,38]. To visualize the green protein expression, EGFP plasmid was used for polypeplex formation at different C/P ratios calculated on the basis of 0.4 μg plasmid per well. Cultured cells (8 × 104 cells/well) were treated with polyplexes for 4 h at 37 °C. After replacement of the medium with serum-supplemented fresh DMEM and incubation for 48 h at 37 °C, the EGFP expression in transformed cells was studied qualitatively using in a JuLi Smart Fluorescence Cell Analyzer (Ruskin Technology Ltd., Bridgend, UK).

3.8.5. In vivo gene delivery

Male Balb/c mice (6 weeks old, 20 to 22.3 g) were purchased from Pasteur Institute (Tehran, Iran) and acclimatized before performing according to the Institutional Ethical 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/ECC Directives of European Community. pEGFP plasmid (60 μg/mouse) was complexed with PEI25C50PMX10 that showed optimal transfection activity in vivo 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) [39,40].

3.8.6. Statistical analysis

All statistical analysis was performed using JAMP (version 8). The data were presented as means ± standard deviation (s.d.), and analyzed by the method of analysis of variation (ANOVA) using student's t-test. P values of ≤0.05 were considered significant.

4. Results and discussion

Gene delivery to the kidney is a promising treatment for the intractable renal disease. Recently, systemic side effects of the kidney medicines 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 [40]. 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. [41] 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 formed non-covalent complexes with siRNA, not only protecting siRNA from nucleic breaking down but facilitating endocytic uptake and following endosomal escape after administration [42]. Although the glomerular filtration of compounds may be similar to the extravasation of macromolecules in leaky tumor vessels, the subsequent uptake of compounds in the PTECs is a highly active process. Different receptors and transporters are located in PTECs that can be utilized for the active targeting. However these transporters will not be suitable targets for renal specific uptake of the delivered compound, because they are involved in the uptake of small endogenous molecules and drugs [19]. Furthermore unwanted excretion of the intracellularly accumulated substrates from the cells into the urine or into the renal interstitium, by the same transporter or via a different transporter expressed in the tubular cell membrane is an important disadvantage for using these transporters. In addition, they are not expressed only in the kidney and other organs also express them. Therefore, drug delivery into the PTECs through receptor-mediated endocytosis is an attractive approach. PTECs contain different internalizing receptors facilitating to internalize small molecules and macromolecules like proteins that are filtered into urine [19]. Targeting of medicines to the luminal side of the tubular cells through carriers that attach to these receptors is a suitable and applicable policy to uptake of the medicines. It has been shown that uptake of substrate from the tubular lumen side of PTECs is often mediated by two receptors, megalin and cubulin, which are responsible for the receptor-mediated endocytic uptake of various substances from the glomerular ultrafiltrate. Megalin, in cooperation with the receptor protein cubulin, mediates the rescue of ligands, including proteins like albumin, biomarkers, vitamins, hormones, and medicines like aminoglycosides and polymyxin B. Aminoglycosides are taken up in the proximal tubules (SI and S2 segments) by a receptor-mediated mechanism as demonstrated by electron microscopic localization of aminoglycosides in coated pits and endocytic compartments [43]. In our previous work we synthesized aminoglycoside-carboxyalkylated-PEI that significantly increased EGFP gene transfection efficiency in vitro and in vivo [33]. The transfection efficiency of polyplexes was highly dependent on the weight ratio of aminoglycoside-containing ligand in the carrier, which was inhibited in the presence of a range of concentrations of human serum, which competes for megalin binding, aminoglycoside-carboxyalkylated-PEI-mediated transfection was reduced to background levels. These results also were confirmed in the present work although the transfection efficiency was lower than gentamicin-PEI conjugates.

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 hypoxic band for alkyl chain at 1100 cm\(^{-1}\) disappeared (Fig. 3B) [35]. After treatment with polymyxin acylase, the characteristic bands for alkyl chain at 1100 cm\(^{-1}\) which confirmed by 1HN M R .

4.1. Surface modification of PEIs

It has been reported that some kidney medications have limitations such as low aqueous solubility, short retention time and non-specific distribution, thus in order to improve their therapeutic efficiency, water soluble drug delivery systems with specific renal targeting potential are required [20]. In this study, in order to increase the water solubility features and reduce the cytotoxicity effects of PEI, high molecular weight branched PEIs (either 10 or 25 kDa) were initially modified by carboxyalkylation of primary amine groups at the molar ratios calculated to give 10%, 30% and 50% primary amine substitutions (Fig. 2). We selected 6-bromohexanoic acid as the linker in different percentages (10%, 30% and 50%), because the optimal alkyl chain length was found to be six carbons (hexanoate) in our previous study [36]. These modifications also provided terminal functional carboxylate groups, to which polymyxin B could be attached through amide bond formation in the next step.

4.2. Characterization

4.2.1. FTIR spectroscopy

FTIR spectra of Fmoc-PMX product showed the characteristic peaks of Fmoc at 1250 cm\(^{-1}\), 2934 cm\(^{-1}\) and 3040 cm\(^{-1}\) which confirms the protection of amine group with Fmoc (Fig. 3A). Melting point of FmocPMX was 236 °C [34]. After treatment with polymyxin acylase, the characteristic band for alkyl chain at 1100 cm\(^{-1}\) disappeared (Fig. 3B) [35].

4.2.2. NMR spectroscopy

Chemical structure of final products was confirmed by \(^{1}H\) NMR. Spectra of PEI\(_{25}\)C\(_{50}\)PMX\(_{10}\) and PEI\(_{25}\)C\(_{50}\) recorded in D\(_{2}\)O were presented in Fig. 4A-B. CH\(_{2}\) protons of PEI were observed in the range of 2.50–3.00 ppm. Usually CH\(_{2}\) protons in the spectrum of unmodified PEI appeared in 2.50 and 2.60 ppm and shifting of these peaks to lower
magnetic fields in the final products was related to change in proton environment due to alkylation of PEI (Fig. 4A). The signals for protons present in polymyxin B moiety were clearly observed in the range 3.10–4.90 ppm (Fig. 4B).

4.2.3. TNBS assay

The TNBS assay was used to measure (i) the extent of PEI primary amine group substitution by carboxyalkyl moieties; and (ii) the number of primary amine groups added back by coupling of oligoamines to carboxylate groups (Table 1). Synthesis was carried out with amounts of amines calculated for derivatization of 10%, 30% and 50% of the primary amines of PEIs. The results indicated that obtained degree of substitution by carboxyalkyl chain was within the range of 7.5%, 22.47% and 39.9% mol% (PEI10 kDa) and 8.3%, 26.47% and 47.3% (PEI25 kDa) for 10%, 30% and 50% theoretical substitution, respectively. The reproducibility of the chemical synthesis procedures was ascertained by carrying out several independent preparations (Table 1).

4.2.4. Buffering capacity

The proton sponge effect is the main mechanism of endosomal escape in polycation-mediated gene delivery. Polymers with higher buffering capacity showed better transfection efficiency [47]. The buffering capacity

![Fig. 2. Schematic representation of the chemical modification steps used for PEI decoration with polymyxin B.](image)

![Fig. 3. FTIR spectra of A) Fmoc- PMX, and B) Acylase treated Fmoc-PMX.](image)
capacity of PEIs and their derivatives was evaluated by acid titration over a pH range of 2–12 (covering the endosomal pH range of pH 4.5–7.5) via titration of PEI conjugates stock (1 mg/mL) with 1 M HCl solution. Among modified-polymyxin-PEI conjugates, PEI25C50 and PEI10C50 were the most efficient carriers, thus their buffering capacity was determined as shown in Fig. 5. In general, increased amounts of amide bound due to carboxyalkylation of either PEI 10 or 25 kDa resulted in increased buffering capacity at the endosomal pH range. As shown in Fig. 5, buffering capacity increased as the percentage of polymyxin B grafting increased.

4.2.5. Ethidium bromide assay
Ethidium bromide (EtBr) exclusion assay 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. The results of EtBr exclusion presented in Fig. 6(A–D) showed that all PEI derivatives prepared in this study were able to efficiently condense plasmid DNA. For the modified-polymyxin-PEI25 kDa (Fig. 6A, C) and 10 kDa (Fig. 6B, D) conjugates, condensations were completed at C/P ratios of 1.5. The condensing activity of unmodified PEI25 was significantly greater than that of the conjugated vectors at C/P ratio 1.5 (P-value < 0.001). There were no significant differences between condensing activity of unmodified PEI25 kDa and their corresponding conjugates at C/P ratios above 1.5.

4.2.6. Particle size and zeta potential measurements
The size of polyplexes was determined using dynamic light scattering (DLS). The ability of the polyplexes to condense pDNA into nanosized particles is an important property which effects cell uptake efficiency. The size and zeta potential measurements for polymyxin B conjugated carboxyalkylated PEIs were presented in Fig. 7. According to

<table>
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<tr>
<th>PEI derivatives</th>
<th>Theoretical (%)</th>
<th>Observed grafting (%)</th>
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<tr>
<td>PEI25C10</td>
<td>10%</td>
<td>8.3 ± 1.8</td>
</tr>
<tr>
<td>PEI25C30</td>
<td>30%</td>
<td>26.47 ± 2.5</td>
</tr>
<tr>
<td>PEI25C50</td>
<td>50%</td>
<td>47.3 ± 3.08</td>
</tr>
<tr>
<td>PEI10C10</td>
<td>10%</td>
<td>7.5 ± 1.24</td>
</tr>
<tr>
<td>PEI10C30</td>
<td>30%</td>
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<tr>
<td>PEI10C50</td>
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Fig. 4. NMR spectra of A) alkylcarboxylated PEI (PEI25C50) and, B) Modified-polymyxin-PEI 25 kDa conjugate (PEI25C50PMX10).

Fig. 5. Buffering capacities of PEI25 and PEI10 modified with 50% 6-bromohexanoic acid and and PMX 5% and 10%. At first, pH adjusted to 12 using 1 M NaOH then pH changes were measured after adding five microliter aliquots of 1 M HCl.
these results, polymyxin B-carboxyalkylate PEI resulted in smaller nanoplexes in comparison to unmodified PEIs. All resulting nanoplexes (C/P = 4) showed the average size about 143–180 nm (modified-polymyxin-PEI25 kDa), and 115–194 nm (modified-polymyxin-PEI10 kDa) (Fig. 7). The results indicated that addition of polymyxin B ligand to both the carboxyalkylated PEI25 and PEI10 kDa reduced the particle size from 230 to 143 nm and from 205 to 115 nm, respectively. Previously, it has been shown that carboxyalkylated PEI coupled with oligoamines was able to form complexes with diameters of 56–97 nm [36]. Because the polymyxin B was coupled to the carboxyalkylated PEIs, the net positive charge and primary amine density increased, resulting in the formation of nanoparticles with smaller sizes (Fig. 7). It has been reported that adding oligoamine to carboxyalkylated PEI resulted in smaller size nanoparticles [36]. On the other hand, zeta potential of polyplexes was determined by Laser Doppler velocimetry. The zeta potentials were in the range of 16.4 ± 1.87 to 23.43 ± 1.25 mV and 11.3 ± 1.4 to 19.3 ± 2.1 mV for conjugates based on PEI25 and 10 kDa, respectively (Fig. 5). Grafting of polymyxin B to both carboxyalkylated PEI25 and 10 kDa increased the surface zeta potential from 10.5 ± 1.29 to 23.43 ± 1.25 mV and from 7.6 ± 1.6 to 19.3 ± 2.1 mV in PEI25C50PMX10. As expected, by coupling of polymyxin B to carboxyalkylated PEI structures, zeta potential increased to value close
to or even higher than those of unmodified PEIs. This positive charge facilitates electrostatic association of the polyplexes with the negatively charged membranes of cells [48] and thereby enhances the cellular uptake.

4.2.7. Atomic force microscopy measurement

The particle size and morphology of PEI25C50PMX10 and PEI10C50PMX10 nanoplexes were further characterized by AFM analysis (Fig. 8). The AFM images revealed that the complexes were spherical and nano-sized at C/P ratio of 2. The size of PEI25C50PMX10 nanoparticles was 163.2 nm. In overall, all results were in good agreement with the particle sizes obtained by DLS analysis.

4.3. Cell toxicity evaluation

The cytotoxicity of polymyxin B-conjugated PEI was evaluated in MDCK (megalin positive) and HepG2 (megalin negative) cells at different C/P ratios [2,4,6] by measuring their viabilities using commercial MTT colorimetric assay. The cytotoxicity of various PEI derivatives was compared with that of polyplexes formed with either unmodified 10 kDa or 25 kDa branched PEIs (Fig. 9). Unmodified PEI10 kDa and

![Fig. 8. Atomic force microscopy (AFM) micrographs of PEI25C50PMX10 polyplex at C/P = 4.](image)

![Fig. 9. Comparison of the cytotoxicity of polyplexes prepared from underivatized PEI25 and PEI10 kDa with polyplexes prepared from modified-polymyxin-PEIs conjugates in MDCK cells (megalin positive cells) and HepG2 (megalin negative cells). (A): modified-polymyxin-PEI25 kDa conjugates in MDCK cells, (B): modified-polymyxin-PEI 10 conjugates in MDCK cells, (C): modified-polymyxin-PEI25 kDa conjugates in HepG2 cells, (D): modified-polymyxin-PEI10 conjugates in HepG2 cells. Polyplexes were prepared with EGFP plasmid DNA at the indicated C/P ratios. Cells were treated with polyplexes for 4 h under the condition used in transfection assays and then the medium changed to fresh medium without polyplexes. Cell viability was assessed at 24 h using the MTT assay. Polyplexes prepared from modified-polymyxin-PEI s with significantly lower cytotoxicity than polyplexes prepared from underivatized PEI25 and PEI10 are indicated by * for P < 0.05 or ** for P < 0.01 (t-test).](image)
Fig. 10. Transfection activity of polyplexes prepared from PEI25 kDa: A) polymyxin B conjugates in MDCK cells. B) polymyxin B conjugates in HepG2 cells. Transfection activity of polyplexes prepared from PEI10 kDa: C) polymyxin B conjugates in MDCK cells, D) polymyxin B conjugates in HepG2 cells. MDCK and HepG2 cells were used as megalin expressing (positive control) and non megalin expressing (negative control), respectively. Values were represented as mean ± SD of triplicate measurements.

Fig. 11. Live cell fluorescent imaging for evaluation of EGFP expression levels in MDCK cells. Expression of green fluorescent protein in MDCK cells transfected with polyplexes prepared from an EGFP-expressing plasmid DNA and PEI25 kDa (C1), PEI10 kDa (C2) or their polymyxin B derivatives that were most active in gene transfection study. Green protein expression was measured by fluorescent microscopy at incidence of strongly fluorescent cells in sparse cultures 48 h after exposure to the polyplexes. (A1) PEI25-C50PMX10 at C/P = 2, (A2) PEI25-C50PMX10 at C/P = 4, (A3) PEI25-C50PMX10 at C/P = 6, (A4) PEI25-C50PMX5 at C/P = 2, (A5) PEI25-C50PMX5 at C/P = 4, (A6) PEI25-C50PMX5 at C/P = 6. (B1) PEI10-C50PMX10 at C/P = 2, (B2) PEI10-C50PMX10 at C/P = 4, (B3) PEI10-C50PMX10 at C/P = 6, (B4) PEI10-C50PMX5 at C/P = 2, (B5) PEI10-C50PMX5 at C/P = 4, (B6) PEI10-C50PMX5 at C/P = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
PEI25 branched PEIs exhibited the highest toxicities at C/P = 6, in which the cell viabilities decreased to 20 and 40%, respectively. The polyplexes exhibited low cytotoxicity in both positive and negative cell lines. Cell viability for polymyxin B-conjugated carboxyalkylated (50%) PEIs was in the range of 71 to 90% at C/P ratio of 6 showing a significant cytotoxicity reduction compared to unmodified PEIs. However, no significant difference was observed between the cytotoxicity induced among different polymyxin B-conjugated PEIs. It is possible that the presence of free carboxylate groups on the surface of polymyxin-PEI conjugates would reduce the net charge of the PEI derivatives resulting in reduced cytotoxicity.

### 4.4. Transfection efficiency in proximal tubular epithelial cells (PTECs)

#### 4.4.1. Flow cytometry

Megalin–mediated targeted delivery of siRNA has been reported by other researchers [20,29–31]. According to flow cytometry results shown in Fig. 10, the transfection efficiency of polyplexes prepared from both polymyxin B-conjugated PEI25 and PEI10 kDa at different C/P ratios were evaluated in MDCK as a megalin expressing cell line, as well as HepG2 as non-megalin expressing cells using EGFP as reporter gene. The results were compared to those obtained with unmodified PEI25 and PEI10 kDa polyplexes. The data demonstrated that the modified PEIs conjugated to polymyxin B showed enhanced ability to transfer EGFP into megalin expressing cells at C/P ratio above 2. Among all vectors synthesized based on PEI25 kDa, PEI25C50PMX10 yielded polyplexes with the highest transfection efficiency at C/P = 4 (P ≤ 0.01, t-test). Increasing the ligands grafting increased gene transfection efficiency at different substitution values (2.5, 5 and 10%) for both PEI25 and PEI10 kDa series (P ≤ 0.01). According to the results obtained with polyplexes prepared with PEI25C50PMX10 (C/P = 4), as the engraftment percentage of the ligands increased, the significant improvement was observed in efficiency of gene transfection for both PEI25 and PEI10 kDa conjugates (P ≤ 0.01). Comparison of gene transfection efficiency levels between formulations bearing different ligand percentage showed that about 12 folds increase in gene transfection for PEI25C50PMX10 (C/P = 2) was obtained, in comparison with PEI25C50PMX2.5 (P ≤ 0.0001). The same increasing trend was observed for polymyxin B conjugates of PEI10C50-based carriers as PEI10C50PMX2.5 exhibited higher transfection efficiency about 8 folds at C/P = 6 (P ≤ 0.01). These results were observed in MDCK as positive cells whereas in HepG2 as negative control, increasing of the polymyxin B ligands had no significant effects on gene transfection efficiency (P ≤ 0.01).

#### 4.4.2. Live cell fluorescence imaging

Transfection expression levels were qualitatively illustrated by fluorescent microscopy imaging when EGFP plasmid was applied as reporter gene. Images were taken for each polyplex at different C/P ratios. The polyplexes with the highest transfection efficiency were only shown, and the results were compared with corresponding PEI25 and PEI25 kDa. The results were shown in Fig. 11 (A and B). As it is clearly illustrated, PEI25C50PMX10 (Fig. 11A1–A3), and PEI10C50PMX10 (Fig. 11B1–B3), transferred EGFP more efficiently than corresponding PEI25 and PEI10 kDa.

#### 4.5. In vivo transfection efficiency

The transfection efficiency of polyplexes prepared with either PEI25C50 or PEI25C50PMX10 as a Polymyxin derivative, was further compared in vivo in groups of twelve Balb/c mice. PEI25C50PMX10 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, lungen, spleen and heart were determined 36 h post injection. Significant EGFP gene expression was observed following the administration of PEI25C50PMX10/pDNA polyplex which resulted in much higher EGFP gene expression compared to polyplex prepared with PEI25 in kidneys (Fig. 12). In our previous work we showed a higher EGFP expression in PEI25C50G10. That it confirmed gentamicin is a more efficient ligand for targeting PTECs in the kidney. 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 Modified polymyxin B which could be attributed to lower expression of megalin in lung and PEI clearance pathway in liver.

5. Conclusion

In many conditions, kidney diseases requires, long term administration of therapeutics; consequently, they are accompanied with systemic toxicities either adverse side effects. Therefore developing a kidney targeted drug delivery system for overcoming these obstacles is necessary in treatment of kidney disease. Some efforts have been conducted aiming to target the kidneys. A majority of kidney- specific delivery systems targets renal proximal tubular cells to treatment of renal disease. Megalin functions as one of the most important receptors on PTECs in the kidney.

For an efficient polymeric delivery system, it is essential to precisely deliver the gene/polymer complex (polyplex) or drugs into a targeted tissue or organ. To solve this issue, we have demonstrated that
polymyxin B as a decorating ligand on polycation could efficiently target the specific receptor, megalin, overexpressed on PTECs. In this study, for the first time, we successfully prepared a series of modified-polymyxin-PEI polycationic conjugates as a novel, non-viral megalin targeting gene carrier. By mediating endocytosis of a numerous ligands, such as aminglycosides and polymyxin B, and low molecular weight proteins, megalin functions as one of the most important receptors in EPTCs. Ad-dition of megalin targeting ligands to PEI25 and PEI10 kDa improved gene transfection efficiency in PTEC cell lines. Modified-polymyxin-PEI/DNA complexes could be specifically delivered into PTECs. Gene transfection efficiency increased with increasing the ligand substitution values. Therefore, polymyxin B can be applied as a small molecule ligand with high efficiency and safety for renal gene targeting that holds promise in using upregulates genes for clinical application. To the best of our knowledge, no other research has reported the use of polymyxin B as a targeting ligands for proximal tubular epithelial cells (PTECs). Polymyxin in B ligand can offer advantages over macromolecules, including well-defined molecular structures, low cost and an extensive history of use in therapy. Additionally, combination of other pharmaceuticals to polymyxin B may also facilitate the development of treatments for kidney disease.

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