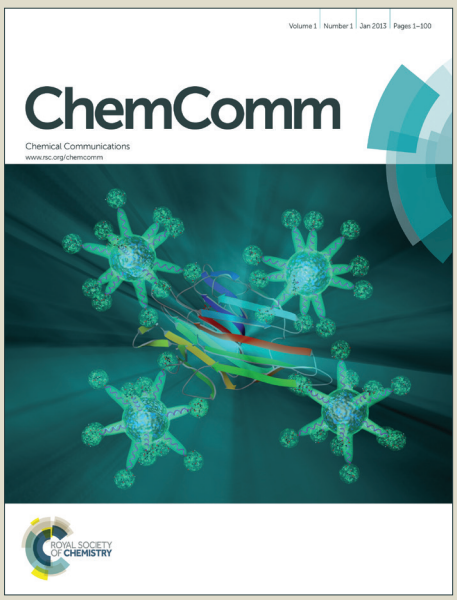
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

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

Cite this: DOI: 10.1039/xoxxoooooox

Received XXXX,
Accepted XXXX

DOI: 10.1039/xoxxoooooox

www.rsc.org/

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,*]

Effects of repeated number (RN) of aminoethylene (AE) units in polyaspartamide side chains were investigated for polyion complex (PIC)-based siRNA delivery. Reduction of apparent RN from 3 to 2 by thiourea introduction increased a protonatable amine fraction in AE units at endosomal pH, leading to efficient endosomal escape of siRNA-loaded PICs.

Small interfering RNA (siRNA) has attracted a great deal of interest because of its sequence-specific gene silencing ability for potential treatment of diverse diseases, such as cancer.[1–4] However, the poor bioavailability in siRNA-based therapies, resulting from rapid enzymatic degradation and inefficient cellular uptake of siRNA, has created an urgent need for delivery carriers.[3,4] In this regard, polyion complexes (PICs) are one of the most promising delivery platforms because they can protect siRNA from enzymatic degradation and dramatically facilitate the cellular internalization of siRNA.[5–9]

Nucleic acid carriers, including PICs, can be internalized into cells through endocytosis, and thus they should be trapped by digestive organelle lysosome, resulting in deactivation of cargo compounds. Therefore, these carriers need to translocate their siRNA cargo from the endosome to the cytosol toward the gene silencing pathway. To this end, polycations bearing low pKa amines, e.g., polyethylenimine (PEI), have been extensively developed to promote the endosomal escape of PICs because these polycations may destabilize the endosomal membrane through direct electrostatic interactions and/or increased osmotic pressure associated with the amine protonation in the acidic compartment.[9–11] Cytotoxicity is another criterion in polycation design. PEI and its derivatives comprising highly charged structures even at extracellular neutral pH have been reported to induce significant cytotoxicity mainly because of their strong interactions with cytoplasmic and mitochondrial membranes.[11–14] Therefore, the protonation of polycations needs to be optimized to enhance siRNA delivery efficiency while reducing cytotoxicity.

Our previous studies revealed that the protonation behaviour of repeated aminoethylene (AE) structures (–$NHCH_2CH_2$–) follows a distinct odd–even tendency in the repeated number. Particularly, polyaspartamide derivatives bearing two (PAsp(DET)) and four AE units (PAsp(TEP)) in side chains exhibited an appreciably large change in the protonation degree (α) between pH 7.4 and 5.5 (Δα), whereas a much smaller Δα was observed for their counterparts comprising one (PAsp(EDA)) and three AE units (PAsp(TET)).[15–17] Consequently, PAsp(DET) and PAsp(TEP) exhibited a modest cationic charge density at pH 7.4, which grew significantly at pH 5.5, inducing an acidic pH-selective membrane destabilization for a less toxic endosomal escape.[14–18] Compared with PAsp(DET), PAsp(TEP) had significant advantage in construction of stable siRNA-loaded PICs (siRNA PICs) in serum-containing media because of its relatively greater charge density, leading to efficient gene silencing in cultured cells but accompanied with a slightly higher cytotoxicity.[16,19] Therefore, the side chain structure of polyaspartamide was further explored in this study to optimize siRNA delivery.

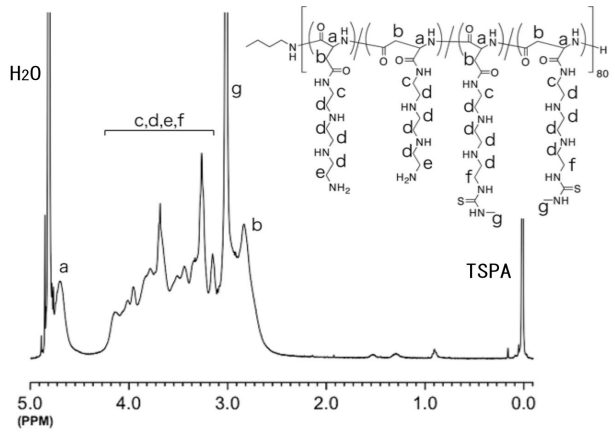


Figure 1. ^{1}H NMR spectrum of PAsp(TET-T) in D_2O at 25 °C.

Herein, PAsp(TET) was modified by introducing a thiourea moiety into primary amines in the side chains for tuning its protonation behaviour (Figure 1 and Supporting Scheme S1). This modification decreases the apparent number of repeated AE units from 3 to 2 in each side chain, thereby assumed to amplify Δα of PAsp(TET) for destabilizing the endosomal membrane as analogized from PAsp(DET). Another motivation for this modification was the unique hydrogen bonding between thiourea and siRNA phosphate groups, which might enhance the stability of siRNA PICs in

extracellular milieu for improving the cellular uptake of siRNA PICs, as previously evidenced for plasmid DNA-loaded PICs.[20] The thiourea-modified PAsp(TET) (PAsp(TET-T), Figure 1) was thus employed to provide siRNA PICs with stability as well as endosome-escaping functionality, directed toward successful cellular delivery of siRNA.

Thiourea modification was performed by reacting side-chain primary amines of PAsp(TET) (degree of polymerization (DP): 80, molecular weight distribution (M_w/M_n): 1.1) with 2 molar equivalents of methyl isothiocyanate (Supporting Scheme S1). The ^{1}H NMR spectrum of the resulting polymer (Figure 1) confirmed successful thiourea modification from the appearance of the new peak assigned to the methyl protons in thiourea moiety at 3.0 ppm (peak g). Particularly, the introduction rate of thiourea moiety was determined to be 70 units per polymer, which are corresponding to 90% of primary amines in the parent polymer. Simultaneously, a colorimetric assay using 2,4,6-trinitrobenzenesulfonic acid confirmed that 90% of primary amines in PAsp(TET) were reacted with methyl isothiocyanate, demonstrating the selective thiourea modification of primary amines. To elucidate the effect of thiourea modification on the protonation behaviour, the obtained polymer was analysed by a potentiometric titration at 37 °C, and resulting titration curve was converted to α plots as a function of pH (Figure 2). The thiourea modification altered the protonation behaviour of PAsp(TET) especially at acidic pH. Whereas an α value of PAsp(TET-T) was similar to that of PAsp(TET) at pH 7.4, PAsp(TET-T) exhibited a larger α value at pH 5.5, compared with PAsp(TET), allowing an appreciably large Δα (= 0.28). This large Δα indicates that the thiourea modification substantially amplified the acidic pH-responsiveness of PAsp(TET). Interestingly, this Δα value is comparable to that of PAsp(DET) (Δα = 0.31),[15] suggesting that PAsp(TET-T) may contribute to acidic pH-selective membrane destabilization.

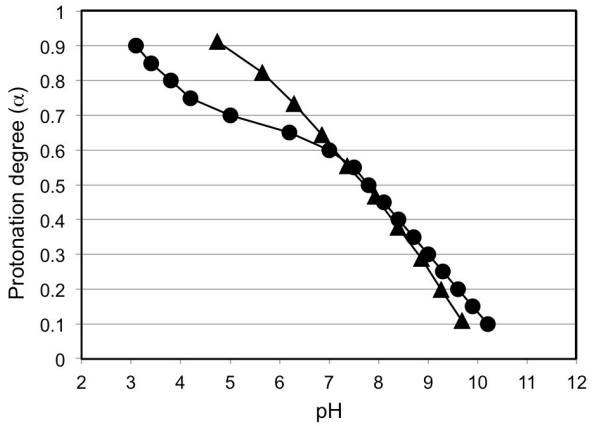


Figure 2. pH-dependent α plots of PAsp(TET) (closed circle) and PAsp(TET-T) (closed triangle) at 37 °C.

Table 1. Cumulant size and polydispersity index (PDI) of siRNA PICs with standard deviation (S.D.) (n = 3).

Polymer	N/P	Cumulant diameter ± S.D. (nm)	PDI ± S.D.
PAsp(TET)	10	107 ± 1	0.19 ± 0.01
	20	103 ± 1	0.15 ± 0.02
PAsp(TET-T)	10	125 ± 37	0.22 ± 0.05
	20	117 ± 18	0.19 ± 0.07

siRNA PICs were prepared by mixing PAsp(TET) or PAsp(TET-T) with siRNA in 10 mM HEPES buffer (pH 7.4) in the presence of an excess amount of the polycations, particularly at residual molar ratios of polycation amines to siRNA phosphates (N/P) of 10 and 20, because PAsp(TET) enabled significant cellular uptake of siRNA in cultured cells at these N/P ratios in a previous study.[16] The prepared siRNA PICs were characterized by dynamic light scattering (DLS, Table 1). Compared with PAsp(TET), PAsp(TET-T) formed relatively larger complexes displaying a narrow size distribution. This increase in size may be attributed to facilitated associations between thiourea moieties in PAsp(TET-T) and phosphates in siRNA, promoting the formation of PICs comprising higher numbers of associated PAsp(TET-T) and siRNA molecules.

siRNA PICs prepared with PAsp(TET) or PAsp(TET-T) were applied to a human hepatoma cell line stably expressing luciferase (HuH7-Luc). First, the cytotoxicity of siRNA PICs was examined in terms of cell viability through a colorimetric assay using Cell Counting Kit-8. Cells treated with PAsp(TET-T)-based PICs exhibited higher cell viability than those treated with PAsp(TET)-based PICs (Figure 3A), indicating that thiourea modification alleviates cytotoxicity. The reduced cytotoxicity may stem from the lower number of protonated amines (or lower cationic charge density) in PAsp(TET-T) at an extracellular pH of 7.4 (90 amines/polymer) than that in the parent PAsp(TET) (134 amines/polymer), as calculated from Figure 2. The gene silencing efficiency of siRNA PICs was then evaluated in HuH7-Luc cells by a luciferase assay, in which a luciferase-targeted siRNA (siLuc) and a control siRNA with a scrambled sequence (siScramble) were used to verify the sequence specificity of siRNA. The gene silencing efficiency was calculated by measuring and normalizing the luciferase-based luminescence intensities to that from nontreated control cells (Figure 3B). siLuc PICs prepared with PAsp(TET-T) induced ~40% and ~70% luciferase silencing at N/P ratios of 10 and 20, respectively. These efficiencies significantly surpassed those obtained from siLuc PICs prepared with PAsp(TET) (~10% at N/P = 10 and ~20% at N/P = 20). This demonstrates that the thiourea modification of PAsp(TET) significantly enhances the gene silencing efficiency of siRNA PICs. Furthermore, control siScramble PICs did not present a significant drop in luminescence intensity, confirming the sequence-specific gene silencing efficiency of siRNA PICs prepared using PAsp(TET-T) and PAsp(TET).

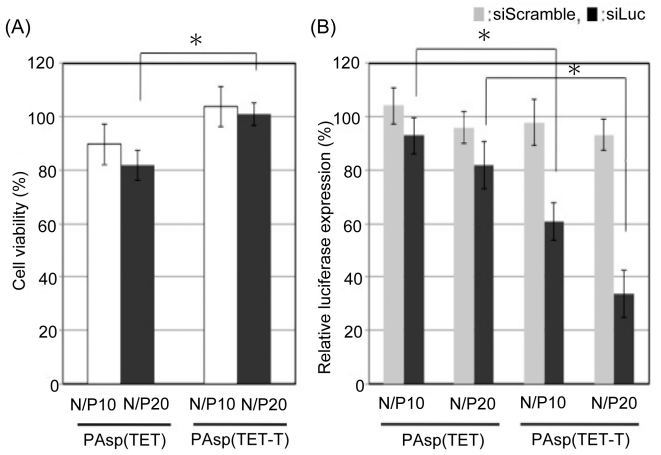


Figure 3. A) Cytotoxicity of siRNA PICs prepared with PAsp(TET) or PAsp(TET-T) against HuH7-Luc cells. B) Gene silencing efficiency of siRNA PICs prepared with PAsp(TET) or PAsp(TET-T) against HuH7-Luc cells. In both experiments, siRNA

concentration was fixed at 100 nM. All results are expressed as mean ± standard deviation (n = 4, *p < 0.05).

To elucidate the underlying mechanism of the enhanced gene silencing with PAsp(TET-T), the cellular uptake and intracellular trafficking of siRNA cargos were evaluated further. In the cellular uptake study, siRNA PICs were prepared using Alexa 546-labeled siRNA at an N/P value of 20 and incubated with HuH7-Luc cells for 24 h. The cellular uptake efficiency of siRNA was subsequently quantified by measuring the mean fluorescence intensity from the cells using a flow cytometer (Supporting Figure S2). Cells treated with PAsp(TET)- and PAsp(TET-T)-based PICs displayed remarkably higher fluorescence intensities than those treated with naked Alexa 546-labeled siRNA, indicating that PIC formation dramatically facilitates cellular uptake of siRNA. Meanwhile, identical fluorescence intensities were observed for PAsp(TET)- and PAsp(TET-T)-based PICs, suggesting their similar uptake efficacy. Next, the intracellular trafficking of siRNA cargos using PAsp(TET) and PAsp(TET-T) was compared. The PICs were prepared with Alexa 546-labeled siRNA before incubation with HuH7-Luc cells for 24 h and subsequent analysis by confocal laser scanning microscopy (CLSM, Figure 4). Alexa 546-labeled siRNA, late-endosomes/lysosomes stained with LysoTracker Green, and nuclei stained with Hoechst 33342 appear red, green, and blue, respectively. Thus, yellow pixels, resulting from the merging of red and green pixels, represent the colocalization of Alexa 546-labeled siRNA with late-endosomes/lysosomes. More red pixels were observed in the cells treated with PAsp(TET-T)-based PICs (Figure 4B), compared with PAsp(TET)-based PICs (Figure 4A), suggesting that Alexa 546-labeled siRNA delivered by PAsp(TET-T) was less efficiently entrapped by late-endosomes/lysosomes.

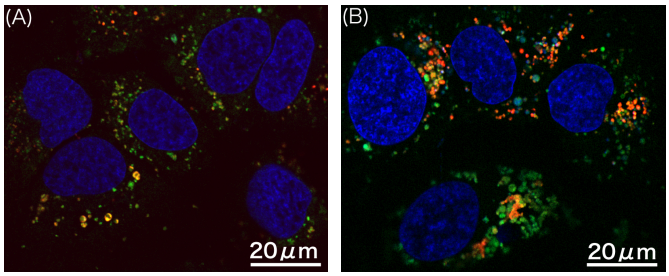


Figure 4. Intracellular distribution of Alexa 546-labeled siRNA PICs in HuH7-Luc cells observed by CLSM. A) PAsp(TET)-based PICs and B) PAsp(TET-T)-based PICs prepared at N/P = 20. Alexa 546-labeled siRNA, late-endosomes/lysosomes stained with LysoTracker Green, and nuclei stained with Hoechst 33342 appear red, green, and blue, respectively.

The endosomal entrapment efficiency of siRNA was quantitatively evaluated by calculating a colocalization ratio, i.e., a pixel ratio of yellow to the sum of yellow and red.[15,18] The colocalization ratios were calculated to be ~53% and ~25% for PAsp(TET)- and PAsp(TET-T)-based PICs, respectively. The significantly lower colocalization ratio of the PAsp(TET-T)-based PICs ($p < 0.05$ for PAsp(TET)-based PICs) demonstrates more efficient endosomal escape of siRNA cargos, which is consistent with the enhanced gene silencing efficiency (Figure 3B). This efficient endosomal escape may originate from the modulated protonation behaviour of PAsp(TET-T). The thiourea modification afforded a high α at pH 5.5 and a large Δα (Figure 2), generating a highly charged diprotonated diaminoethane structure (–$NH_2^+CH_2CH_2NH_2^+$–) in the side chains at pH 5.5 for acidic pH-selective membrane destabilization, similar to PAsp(DET).[15,18] Unlike PAsp(DET), PAsp(TET-T) was expected to form stable siRNA PICs, presumably due to the hydrogen bonding between thiourea moieties and siRNA phosphates. Thus, the stability of PAsp(TET-T)-based PICs was further verified by fluorescence correlation spectroscopy. The diffusion coefficient of fluorescently labeled siRNA loaded in PAsp(TET-T)-based PICs at N/P = 20 was determined to be 1.5 ± 0.4 μm^2/sec after 1 h incubation in 10% fetal bovine serum-containing medium. This value was substantially smaller than that of naked siRNA (101 ± 7 μm^2/sec), indicating that PAsp(TET-T)-based PICs were maintained without dissociation (or siRNA release) in the transfection medium. This significant PIC stability is in sharp contrast with the prompt dissociation of PAsp(DET)-based PICs within 10 min.[19]

In summary, the construction of siRNA carriers that enable safe and efficient siRNA delivery relies on the optimization of protonation behaviour of polycations. Herein, PAsp(TET) was functionalized with thiourea moieties to tune its protonation based on the repeated number of AE units. A potentiometric titration revealed that the thiourea modification dramatically increased the fraction of protonatable amines at endosomal acidic pH by reducing the apparent repeated number of AE units. Consequently, PAsp(TET-T) significantly enhanced the gene silencing activity of siRNA PICs without cytotoxicity, presumably because of the facilitated endosomal escape of siRNA cargos. Therefore, a strategic regulation of the repeated number of AE units in polyaspartamide side chains was demonstrated to improve the delivery efficacy of siRNA PICs.

This research was financially supported by the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST) from Japan Society for the Promotion of Science (JSPS) and the Center of Innovation (COI) program from Japan Science and Technology Agency (JST). H. Gao would like to express her thanks to JSPS Fellowship for Research in Japan (NO. L11524).

Notes and references

[a] *School of Chemistry and Chemical Engineering, Tianjin University of Technology, Tianjin, 300384 China*

[b] *Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.*

[c] *Polymer Chemistry Division, Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan*

[d] *Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

[e] *Kawasaki Institute of Industry Promotion, 66-20 Horikawa-cho, Saiwai-ku, Kawasaki 212-0013, Japan*

** To whom correspondence should be addressed:*

K. Kataoka, kataoka@bmw.t.u-tokyo.ac.jp; K. Miyata, miyata@bmw.t.u-tokyo.ac.jp

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

1 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S .E. Driver, C. C. Mello, *Nature* 1998, **391**, 806.
2 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* 2001, **411**, 494.
3 H. Wang, W. Chen, H. Xie, X. Wei, S. Yin, L. Zhou, X. Xu, S. Zheng, *Chem. Commun.,* 2014, **50***,* 7806.
4 R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat. Mater.* 2013, **12**, 967.
5 K. Itaka, N. Kanayama, N. Nishiyama, W. -D. Jang, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, *J. Am. Chem. Soc.* 2004, **126**, 136.
6 R. M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Strom, G. Molema, P. Y. Lu, P. V. Scaria, M. C. Woodle, *Nucleic Acids Res.* 2004, **32**, e149.
7 P. Kumar, H. Wu, J. L. McBride, K. -E. Jung, M. H. Kim, B. L. Davidson, S. K. Lee, P. Shankar, N. Manijunath, *Nature* 2007, **448**, 39.
8 M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel, A. Ribas, *Nature* 2010, **464**, 1067.
9 K. Miyata, N. Nishiyama, K. Kataoka, *Chem. Soc. Rev.* 2012, **41**, 2521.
10 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. U.S.A.* 1995, **92**, 7297.
11 M. Neu, D. Fischer, T. Kissel, *J. Gene. Med.* 2005, **7**, 992.
12 K. L. Kozielski, S. Y. Tzeng, J. J. Green *Chem. Commun.,* 2013, **49**, 5319.
13 A.C. Hunter, *Adv. Drug Deliv. Rev.* 2006, **58**, 1523.
14 K. Masago, K. Itaka, N. Nishiyama, U. –I. Chung, K. Kataoka, *Biomaterials* 2007, **28**, 5169.
15 H. Uchida, K. Miyata, M. Oba, T. Ishii, T. Suma, K. Itaka, N. Nishiyama, K. Kataoka *J. Am. Chem. Soc.* 2011, **133**, 15524.
16 K. Miyata, R. J. Christie, T. Suma, H. Takemoto, H. Uchida, N. Nishiyama, K. Kataoka, *ACS Symposium Series* 2013, **1135**, 189.
17 H. Uchida, K. Itaka, T. Nomoto, T. Ishii, T. Suma, M. Ikegami, K. Miyata, M. Oba, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* 2014, **136**, 12396.
18 K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* 2008, **130**, 16287.
19 T. Suma, K. Miyata, T. Ishii, S. Uchida, H. Uchida, K. Itaka, N. Nobuhiro, K. Kataoka, *Biomaterials* 2012, **33**, 2770.
20 C. Li, Y.-W. Yang, Z. Liang, G. Wu, H. Gao, *Polym. Chem.* 2013, **4**, 4366.

