Electronic Supplementary Information

Regulated protonation of polyaspartamide derivatives bearing repeated aminoethylene side chains for efficient intracellular siRNA delivery with minimal cytotoxicity

Hui Gao,^{a,b} Hiroyasu Takemoto,^c Qixian Chen,^b Mitsuru Naito,^b Hirokuni Uchida,^d

Xueying Liu,^d Kanjiro Miyata,^{d,*} and Kazunori Kataoka^{b,d,e,*}

^aSchool of Chemistry and Chemical Engineering, Tianjin University of Technology, Tianjin 300384, China

^bDepartment of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

^cPolymer Chemistry Division, Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan

^dCenter for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 Japan

^eKawasaki Institute of Industry Promotion, 66-20 Horikawa-cho, Saiwai-ku, Kawasaki 212-0013, Japan

AUTHOR EMAIL ADDRESS: kataoka@bmw.t.u-tokyo.ac.jp; miyata@bmw.t.u-

tokyo.ac.jp

Materials and Methods

1. Materials

derivative. $poly{N''-(N''-{N'-[N-2-aminoethyl]-2-}$ А polyaspartamide aminoethyl}-2-aminoethyl)aspartamide} (PAsp(TET)), degree with а of polymerization (DP) of 80 and a molecular weight distribution (Mw/Mn) of 1.1 was synthesized as described previously.^[1] Methyl isothiocyanate and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS) was supplied by Dainippon Sumitomo Pharma Co, Ltd. (Osaka, Japan). Luciferase Assay System was procured from Promega Co. (Madison, WI). Nonlabeled siRNA molecules were synthesized by Hokkaido System Science (Hokkaido, Japan). Sequences for GL3 luciferase siRNA (siLuc) consisted of 5'-CUUACGCUGAGUACUUCGAdTdT-3' and antisense 5'sense UCGAAGUACUCAGCGUAAGdTdT-3'. Sequences for scrambled siRNA (siScramble) comprised sense 5'-UUCUCCGAACGUGUCACGUdTdT-3' and antisense 5'-ACGUGACACGUUCGGAGAAdTdT-3'. A 5'-Alexa 546-labeled sense strand-containing siLuc was synthesized by Invitrogen (Carlsbad, CA).

2. Synthesis and characterization of thiourea-modified PAsp(TET)

The synthesis route is shown in **Scheme S1**. Essentially, thiourea-modified PAsp(TET) (PAsp(TET-T)) was synthesized according to a previous method^[2] with some minor modifications considering the biodegradable nature of PAsp(TET). PAsp(TET) (50 mg) was dissolved in cool methanol. Methyl isothiocyanate (2 molar equivalent of the primary amine of PAsp(TET)) was added to the methanol solution before K₂CO₃ addition (50 mg). The reaction mixture was stirred overnight at 4 °C and subsequently dialyzed against 0.01 N acetic acid buffer at 4 °C for 24 h and then

against deionized water at 4 °C for another 12 h. The final solution was lyophilized to give final product PAsp(TET-T) (yield: 80%). The ¹H NMR spectrum was recorded with a JEOL ECS400 spectrometer (JEOL, Tokyo, Japan). The introduction rate of thiourea moiety in the polymer was calculated from the peak intensity ratio of methyl protons in thiourea moiety and methylene protons in the polyaspartamide side chains in ¹H NMR spectrum. Furthermore, the residual primary amine content in the polymer was quantified according to the 2,4,6-trinitrobenzenesulfonic acid method.^[3] A potentiometric titration was conducted to determine the protonation profiles of PAsp(TET) and PAsp(TET-T). The polymer was dissolved in 0.1 N HCl containing 50 mM NaCl and subsequently titrated with 0.05 N NaOH containing 50 mM NaCl at 37 °C (3-min intervals for stabilization of pH values). The titration curve was collected from pH 2.0 to 10.0. The obtained titration curves were converted to the corresponding pH-dependent α curves, as described previously.^[1]

3. Preparation of polyion complexes (PICs).

PAsp(TET-T) (or PAsp(TET)) was dissolved in 10 mM HEPES buffer (pH 7.4) and subsequently mixed with a twofold volume of siRNA solution (15 μ M in 10 mM HEPES buffer (pH 7.4)) at N/P ratios of 10 and 20.

4. Dynamic light scattering (DLS).

Particle size and polydispersity (PDI) of siRNA-loaded PICs were measured by DLS measurement at 25 °C using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) equipped with a He–Ne ion laser ($\lambda = 633$ nm). The scattering angle was fixed at 173° in all measurements. The PICs (N/P = 10 and 20) prepared as above using 10 μ M siRNA were diluted to 4 μ M siRNA before measurement.

5. Cell viability assay.

HuH7-Luc cells were deposited on a 24-well plate at a cell density of 10,000 cells/well in DMEM containing 10% FBS (0.5 mL/well) and incubated for 24 h. PICs loaded with siLuc at N/P values of 10 and 20 were added to each well to obtain a final concentration of 100 nM siRNA. After 48 h incubation with PICs, cell viability was measured using Cell Counting Kit-8 (DOJINDO Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The absorbance was measured using a microplate reader equipped with a long-pass filter of 450 nm (Model 680, BIO-RAD, Hercules, CA). The cell viability was determined as a percentage of nontreated control wells. Results were expressed as a mean and standard deviation obtained from four samples.

6. Luciferase gene silencing assay.

HuH7-Luc cells were deposited on a 24-well plate at a cell density of 10,000 cells/well in DMEM containing 10% FBS (0.5 mL/well) before 24-h incubation. The PICs loaded with siLuc or siScramble at N/P values of 10 and 20 were added to each well to obtain a final concentration of 100 nM siRNA. After 48-h incubation, the medium was removed and cells were washed with PBS (0.5 mL) followed by the addition of lysis buffer (0.2 mL, Promega). The luciferase activity of the lysates was determined from the photoluminescence intensity determined using the Luciferase Assay System (Promega) and a luminescence microplate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). The relative luminescence unit

(RLU) was calculated by normalizing the obtained luminescence intensity to that from nontreated cells. Results were presented as a mean and standard deviation obtained from four samples.

7. Flow cytometric analysis.

HuH7-Luc cells were deposited on a 6-well plate at a cell density of 50,000 cells/well in DMEM containing 10% FBS and incubated for 24 h. PICs prepared with Alexa 546-labeled siRNA at an N/P value of 20 were added to each well to obtain a final concentration of 100 nM siRNA. After 24-h incubation, cells were rinsed three times with PBS (2 mL), treated with a trypsin–EDTA solution, and resuspended in PBS. The fluorescence intensity of the suspended cells was measured using a BDTM LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with FACSDiva software (BD Biosciences). Results were expressed as a mean and standard deviation obtained from three samples.

8. Confocal laser scanning microscopic (CLSM) analysis.

HuH7-Luc cells were deposited on a 35-mm glass-based dish (Iwaki, Tokyo, Japan) at a cell density of 50,000 cells/well in 2 mL DMEM containing 10% FBS. After 24-h incubation, the medium was replaced and PICs prepared with Alexa 546-labeled siRNA at an N/P value of 20 were added to a final concentration of 100 nM siRNA. After 24-h incubation, late endosomes/lysosomes and cell nuclei were stained with LysoTracker Green (Molecular Probes, Eugene, OR) and Hoechst 33342 (Dojindo, Japan), respectively. A CLSM observation was conducted to determine the intracellular distribution of PICs using the LSM 780 (Carl Zeiss, Oberlochen, Germany) equipped with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective.

Excitation wavelengths were 488 nm (Ar laser) for LysoTracker Green, 514 nm (Ar laser) for Alexa 546-labeled siRNA, and 405 nm (Diode laser) for Hoechst 33342. The endosomal entrapment of Alexa 546-labeled siRNA was quantified by calculating its colocalization ratio with LysoTracker Green using the following expression:

Colocalization ratio (%) = $100 \times$ [yellow pixels (colocalization of Alexa 546-labeled siRNA with LysoTracker Green)/yellow and red pixels (all the Alexa 546-labeled siRNA in cells)].

Results were represented as the mean colocalization ratio obtained from 25 cells.

9. Fluorescence correlation spectroscopy (FCS).

Stability of siRNA PICs was examined by FCS using a confocal laser scanning microscope, LSM510 equipped with a ConfoCor3 module (Carl Zeiss, Oberkochen, Germany).^[4] Each PIC sample was prepared with Cy3-labeled siRNA (Hokkaido System Science, Hokkaido, Japan) and put into an eight-well Laboratory-Tek chamber (Nalgene Nunc International, Rochester, NY) immediately after dilution to 100 nM Cy3-labeled siRNA with FBS-containing medium. The measurements were conducted with a sampling time of 20 sec and a repeating time of 10 under excitation by He-Ne laser (543 nm). The obtained autocorrelation curves were converted to diffusion times using ConfoCor3 software, followed by the calculation of the diffusion coefficients based on a reference of rhodamine 6G. The diffusion coefficients were determined to be 1.2 ± 0.9 and $1.5 \pm 0.4 \,\mu\text{m}^2/\text{sec}$ for PAsp(TET-T)-based PICs at N/P = 10 and 20, respectively.

10. Statistics analysis.

Statistical significance was evaluated by Student's *t*-test.



Supporting Scheme S1. Synthesis of PAsp(TET-T) by the reaction of PAsp(TET) with methyl isothiocyanate.



Supporting Figure S1. ¹H NMR spectrum of PAsp(TET) in D_2O at room temperature. This spectrum was recorded by AV-400 spectrometer (Bruker, Freemont, CA).



Supporting Figure S2. Cellular uptake of naked siRNA and siRNA PICs prepared at an N/P value of 20 using HuH7-Luc cells ([siRNA] = 100 nM, incubation time = 24 h). Results are expressed as mean \pm standard deviation (n = 3).

References

- [1] H. Uchida, K. Miyata, M. Oba, T. Ishii, T. Suma, K. Itaka, N. Nishiyama, K.
- Kataoka, J. Am. Chem. Soc. 2011, 133, 15524.
- [2] C. Li, Y.-W. Yang, Z. Liang, G. Wu, H. Gao, Polym. Chem. 2013, 4, 4366.
- [3] S. L. Snyder, P. Z. Sobocinski, Anal. Biochem. 1975, 64, 284.
- [4] T. Suma, K. Miyata, T. Ishii, S. Uchida, H. Uchida, K. Itaka, N. Nishiyama, K. Kataoka, *Biomaterials* 2012, 33, 2770.