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Synthesis and characterization of oligo aminoglycosides and polyethylenimine conjugates as polymeric gene carriers

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Abstract−In this study, a new kind of antibiotic drug-polymer conjugate, the kanamycin-methyl acrylate-polyethylenimine (KMP) polymer, was synthesized and characterized as gene carrier. Previous reports have shown that the modification of low molecular weight polyethylenimine (LMW-PEI) improves the transfection efficiency and decreases the toxicity. Kanamycin can be hydrolyzed under an acidic environment owing to the glycosidic bond. In this physicochemical study, the properties of the kanamycin-based hybrid polymers conjugated with various LMW-PEIs, such as 0.6, 1.2, and 2 kDa polyethylenimine (PEI), were investigated by ^{1}H NMR, FT-IR, and their buffering capacity was assayed by acid-base titration method. Gel electrophoresis and picogreen assay were performed to evaluate the ability to bind with plasmid DNA. The polyplexes were nanosized with a net positive charge. The cytotoxicity and transfection efficiency were evaluated using human cervical carcinoma (HeLa) cells. KMPs exhibited lower toxicity than PEI 25 kDa, and higher transfection efficiency compared to the native LMW-PEIs.

Keywords: Kanamycin, PEI, Transfection, Gene Delivery, Polyplexes

INTRODUCTION

In gene therapy, the development of techniques for stable and efficient gene delivery is crucial to suitably introduce therapeutic genes such as deoxyribonucleic acid (DNA), small interfering ribonucleic acid (siRNA), and messenger RNA (mRNA) into cells for compensating abnormal genes or producing a target protein [1-4]. Over the last decades, gene therapy has been exploited as an alternative treatment of inherited genetic disorders [5-7]. Nowadays, it is applied in various diseases including diabetes, neurodegenerative diseases, and cancer [8-13]. However, genetic material has low cellular uptake and transfection efficiency, because of its high anionic charge density and sensitivity to nuclease.

Viral vectors are well known for their high gene transfection efficacy, but their limitations include inflammation responses and difficulty in production. To overcome these drawbacks, non-viral vectors have been applied as alternative gene carriers [14-16]. Compared to viral vectors, non-viral vectors are safer, but further investigation is necessary to overcome issues such as its cytotoxicity, poor biocompatibility, and limited transfection efficiency [14]. Therefore, several studies focused on the development of non-viral carriers for gene delivery. Polysaccharides, cationic polymers, and liposomes form complexes with DNA, resulting in nanoparticles that can easily uptake into the cell [17-22]. Among the cationic polymers, the branched polyethylenimine 25 kDa (PEI 25 kDa) has been studied in gene delivery because of its high transfection efficiency. Both linear form and branched form of PEI have been applied as gene carriers, because they contain primary, secondary, and tertiary amines that facilitate their effective condensing with plasmid DNA to form nano-sized polyplexes [23]. Moreover, PEI has a high buffering capacity that helps the complexes to be released from endosomes into cytoplasm by the "proton sponge effect" mechanism [24,25]. Moreover, the high molecular weight of PEI contributes to the transfection efficiency. Therefore, PEI 25 kDa is a gold standard polymeric gene carrier. However, this non-degradable polymer is highly toxic for cells [26]. Nevertheless, the low molecular weight PEIs exhibit a low capacity to condense with plasmid DNA and insufficient gene transfection efficiency compared to PEI 25 kDa. To overcome this problem, several studies focused on low molecular weight polyethylenimine (LMW-PEI) cross-linking and chemical modification with functional components. Various branched PEI polymer derivatives were prepared with LMW-PEI using degradable chemical linkage (disulfide, ester, carbamate, amide, and ketal groups) and cross-linkers, such as dithiobis succinimidyl propionate (DSP) and dimethyl 3,3′-dithiobispropionimidate (DTBP) [27,28]. Recently, LMW-PEIs were grafted on LMW-PEI or glycol chitosan, and the conjugate polymers showed enhanced transfection efficacy and lower toxicity to cells compared to PEI 25 kDa [29,30]. Therefore, we developed a new oligosaccharide-based polymer grafted with LMP-PEI as an efficient gene transfection agent. Kanamycin is an aminoglycoside bactericidal antibiotic available in oral, intravenous, and intramuscular forms used to treat a wide variety of bacterial infections. Kanamycin can target the cell membrane by electrostatic and hydrogen bonding. Upon bacterial uptake, kanamycin binds to the ribosome and reduces the mRNA translation followed by the inhibition of protein synthesis. Previous studies showed that kanamycin derivatives are applicable for gene delivery [31-34]. Kanamycin was conjugated with a hydropho-

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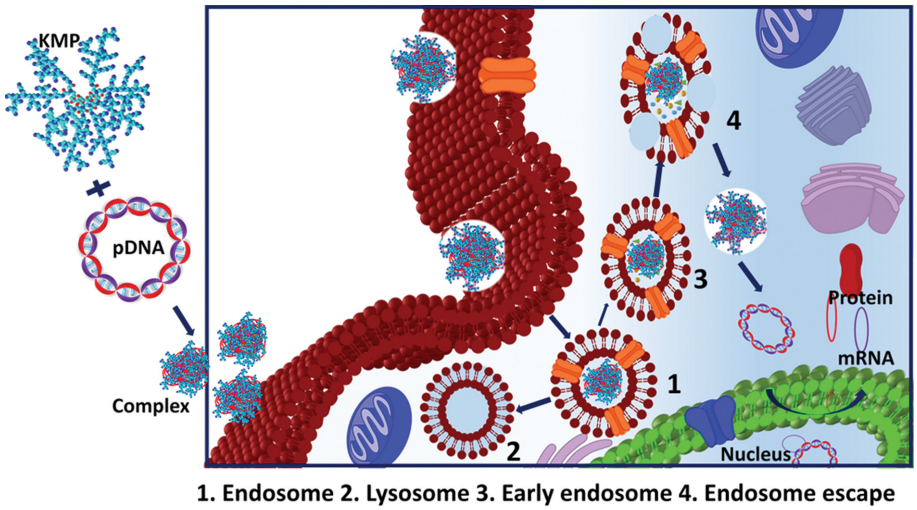


Fig. 1. Schematic of KMPs for gene delivery. First, KMPs form complexes with pDNA by electrostatic charge interaction. Second, polyplexes enter the cell.

bic component to form a cationic liposome [33]. Moreover, kanamycin can be hydrolyzed under an acid environment due to the glycosidic bond [34].

In this study, we developed a new family of cationic conjugated polymers using kanamycin as the biodegradable core. Various low molecular weight PEIs (0.6, 1.2, and 2 kDa PEI) were conjugated with kanamycin to produce KMP derivatives (KMP 0.6, KMP 1.2, and KMP 2). First, methyl acrylate reacted with kanamycin by Michael reaction, followed by amidation reaction with PEI. The amine group of KMPs can bind with DNA to form nano-sized polyplexes (Fig. 1). The physicochemical properties of the polyplexes were studied and in vitro cell assays were examined to demonstrate that the KMP polymer has a potential as a promising biocompatible gene carrier.

MATERIALS AND METHODS

1. Materials

Kanamycin A monosulfate from Duchefa, polyethylenimine (branched 25 kDa), polyethylenimine (branched 2 kDa), polyethylenimine (branched 1,200 Da), ethidium bromide, Methyl acrylate, and Methanol were purchased from Sigma-Aldrich (Seoul, South Korea). Polyethylenimine (branched 600 Da) and polyethylenimine (branched 10 kDa) were purchased from polysciences, Inc. EZ-Cytox reagent was purchased from Daeillab Service (Seoul, South Korea). Luciferase 1000 assay kit and 5X Reporter Lysis Buffer were purchased from Promega (Madison, WI, USA). The Micro BCA Protein Assay kit was purchased from Pierce (Rockford, IL, USA). The luciferase reporter plasmid DNA (pCN-Luci) was prepared as reported previously. Picogreen reagent, Alexa flour 488 TFP, Alexa flour 546, Hoechst 33342 were obtained from Invitrogen (Seoul, South Korea). Fetal bovine serum (FBS), 100X Antibiotic-antimycotic agent and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD, USA). NIH3T3 and HeLa cell lines were obtained from Korea Cell line Bank. Micro BCA™ protein assay kit was purchased from Pierce (Rockford, IL, USA).

2. Synthesis of Kanamycin-Methyl Acrylate-polyethylenimine (KMP)

Kanamycin A monosulfate (10 mg) was desalted and dissolved in 3 mL of water with 27 mL of methanol containing 500-fold molar excess of methyl acrylate. The solution was stirred in a nitrogen atmosphere at 37 °C for 3 days, and the sample was evaporated under reduced pressure to remove the solvent and freeze dry. The sample (KAN-MA) was dissolved in 2 mL water with 18 mL of methanol containing 50-fold molar excess of PEI 2 kDa, PEI 1.2 kDa and PEI 0.6 kDa. The solution was stirred in a nitrogen atmosphere at 37 °C for 3 days, and the sample was evaporated under reduced pressure to remove the solvent. The raw product was dialyzed for 1 day against distilled water using a dialysis membrane (MWCO 3500, Spectra/por). The dialyzed materials were lyophilized and characterized by ^{1}H NMR (400 MHz, D2O) and FT-IR spectroscopy. The synthesis scheme of KMP is shown in Fig. 2.

3. Size Measurement

The size of polyplexes prepared at different weight ratios of polymer/pDNA was measured by Particle size analyzer ELS-Z (Photal, Otuka Electonics, Tokyo, Japan). The measurement was taken in triplicate and evaluated as z-average (size).

4. Agarose Gel Electrophoresis

The polymers were complexed with pDNA at various weight ratio in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). PEI 25 kDa (weight ratio, 2 : 1) and PEI 2 kDa (weight ratio 2, 3, 4, 5 : 1) were used as the control group. KMP at weight ratios 2 : 1, 3 : 1, 4 : 1, 5 : 1, 8, 12, and 16 : 1 were examined for their DNA condensation ability. The samples were incubated for 1 h and electrophoresed on agarose gel (0.7% w/v) containing ethidium bromide (EtBr, 0.5 μg/mL of the gel) at 100 V for 30 min, room temperature.

5. Picogreen Assay

Picogreen reagent was performed using the HEPES buffer and Picogreen reagent (diluted in TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer). Picogreen reagent (200 μL) was added to prepared polyplexes and incubated for 2 min. Fluorescence (Excitation and

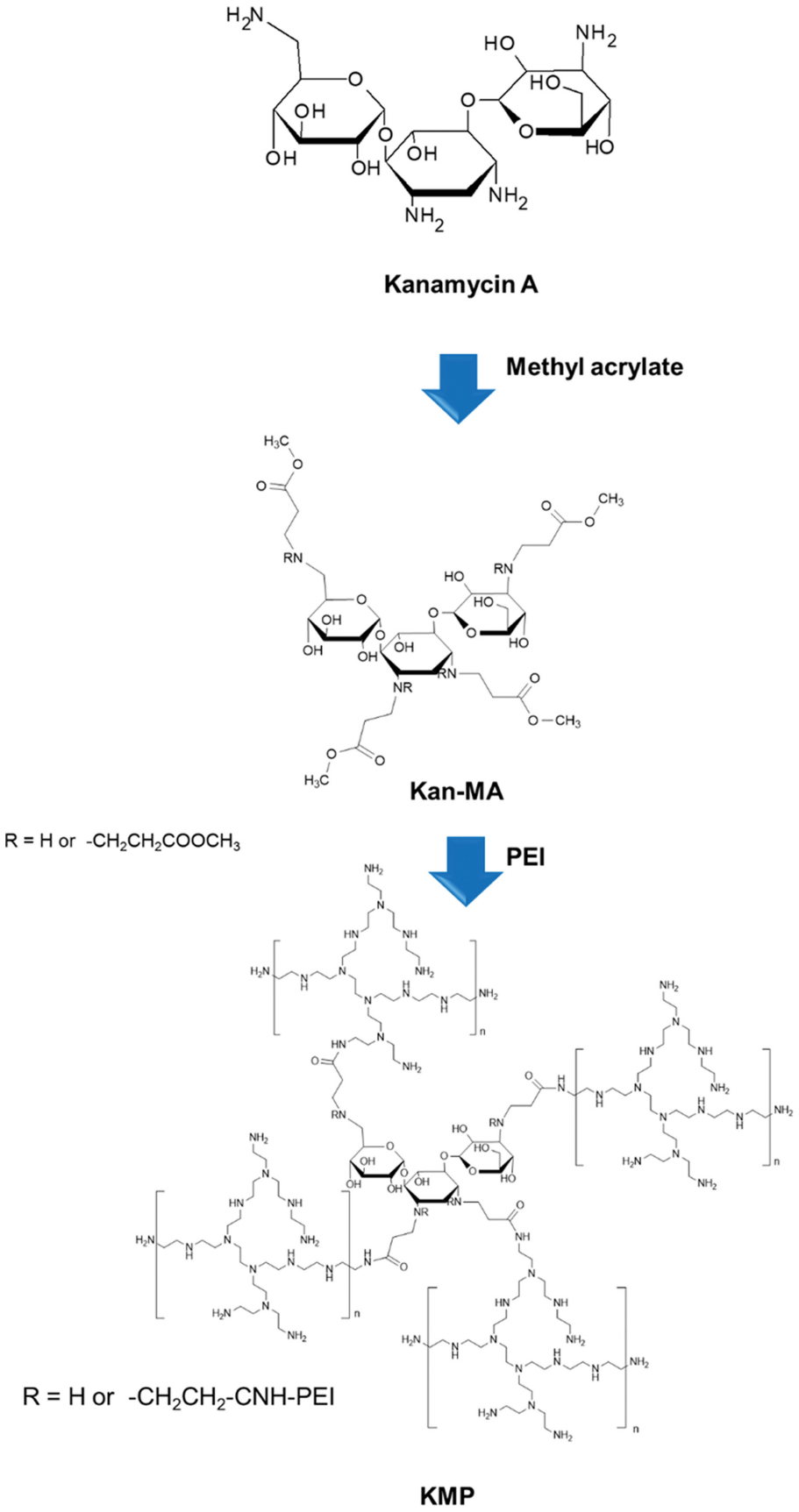


Fig. 2. Schematic diagram of KMP derivatives. The structure of the kanamycin, kanamycin-methyl acrylate (Kan-MA), and the kanamycin-methyl acrylate-polyethylenimine (KMP).

emission wavelengths were 480 nm and 520 nm respectively) was measured with a filter fluorometer (QUANTEC, Thermo Scientific).

6. Plasmid DNA Stability Assay of Polyplexes

Polyplexes were prepared at a certain weight ratio (KMP derivatives/plasmid DNA). Then, each polyplex was incubated with 0.5 unit of DNase I at 37 °C for 1 h. To stop the DNase I reaction, 3 μL of 0.5 M EDTA solution was added. After 5 min, 10 μL of heparin (5 mg/mL) was treated to dissociate polyplexes at 37 °C for 1 h. The polyplexes were analyzed by electrophoresis on a 0.7% (w/v) agarose gel containing ethidium bromide (EtBr, 0.5 μg/mL of the gel) at 100 V for 30 min.

7. Titration

The ability of the polymers to be protonated and become positively charged over the pH range 11-2 was determined by acid-base titration. The solution A (150 mM NaCl 4 mL, 1 N NaOH 0.1 mL) was titrated with 0.1 N HCl to pH 2 as negative control. PEI 25 kDa was used as positive control. The polymers dissolved in solution A (8×10^{-8} M) were titrated with 0.1 N HCl until pH 2 was reached. The pH values were determined using a pH-meter (pH 211 microprocessor pH meter, HANA Instruments, Seoul, South Korea).

8. Cytotoxicity Assay In Vitro

To check the polymer cell viability, colorimetric WST assay was performed. HeLa cells and NIH3T3 were cultured in a Dulbecco's Modified Eagle Medium (DMEM medium) containing 10% (v/v) FBS and 1% (w/v) penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Afterwards, 12×10^3 cells/well was seeded in a 96 well-plate and incubated in 100 μL media for 24 h. Cells were then treated with 10 μL of KMP sample along with positive controls. After 24 h incubation, 10 μL EZ-Cytox reagent was added to each well (except negative control) and incubated 2 h. The absorbance was measured at 450 nm.

9. Confocal Microscopy

Alexa Fluor 546 labeled pDNA and Alexa 488 TFP labeled polymer were prepared according to the manufacturer's protocol. HeLa cells (5×10^3 cells/well) were seeded in confocal dishes (μ slide 8 well, ibiTreat, South Korea) and allowed to adhere in 200 μL DMEM media (10% FBS and 1% w/v penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Polyplexes were prepared at weight ratio 8 : 1 for KMP, whereas PEI 25 kDa and PEI 2 kDa at the weight ratio 2 : 1 and 4 : 1, respectively, were used after 1 h incubation at 25 °C. Then cells were incubated with 10 μL of polyplexes for 4 h and 12 h. The medium was removed and the cells were washed with DPBS twice, the nucleus was stained for 10 min with 100 μL of bisbenzimide (Hoechst 33342). Finally, the cells were washed and examined with a Zeiss LSM 5 Live confocal laser microscope.

10. Flow Cytometry

The quantitative cellular uptake efficiency was determined through FACS analysis using Alexa Fluor 546 labeled pDNA. HeLa cells (3×10^5 cells/well) were seeded in 1,800 μL DMEM media (10% FBS and 1% w/v penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 for 17 h. Polyplexes were prepared as described in paragraph 2.9. After incubating with 200 μL of polyplexes for 4 h and 12 h, the cells were harvested using 0.05% trypsin-EDTA and washed by centrifugation at 4,500×g for 5 min with DPBS. The cells were then dissolved in 500 μL of DPBS, and DNA fluorescence intensity was measured in the cell suspension using a flow cytometer (FACSCalibur, Becton Dickinson, USA).

11. Transfection Experiments

HeLa cells were seeded at a density of 12×10^3 cells/well in 96 well plates and grown in 100 μL of medium containing 10% FBS and 1% (w/v) penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The polyplexes (10 μL) prepared at various weight ratios were injected and incubated for 24 h at 25 °C. The medium was afterward removed and the cells were washed with DPBS and lysed for 30 min at room temperature using 50 μL of Reporter lysis buffer (Promega). The protein content was measured by the Micro BCA assay reagent kit (Pierce, Rockford, IL). The absorption was measured at 570 nm by a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) and compared to a standard curve calibrated with BSA samples of known concentration. The results are expressed as RLU per milligram of cell protein lysate (RLU/mg protein).

RESULTS AND DISCUSSION

1. Synthesis of KMP 0.6 kDa, KMP 1.2 kDa, and KMP 2 kDa

Low-molecule-weights of PEI (PEI 600, PEI 1,200, and PEI 2,000 Da) were first conjugated on the surface of kanamycin A by methyl acrylate link to create the KMP library (Fig. 2). The polymerization of low molecular PEI was performed with kanamycin due to its advantageous abundant positive surface charges. Although high molecular PEI is non-degradable and toxic for the cell, low molecular PEI is not effective for DNA condensing. Kanamycin undergoes hydrolysis under acidic conditions, owing to the degradation of glycolysis bond [34]. Hence, the combination of PEI and kanamycin is suitable to produce the degradation of the KMP polymers to avoid toxicity and enhance the transfection efficiency by improving the condensing with the DNA. The KMP polymer was confirmed by ^{1}H NMR and FT-IR (Fig. 3). Particularly, ^{1}H NMR spectra showed the PEI peak in the range 2-2.5 ppm, with KMPs characterized by peaks in the range 2-4 ppm. KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa exhibited peaks arising from kanamycin, methyl acrylate, and PEI, confirming their successful preparation. The FT-IR confirmed the conjugation bond between PEI and kanamycin. The CO-CH_3 belonging to methyl acrylate was at 1,728.46 cm^{-1} for Kan-MA. However, this peak disappeared after PEI amidation. The amide bond appeared in the range 1,552.81-1,556.43 cm^{-1} in KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa. The vibration bond C-O-C (νC-O-C) of the component containing kanamycin (kanamycin, kanamycin-MA, KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa) was in the range 1,027.90-1,041.04 cm^{-1}. The combination of ^{1}H NMR and FT-IR analyses corroborated the successful Michael addition and amidation reactions in the KMP synthesis. The concentration of primary amine of the KMP derivatives was determined using the ninhydrin test. The 4-5 of LMW-PEI were grafted on the kanamycin core in the case of KMP 2 kDa and KMP 1.2 kDa. The 3-4 of LMW-PEI were attached in the case of KMP 0.6 kDa (Fig. S1, Table S1 and Table S2).

2. Titration

The protonation ability of the polymer is considered a crucial

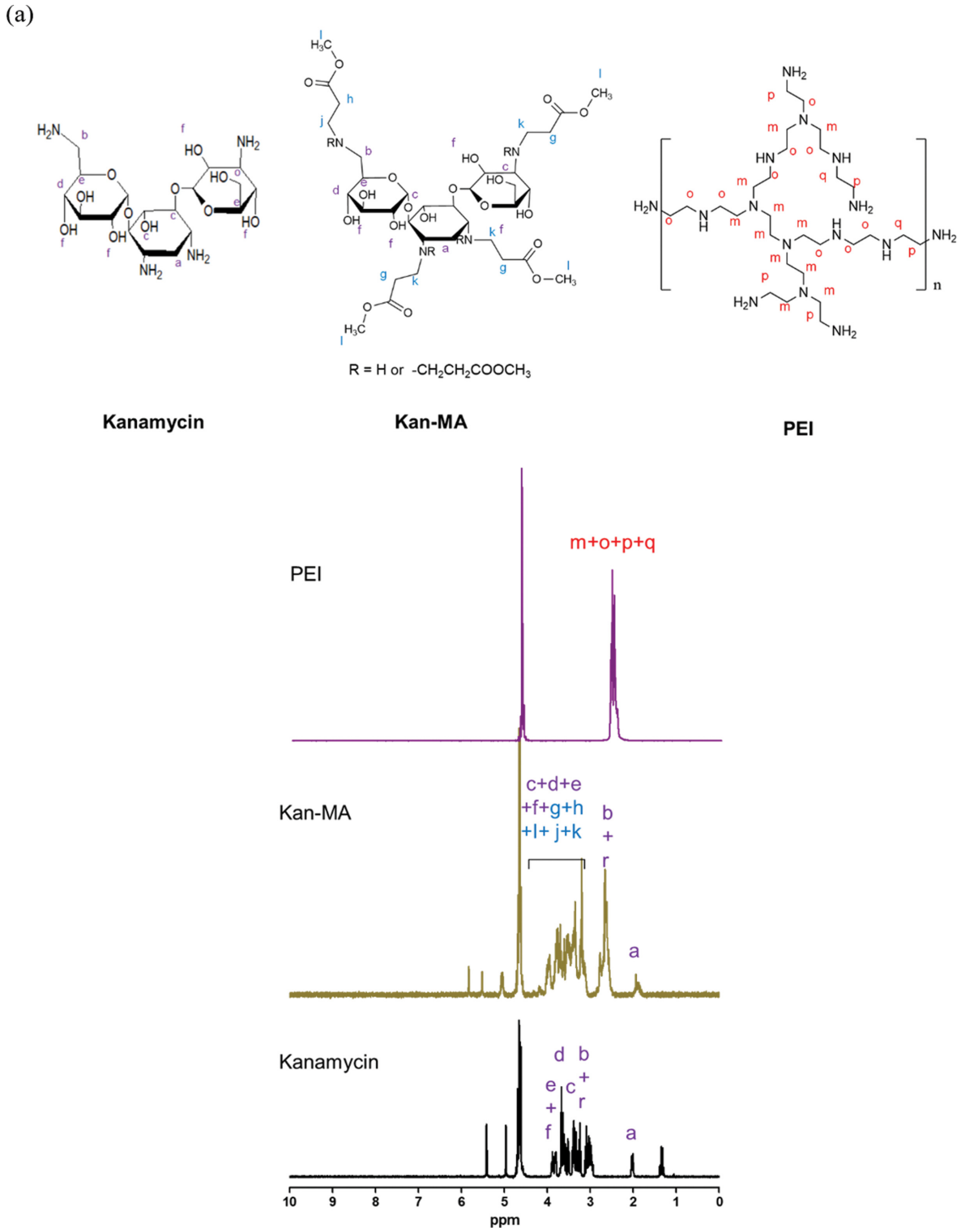
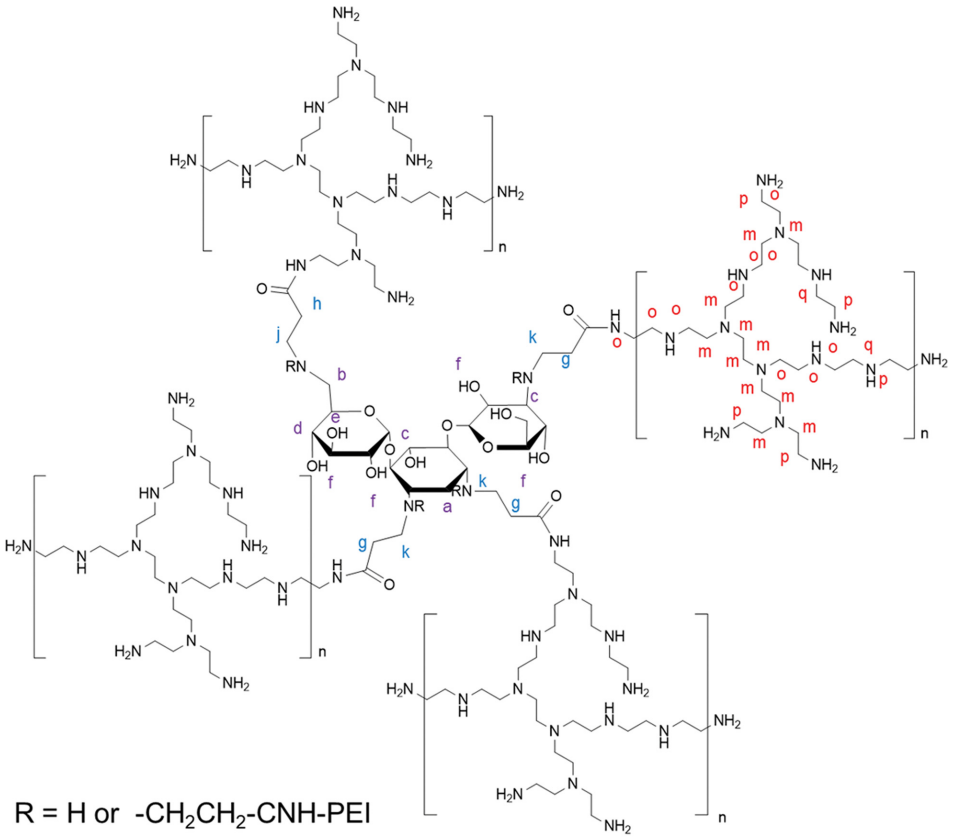


Fig. 3. Characterization of KMP: (a) Proton nuclear magnetic resonance (NMR) data, (b) Fourier-transform infrared spectroscopy (FT-IR) data. The peak of the kanamycin, methyl acrylate, and polyethyleneimine was labeled by purple, blue and red characters.

property of gene carrier candidates because the proton sponge hypothesis explains the endosomal escape of cationic carriers. The proton-buffering capacity of polymers supplies a vital role in high transfection efficiency, because it inhibits the lysosomal nuclease activity and changes the osmotic pressure of acidic vesicles, resulting in endosomal swelling and rupture. Hence, we examined the buffering capacity of KMPs by acid-base titration. The high density of secondary and tertiary amines of PEI contributes to the buffering capacity. PEI 25 kDa was used as a positive control. Fig. 4 showed a slight pH change in the case of PEI 25 kDa, KMP 2 kDa,



KMP

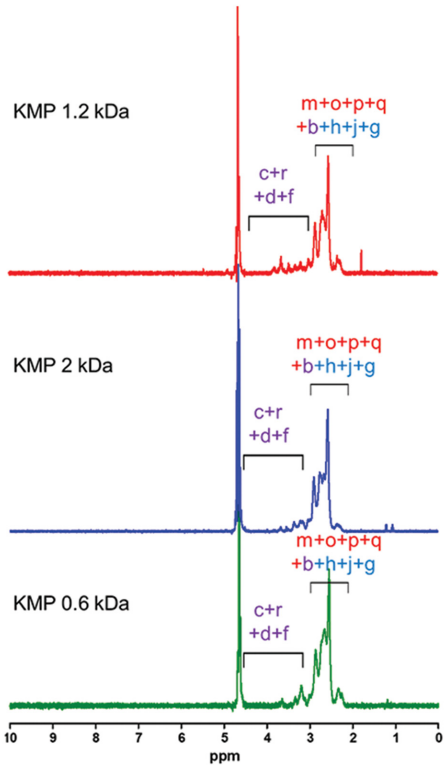


Fig. 3. Continued.

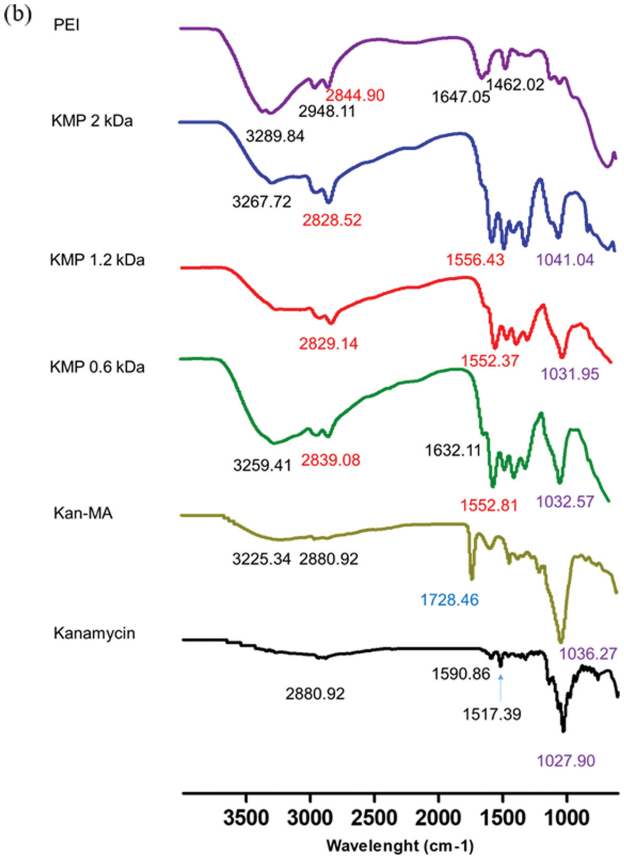


Fig. 3. Continued.

KMP 1.2 kDa, and KMP 0.6 kDa upon HCl addition to the polymer solution. Specifically, KMP 2 kDa and KMP 1.2 kDa showed

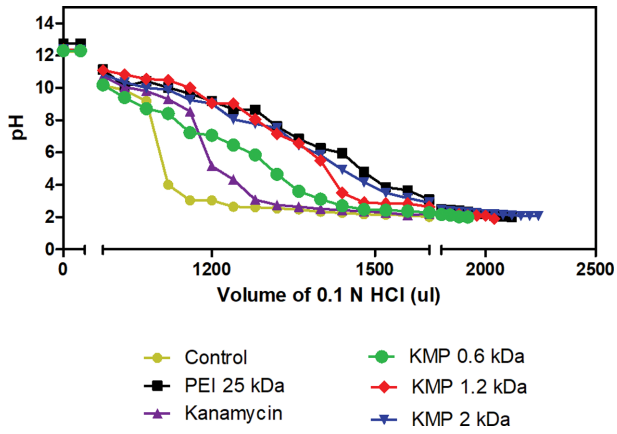


Fig. 4. Acid-base titration profiles of PEI 25 kDa and KMPs in aqueous 0.15 mol/L NaCl solutions (sample 8×10^{-8} M). Abbreviations: PEI, polyethylenimine; KMP, kanamycin-methyl acrylate-polyethylenimine.

almost similar levels with PEI 25 kDa. The buffering capacity is relative to the protonate of the amine group. A high propensity to proton is a strong buffering capacity. The amine from PEI 2 kDa, PEI 1.2 kDa, and PEI 0.6 kDa decreased. Since KMP 2 kDa and KMP 1.2 kDa show higher levels of buffering capacity than KMP 0.6 kDa. The results denoted KMP 2 kDa and KMP 1.2 kDa as promising gene carriers.

3. Complexes Test

The main characteristic of gene carriers is the ability to condense with the DNA. The pDNA binding ability of the KMP polymers was evaluated by agarose gel retardation assay. KMPs were complexed with pDNA at the weight ratios 2 : 1, 3 : 1, 4 : 1, 5 : 1, 8, 12, 16, and 24 : 1 in HEPES buffer and incubated for 1 h. Only DNA

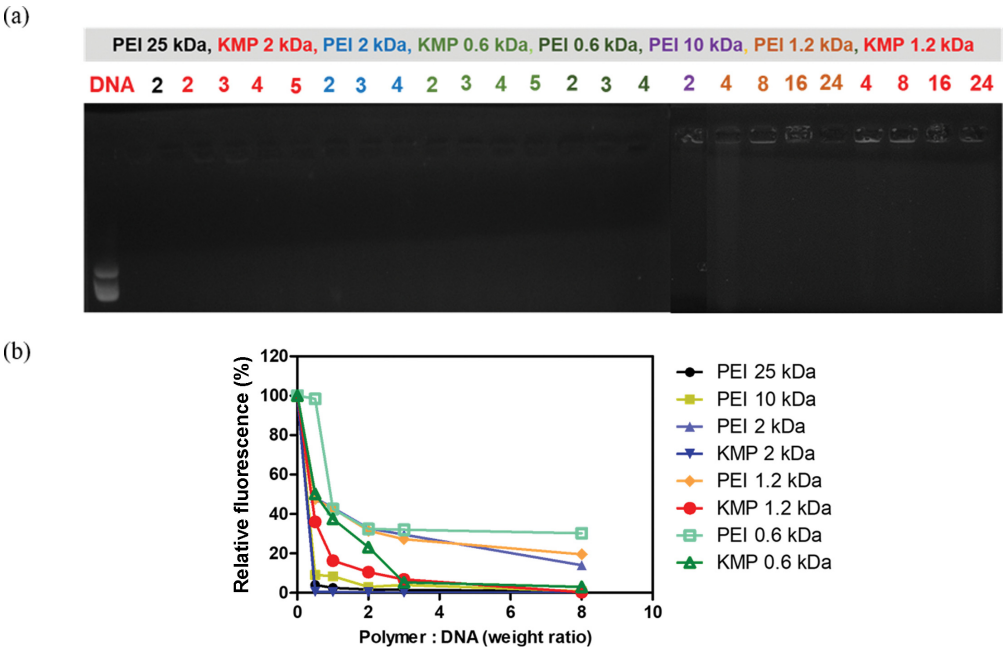


Fig. 5. Complex test of KMP and pDNA. (a) Agarose gel electrophoresis retardation of plasmid DNA by KMP. The labelled number is the weight ratios of polymer/pDNA. (b) Picogreen assay of polyplexes.

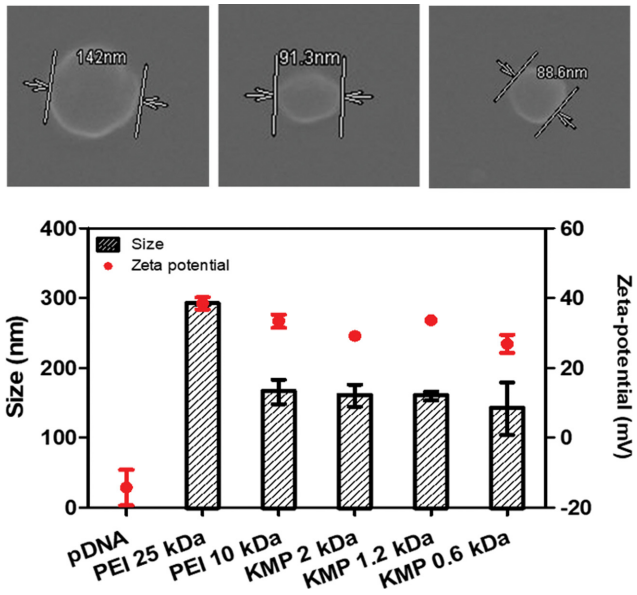


Fig. 6. Polyplex characterization. Morphological analysis of KMP polyplexes using field emission scanning electron microscope (FE-SEM), size and zeta potential.

and PEI 25 kDa (w/w=2) were used at negative and positive control. As shown in Fig. 5, KMPs condensed with DNA at a low ratio. The picogreen was consistent with the gel data. The LMW-PEI PEI 0.6, PEI 1.2, and PEI 2 kDa could not bind to DNA at a low ratio. However, KMPs are able to condense with DNA. The finding is consistent with the LMW-PEI derivatives easily binding to DNA compared to the original LMW-PEI [29,30]. A reasonable explanation of this result resides in the amine content of the polymer.

4. Size, Surface Charge and Shape of Polyplexes

The size, sharpness, and surface charge of the particles directly influence the targeting of nanomaterials [35]. The efficiency of cell uptake, function, and toxicity of the material depends on the size scale. To overcome the blood barrier and pass enhanced permeability and retention (EPR), a small particle size is more effective. Sharpness of the particle was demonstrated relative to the effect of the particle. Positively charged nanoparticles are required owing to their nonspecific interaction with the negatively charged surface protein on the cell membrane leading to cell uptake. However, a high charge density causes toxicity to the cells or other organs due to the interaction with other plasma components. Typically, the appropriate particle size and zeta potential of polyplexes is crucial in cellular uptake and efficient gene transfection. PEI 25 kDa, PEI

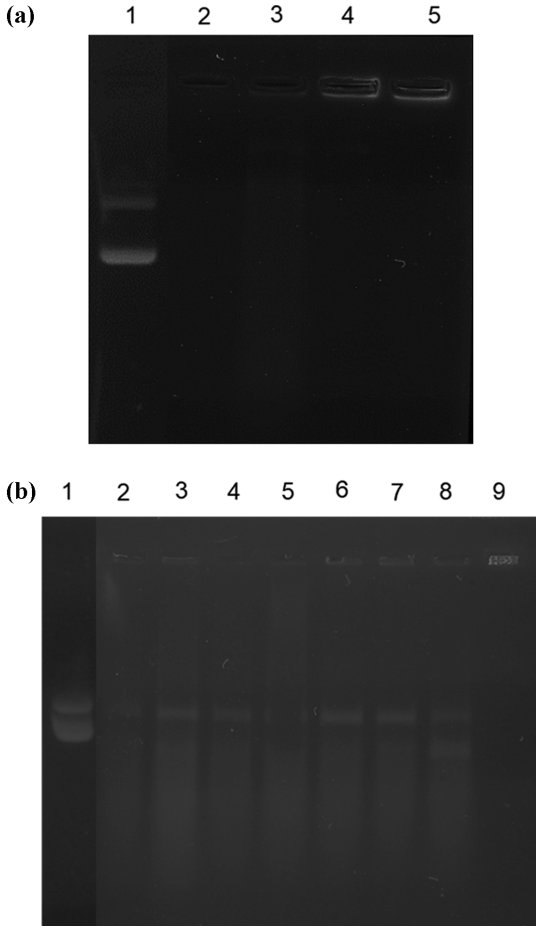


Fig. 7. (a) Stability of pDNA against DNase I degradation. pDNA only (0.5 μg, lane 1), pDNA treated DNase I (lane 2), and polyplexes treated DNases I (KMP 0.6 kDa is lane 3 (Weight ratio of polymer/pDNA=16), KMP 1.2 kDa is lane 4 (Weight ratio of polymer/pDNA=8), and KMP 1.2 kDa is lane 5 (Weight ratio of polymer/pDNA=3)). (b) Stability of pDNA against DNase I degradation plus heparin treatment. pDNA only (lane 1), KMP 2 kDa (lane 2 (w/w=1.5), lane 3 (w/w=2), lane 4 (w/w=3)), KMP 1.2 kDa (lane 5 (w/w=8), lane 6 (w/w=16), lane 7 (w/w=32)), and KMP 0.6 kDa (lane 8 (w/w=32), lane 9 (w/w=50)).

10 kDa, KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa formed positive nanopolyplexes with pDNA (Fig. 6 and Table 1). Spherical nanoparticles were observed with field emission scanning electron microscopes (FE-SEM) (Fig. 6).

Table 1. Characterization of KMP polyplexes

Sample	Zeta potential (mV)	Size (nm)	Polydispersity
pDNA	−14.26±5.09	-	-
PEI 25 kDa	38.5±1.71	291.9±1.35	0.26±0.02
PEI 10 kDa	33.40±1.90	165.73±17.5	0.31±0.02
KMP 2 kDa	26.93±0.83	160.27±15.81	0.30±0.02
KMP 1.2 kDa	33.73±1.35	159.83±6.22	0.29±0.02
KMP 0.6 kDa	26.93±2.57	141.57±37.53	0.48±0.02

5. Stability Test of Plasmid DNA in Polyplexes

The stability of the plasmid DNA is one of the prerequisite requirements for the construction of a non-viral vector for gene delivery. Therefore, we confirmed the stability of the KMP polyplexes against DNase I, which is an endonuclease enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkage in the DNA backbone. The result indicated that the incorporated pDNA in the polyplex was protected against the attack of DNase I enzyme (Fig. 7(a)). While naked pDNA was completely digested by DNase I (lane 2), the pDNA condensed by the KMP derivatives presented in lane 3 (KMP 0.6 kDa), lane 4 (KMP 1.2 kDa), and lane 5 (KMP 2 kDa). KMP 2 kDa and KMP 1.2 kDa showed a better ability to hold the plasmid DNA. The presence of the heparin demonstrated the plasmid DNA was protected in the polyplexes and released after mixing with the competitive agent (Fig. 7(b)). The results indicated that KMP derivatives that have positive charges provide the stability to pDNA by forming polyplexes through electrostatic interaction. However, in the case of KMP 0.6 kDa polyplexes (w/w=50), the plasmid DNA could not release due to the high interaction between KMP and pDNA. The protection and release of pDNA inside a cellular circumstance have an increased probability of transfection efficiency.

6. Cellular Uptake

Each polymer was complexed with Alexa Fluor 546-labeled

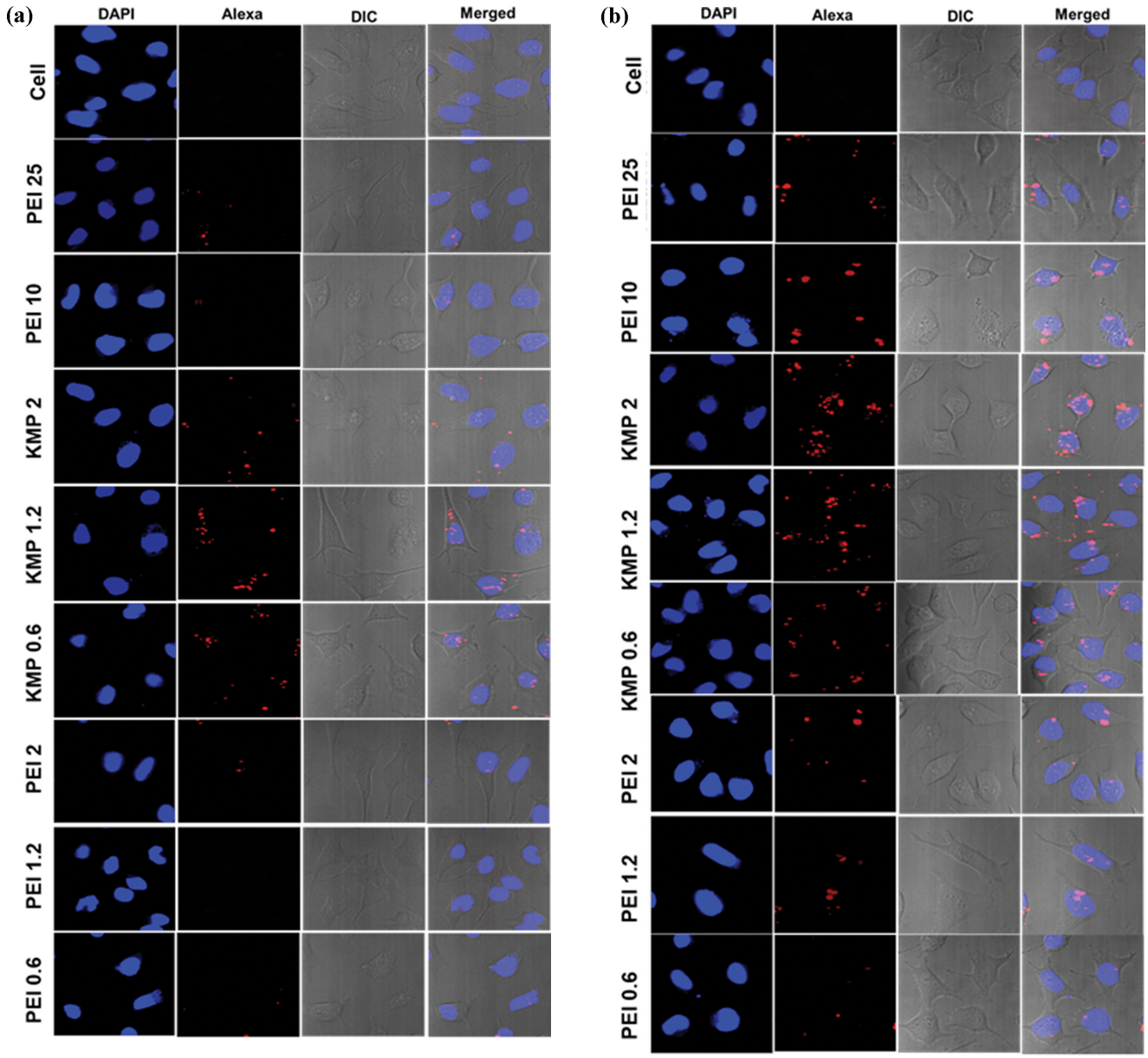


Fig. 8. Confocal laser scanning microscopy image of HeLa cells incubated with polyplexes for (a) 4 h and (b) 12 h. Cells were stained after transfection and observed by confocal microscopy. Hoechst 33342-labeled nuclei (blue), Alexa 546-labeled pDNA (red). (c) Flow cytometry of HeLa cells incubated with polyplexes for 4 h and 12 h. The Alexa 546-labeled pDNA (red) was transfected to the cell and recorded using a flow cytometer (FACSCalibur, Becton Dickinson, USA).

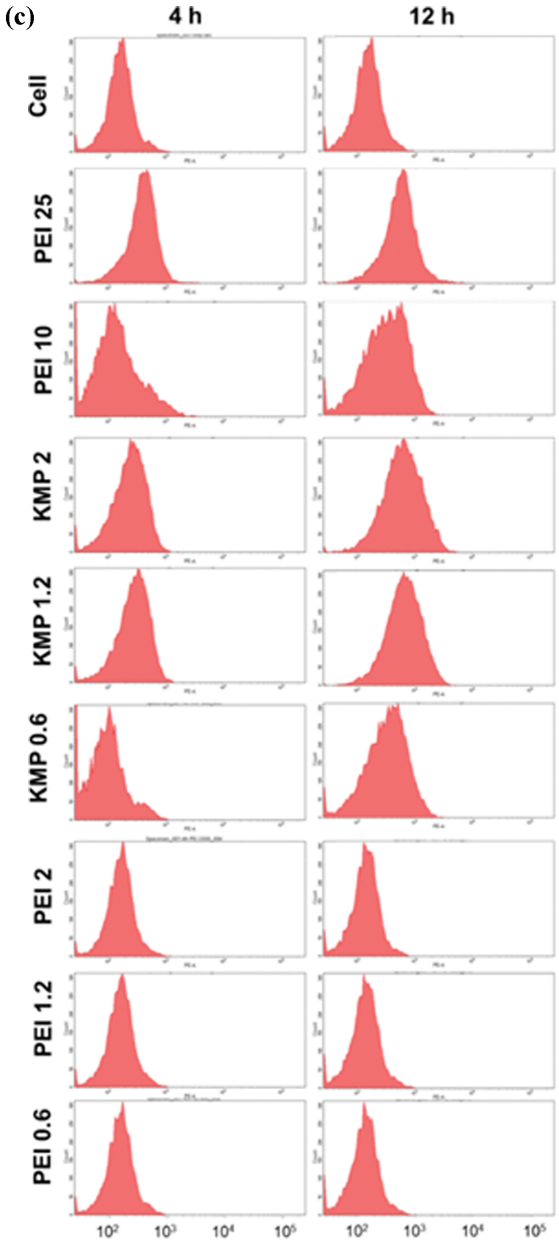


Fig. 8. Continued.

pDNA at an optimal weight ratio and transfected into HeLa cells to analyze the cellular distribution of polyplexes for 4 h and 12 h. The red fluorescence intensity was directly proportional to the cell uptake of polyplexes using a laser scanning confocal microscope. The uptake efficiency of polyplexes depended on the time. The higher red fluorescence was at 12 h incubation compared to 4 h (Fig. 8). Fig. 8 clearly demonstrates that KMPs (KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa) are more effective in DNA uptake than PEI 2 kDa, PEI 1.2 kDa, and PEI 0.6 kDa even after 4 h incubation. Specifically, KMP 2 kDa and KMP 1.2 kDa exhibited the best cellular uptake (Fig. 8(b)), which is the primary factor for enhancing gene transfection. Quantitative fluorescence intensity was proved using flow cytometry (Fig. 8(c)) after the cell incubation with polyplexes for 4 h and 12 h. The fluorescence intensity of KMP derivative polyplexes was higher than that of native LMW-PEI. KMP 2 kDa and KMP 1.2 kDa exhibited the best ability to internal to the cell, which was similar to that of PEI 25 kDa polyplexes. Our data were consistent with those of previous studies on the cell uptake improvement in LMW-PEI derivatives by increasing their amine content [29,30].

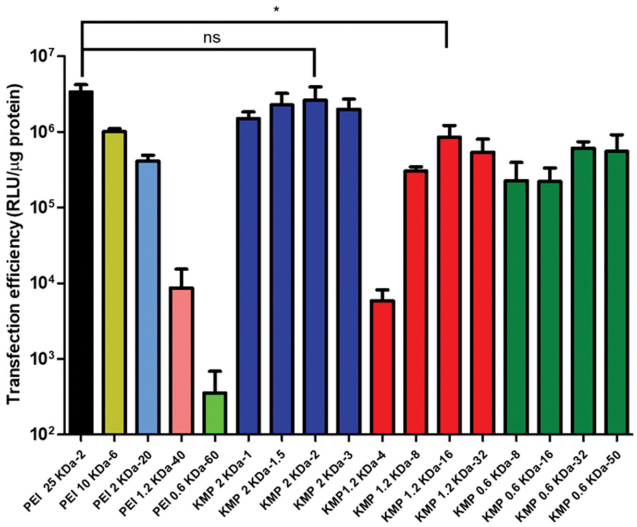


Fig. 9. Polymer transfection efficiency in HeLa. Data are shown as mean±standard deviation (n=3). *p<0.05, ns: not significant.

7. Transfection

To examine the effectiveness of each derivative as gene delivery vehicle, a transfection assay was performed on HeLa cell line. The positive control PEI 25 kDa facilitated the highest transfection efficiency. The transfection efficiency of the KMP 2 kDa polyplexes was comparable to the gold standard PEI 25 kDa (Fig. 9); therefore, our KMP exhibited high transfection efficiency. The gene therapy research focused on the development of optimal PEI derivatives, such as aminoglycoside, as gene carriers. Mingxing et al. [36] demonstrated that aminoglycoside can deliver the antisense morpholino oligonucleotide. According to Goklany [37], aminoglycoside derivative polymers are promising for gene therapy with high efficiency of transfection and low toxicity. Herein, the combination of kanamycin and PEI led to promising results for gene delivery. KMPs showed a higher ability to bind on DNA compared to the LMW-PEI owing to the higher amine group content. In addition, the polyplexes were safe for HeLa cells (Fig. S3). The optimum condition of transfection depended on a different kind of KMP. The KMP 2 kDa showed the highest efficiency at the ratio of 2 (polymer/pDNA, w/w). The ratio of 16 and 32 is for KMP 1.2 kDa and KMP 0.6 kDa, respectively. Moreover, the KMP buffering capacity resulting from the titration results enhanced the transfection efficiency. Particularly, KMP 2 kDa and KMP 1.2 kDa are suitable as gene carriers (Fig. 9).

8. Cell Viability

Toxicity is one of the concerning issues in the application of the polymer as gene delivery. WST-1 assay was used to detect the cell viability on HeLa (Fig. 10) and NIH3T3 cells (Fig. S2). PEI 25 kDa and PEI 10 kDa exhibited high cellular toxicity due to the polymer surface charge and non-degradability. Specifically, the high positive

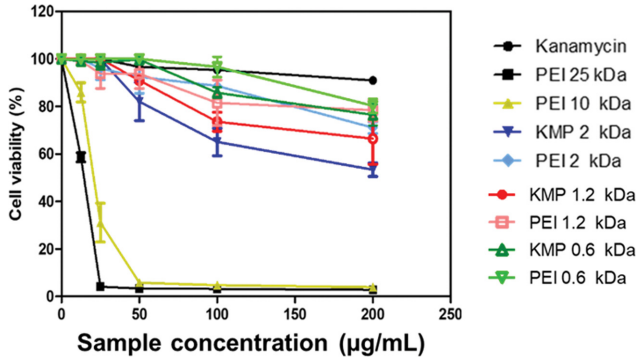


Fig. 10. Cytotoxicity of polymers in HeLa. The data of respective point indicates average and standard deviation from quadruplicate.

charge density caused cell damage. Previous studies showed that degradable polymers overcome this problem [38]. KMPs displayed low cytotoxicity compared to PEI 25 kDa and PEI 10 kDa owing to the peptide bond and glycosidic bond in the polymer. KMPs were less toxic to HeLa cells compared to NIH3T3 at the same concentration ranges. However, the high amine group content led to polymer cytotoxicity at high concentrations. In both cell lines, KMP 2 kDa showed higher toxicity at concentrations reaching 200 μg/mL. For the same concentration, the toxicity level gradually decreased from KMP 2 kDa to KMP 1.2 kDa to KMP 0.6 kDa (Fig. 10). Considering the cytotoxicity data of NIH3T3, the acceptable concentration for the KMP 2 kDa, KMP 1.2 kDa, and KMP 0.6 kDa should be less than 25, 50, and 100 μg/mL, respectively.

CONCLUSIONS

Our study provides evidence of the conjugation ability of KMP with LMW-PEI for gene delivery. The KMP library was successfully created. ^{1}H NMR and FT-IR analyses showed the conjugation of PEIs to the kanamycin. KMP buffering capacity is due to the PEI component, which facilitates to endosome escape of polyplexes. The ability of the polymer to complex with DNA was also studied by gel electrophoresis and picogreen assay. KMPs can condense with pDNA at a low ratio, leading to a special shape. The nanopolyplex positive charges improve the cellular uptake. The gene expression was corroborated by the efficient transfection of KMP 2 kDa and KMP 1.2 kDa with low toxicity. KMP 2 kDa and KMP 1.2 kDa are therefore promising carriers for gene delivery.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Synthesis and characterization of oligo aminoglycosides and polyethylenimine conjugates as polymeric gene carriers

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Method:

Quantities of the amine in the KMP

The PEI 2 kDa was used to prepare for the amine standard curve. The 300 μL of the different concentrations of PEI 2 kDa was reacted with 300 μL ninhydrin (2% in ethanol) for 15 min at 90 °C. Then, the solution was stored on ice and transferred to the 96-well plate to measure the OD_{570} absorbance. For the KMP, the KMP solution (1 mg/mL) was used. The amount of the primary amines was calculated based on the amine standard curve. The weight percentage of primary amine per mg of KMP from the experiment was compared with the estimated value to determine the amount of the primary amine in KMP.

Results

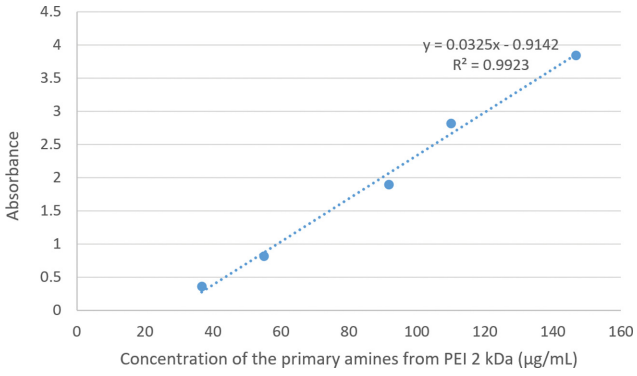

Fig. S1. The standard curve of primary amines.

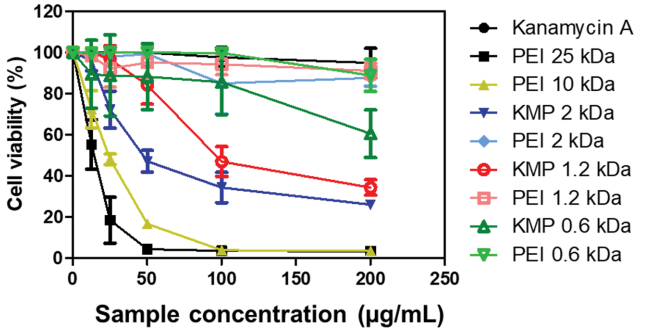

Fig. S2. Cytotoxicity assay in NIH3T3 cells of polymers using WST-1 assay. The data of respective point indicates average and standard deviation from quadruplicate.

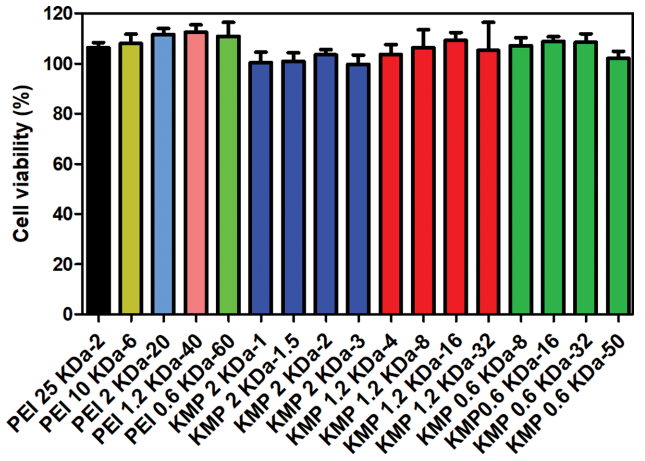

Fig. S3. Cytotoxicity assay in HeLa cells of polyplexes using WST-1 assay. The data of respective point indicates average and standard deviation from triplicate.

Table S1. The estimated weight percentage of primary amine per KMP conjugate based on the various number of PEI conjugated

Number of PEI	Sample name		
	KMP 2 kDa	KMP 1.2 kDa	KMP 0.6 kDa
1	5.39	4.04	2.18
2	6.60	5.36	3.27
3	7.14	6.02	3.93
4	7.44	6.41	4.37
5	7.63	6.67	4.68
6	7.76	6.85	4.91
7	7.86	6.99	5.09
8	7.94	7.10	5.24

Table S2. The weight percentage of the primary amine in the KMP derivatives

	OD_{570}	Concentration of primary amine (μg/mL)	Weight percentage of primary amine (%)
KMP 2 kDa	1.5343	75.34	7.53
KMP 1.2 kDa	1.2371	66.19	6.62
KMP 0.6 kDa	0.4209	41.08	4.11