Controlled Delivery of Plasmid DNA and siRNA to Intracellular Targets Using Ketalized Polyethylenimine

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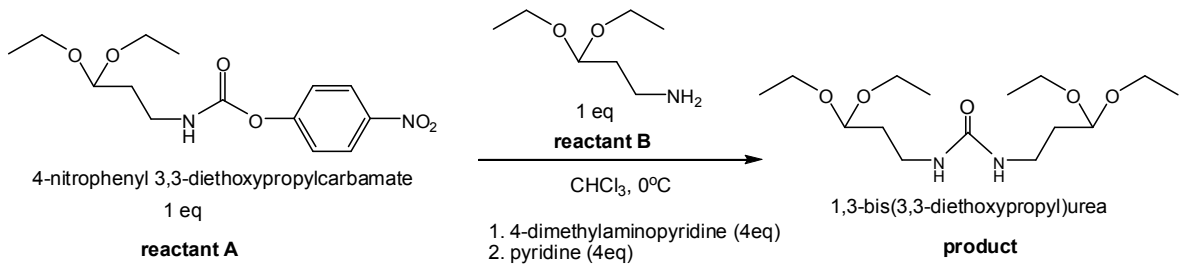
Reactivity of *p*-nitrophenyl carbamate to amines in the presence of pyridine or 4-dimethylaminopyridine (DMAP)



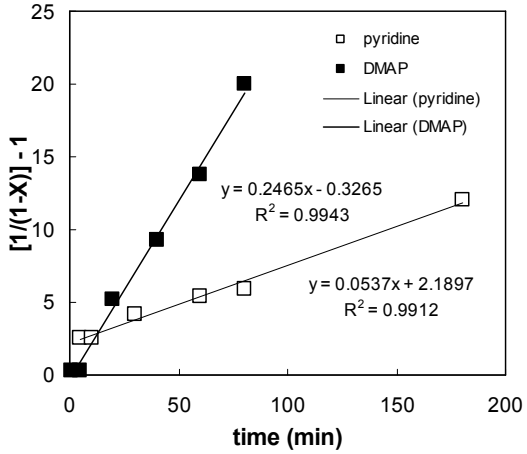


Figure S1. Reaction schemes and quantitative reaction rates of diamide formation by carbamate in the presence of pyridine or DMAP. The reactions were assumed to the first-order for carbamate and amine, respectively.

By assuming that the reaction rate of DMAP-catalyzed amine conjugation of *p*-nitrophenyl 3,3-diethoxypropylcarbamate linearly depends on both reactant A and B in the first-order, the reaction rate equation can be described as following:

$$-\frac{d[\text{reactantA}]}{dt} = k[\text{reactantA}][\text{reactantB}][\text{DMAP or pyridine}]$$

Because the concentration of DMAP is relatively high (i.e., constant), the reaction can be expressed by a pseudo-second-order equation. The concentrations of reactants A and B are always the same during the reaction; the reaction can be expressed as

$$-\frac{d[\text{reactantA}]}{dt} = k_1[\text{reactantA}]^2 \;\rightarrow\; -\frac{d[\text{A}]}{dt} = k_1[\text{A}]^2$$

where [A] and *t* represent concentration of A and reaction time, respectively.

By rearranging the above equation,

$$k_1 = \frac{\frac{1}{\text{A}} - \frac{1}{[\text{A}]_0}}{t} = \frac{\frac{1}{([\text{A}]_0 - [\text{A}]_0\text{X})} - \frac{1}{[\text{A}]_0}}{t} = \frac{\frac{1}{[\text{A}]_0}\left[\frac{1}{1\text{-X}} - 1\right]}{t}$$

where X and $[A]_0$ represent reaction conversion and initial concentration of A, respectively.

By plotting $\left[\frac{1}{1\text{-X}} - 1\right]$ versus *t*, the slope of the straight line yields $k_1[A]_0$ where $[A]_0$ was 0.016 mol/L for both reactants A and B. The reaction conversion was determined by ^{1}H-NMR, and the calculated

reaction rate constants at 0°C were

k_1(pyridine) = 3.356 L/mol·s
k_1(DMAP) = 15.40 L/mol·s

Quantification of free amino groups (TNBS assay)

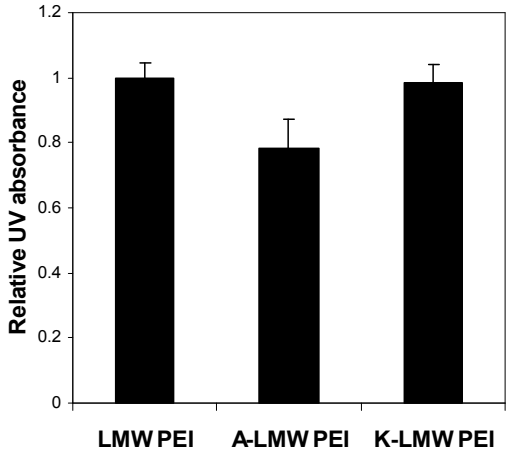


Figure S2. Quantification of relative primary amine ratios of unmodified, activated, and ketalized LMW PEI by TNBS (2,4,6-trinitrobenzenesulfonic acid) assay. Both modified LMW PEIs and unmodified LMW PEI were dissolved in 0.10 M sodium bicarbonate, pH 8.5, at the polymer concentration of 1.3 mM based on molecular weights data from GPC. Then, 25 μL of aqueous 0.05 M TNBS was added to 1 mL of each sample solution followed by being incubated for 2 h at 37 °C. Concentrations of primary amines of A-LMW PEI and K-LMW PEI were quantified by relative UV absorbance at 335 nm, compared with the absorbance of unmodified LMW PEI solution at the same molar concentration.

Hydrolysis kinetics of K-HMW PEI

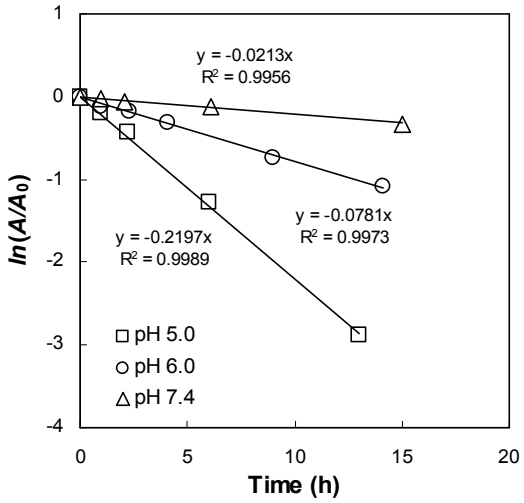


Figure S3. Hydrolysis kinetics of K-HMW PEI at various pHs. The hydrolysis rates of K-HMW PEI at different pHs were investigated by incubating K-HMW PEI in pH 5.0 and 6.0 acetate, and pH 7.4 Tris-HCl buffers in D_2O at 37 °C for various periods of time, respectively. The half-lives at pH 5.0, 6.0 and 7.4 were calculated to be 3.2, 8.9, and 33 h, respectively, which were very similar to the ones of K-LMW PEI at the same pHs. This indicates almost negligible effects of molecular weights of ketalized PEI on hydrolysis.

Zeta-potential analysis of nucleic acids/ketalized PEI polyplexes

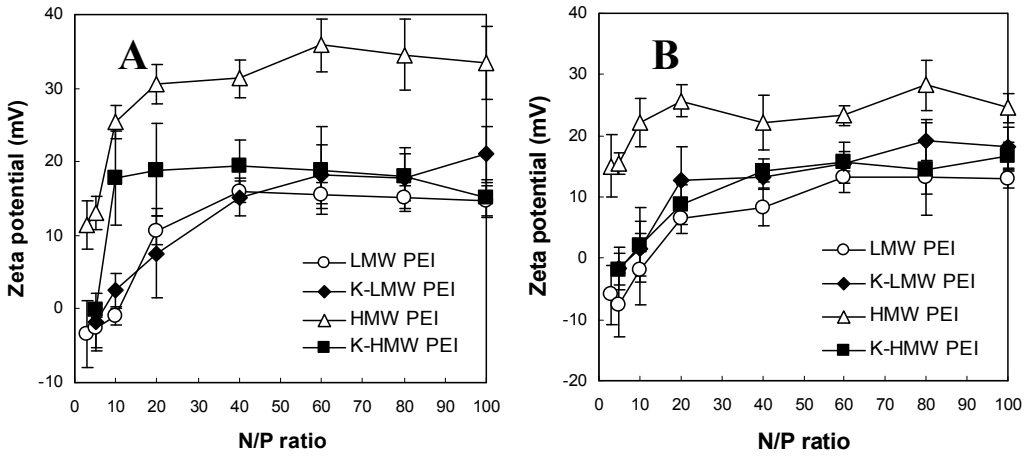


Figure S4. Surface charge of (A) plasmid DNA and (B) siRNA-condensing polyplexes. Different amounts of un-ketalized and ketalized PEI were mixed with 1 μg of DNA in 100 μL of DI water to obtain various N/P ratios. The siRNA/polymer polyplexes were formed by the same procedure using 990 ng of siRNA. Each polyplex solution was diluted with additional 1.4 mL of 1 mM NaCl before the measurement. All experiments were performed at 25 ^{o}C using a zeta potential analyzer (Zeta-Plus, Brookhaven Instruments, Holtsville, NY). The zeta-potential was automatically calculated from the electrophoretic mobility based on Smoluchowski's model, and average values were calculated from 5 runs of each sample. Result showed that surface charges of various polyplexes prepared at various N/P ratios were greatly dependent on the N/P ratios and generally increased with increasing N/P ratios up to 40. Polyplexes synthesized with K-HMW PEI showed lower cationic charges than the ones synthesized with un-ketalized HMW PEI. The polyplexes synthesized with both ketalized and un-ketalized LMW PEI did not show a noticeable difference in surface charges although only K-LMW PEI could successfully condense nucleic acids (Figure 5).The polyplexes of plasmid DNA and siRNA prepared with both K-HMW and K-LMW PEI at high N/P ratios (i.e., 40 through 100) showed the similar zeta-potentials, indicating insignificant effects of surface charges of the polyplexes on processes of cellular internalization.

Plasmid DNA condensation by K-LMW PEI and non-degradable modified LMW PEI

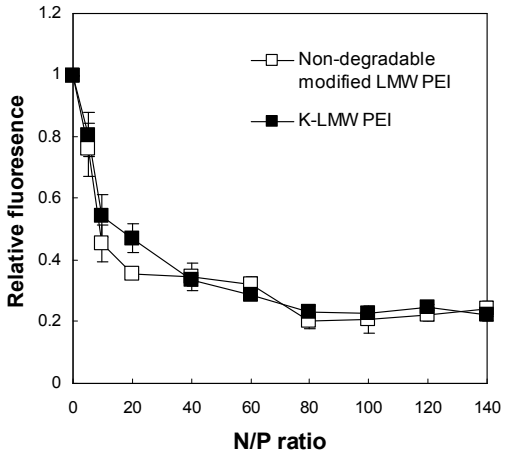


Figure S5. DNA condensation efficiency of K-LMW PEI and modified LMW PEI with non-degradable diamines determined by ethidium bromide exclusion assay. The non-degradable modified LMW PEI was synthesized by conjugating *p*-nitrophenyl carbamate-activated LMW with excess 1,3-diaminopropane instead of ketal diamines. One microgram of ethidium bromide was mixed with 1 μg of plasmid DNA in 40 μL of DI water. After 10 min-incubation at room temperature, ketalized PEI polyplexes and non-degradable modified PEI polyplexes in 60 μL of DI water were added to the DNA/ethidium bromide mixtures to obtain various N/P ratios. After 30 min-incubation,

fluorescence intensity of each solution was measured using a plate reader (Spectramax GeminiXS, Molecular Devices, Sunnyvale, CA).

Effects of molecular weights of ketalized and unketalized PEI on Transfection

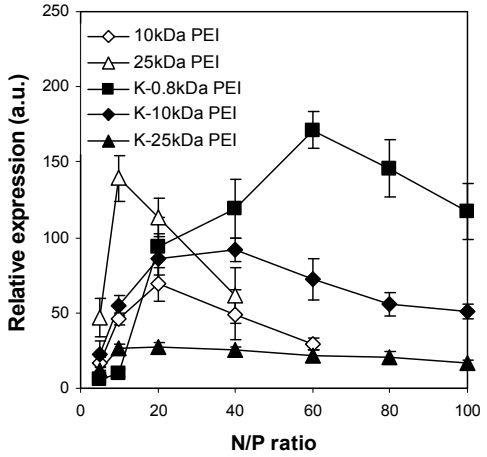


Figure S6. Opposite effects of molecular weights on transfection efficiency by un-ketalized and ketalized PEI. Middle molecular weight (MMW) PEI (i.e., 10 kDa, Polyscience, Inc., Warrington, PA) was ketalized by the same manner used for synthesis of K-HMW PEI, and then ketalization efficiency of primary amines of 10 kDa PEI was determined to be 35% using ^{1}H-NMR. Transfection efficiencies of ketalized PEI polyplexes with different molecular weights and N/P ratios were evaluated using NIH 3T3 cells. Each polyplex solution was prepared in the same manner as described in the DNA transfection experiment. The result showed that transfection efficiency was inversely proportional to molecular weights of ketalized PEI while larger molecular weight of un-ketalized PEI showed higher transfection efficiency.

Transfection of HeLa cells

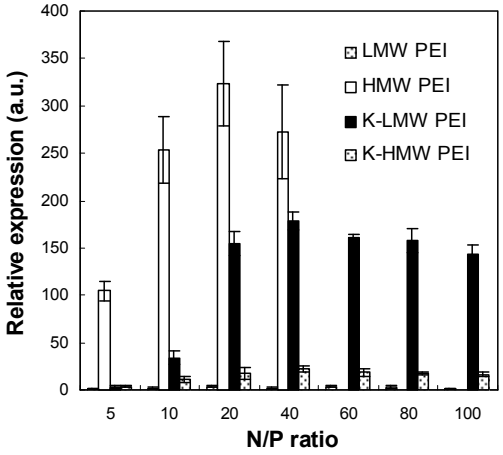


Figure S7. Transfection of human cervical cancer HeLa cells by un-ketalized and ketalized PEI. HeLa cells (ATCC, Rockville, MD) were inoculated in a 12-well plate at a density of 1×10^5 cells/well with DMEM containing 10% FBS and transfected by the same way described for transfecting NIH 3T3 cells. Results showed that transfection efficiency by ketalized PEI was inversely proportional to molecular weights although un-ketalized HMW PEI transfected cells more efficiently than ketalized LMW PEI. Cytotoxicity of ketalized PEI was very consistently low regardless of molecular weights and N/P ratios, while significant cytotoxicity was observed with HMW PEI at the N/P ratio of 60 and higher (data not shown).

Internalization pathways of ketalized and un-ketalized PEI polyplexes

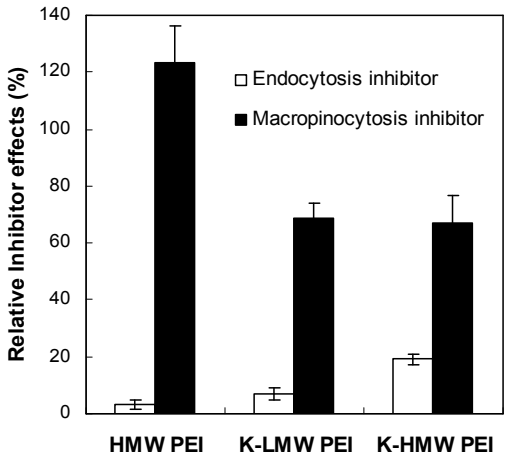


Figure S8. Different internalization pathways for un-ketalized and ketalized PEI. NIH 3T3 cells were inoculated at a density of 1×10^5 cells/well in a 12-well plate, 24 h prior to transfection. The medium was replaced with 1 mL of DMEM containing 10% FBS and inhibitors (500 μM *N*-ethylmaleimide (NEM, Sigma-Aldrich, St. Louis, MO) for endocytosis inhibition or 25 μM 5-(*N*-ethyl *N*-isopropyl)amiloride (EIPA, Sigma-Aldrich, St. Louis, MO) for macropinocytosis inhibition) for pre-treatment.[1] After being incubated for 1 h at 37 ^{o}C, 100 μL of DNA/PEI polyplex solutions was added to a well with 500 μL of DMEM containing inhibitors with the same concentration as used for pre-treatment. Polyplex solutions were prepared by mixing 1 μg of DNA with desired amounts of various polymers achieving different optimal N/P ratios for each polymer (e.g., N/P = 10 for HMW PEI, N/P = 40 for K-LMW PEI and K-HMW PEI). The cells treated only with DNA/PEI polyplex solution (i.e., without inhibitors) were used as a control. Cells were further incubated for 2.5 h, and then the medium was replaced with 1 mL of fresh DMEM containing 10% FBS. After being further incubated for 24 h at 37 °C, the cells were harvested by trysinization and fixed with 2% *p*-formaldehyde solution for 30 min at 4 °C. The expression of eGFP fluorescence was analyzed by flow cytometry. Result showed significant inhibitory effects by endocytosis inhibitor on internalization of both un-ketalized and ketalized PEI polyplexes. However, internalization of ketalized PEI polyplexes was noticeably inhibited by macropinocytosis inhibitor while internalization of un-ketalized PEI was not affected.

Effect of serum on transfection

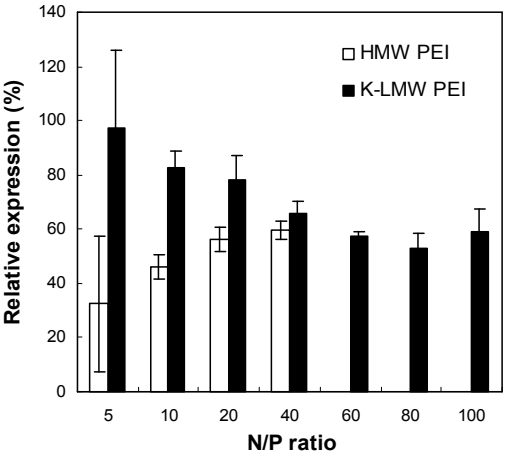


Figure S9. Transfection of NIH 3T3 cells by un-ketalized and ketalized PEI with serum and without serum. DNA/polymer polyplexes were prepared in the same manner as described in DNA transfection part. The polyplex solution was added to a well with 500 μL of DMEM alone or 500 μL of DMEM containing 10% FBS. After 4 h-incubation at 37 °C, the medium was replaced with DMEM containing 10% FBS. After being further incubated for

24 h at 37 °C, the cells were harvested by trysinization and fixed with 2% *p*-formaldehyde solution for 30 min at 4 °C. The expression of eGFP fluorescence was analyzed by flow cytometry. The relative GFP expression of the cells incubated with polyplex solutions in the presence of serum was evaluated in comparison with that after being incubated in the absence of serum. At low N/P ratios up to 40, transfection efficiency of K-LMW PEI was less decreased than the one of un-ketalized HMW PEI. With increasing the N/P ratios, un-ketalized HMW PEI showed improved transfection in the presence of serum, although severe cytotoxicity was observed at the N/P ratio of 60 and higher.

□

References

(1) Khalil, I. A.; Kogure, K.; Akita, H.; Harashima, H. *Pharmacol. Rev.* **2006**, 58, 32-45.