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

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Effects of repeated number (RN) of aminoethylene (AE) units in polyaspartamide side chains were investigated for polycation 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. 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. In this regard, polycation 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.

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. 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. 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 (-NHCH2CH2-) 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 (Δα) 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)). 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. 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. Therefore, the side chain structure of polyaspartamide was further explored in this study to optimize siRNA delivery.

![Figure 1. 1H NMR spectrum of PAsp(TET-T) in D2O at 25 °C.](image-url)

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.\textsuperscript{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\textsubscript{w}/M\textsubscript{n}): 1.1) with 2 molar equivalents of methyl isothiocyanate (Supporting Scheme S1). The \textsuperscript{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 measured 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 a plots as a function of pH (Figure 2). The thiourea modification altered the protonation behaviour of PAsp(TET) especially at acidic pH. Whereas an \( \alpha \) value of PAsp(TET-T) was similar to that of PAsp(TET) at pH 7.4, PAsp(TET-T) exhibited a larger \( \alpha \) value at pH 5.5, compared with PAsp(TET), allowing an appreciably large PAsp(TET-T) α value at pH 5.5, compared with PAsp(TET), 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) exhibited ~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).
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. The colocalization ratios were calculated to be ∼53% and ∼25% for PAsp(TET-) and PAsp(TET)-based PICs, respectively. The significantly lower colocalization ratio of the PAsp(TET)-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 z at pH 5.5 and a large Δα (Figure 2), generating a highly charged diprotinated diaminoethane structure (−NH₂CH₂CH₂NH₂−) in the side chains at pH 5.5 for acidic pH-selective membrane destabilization, similar to PAsp(DET). 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 μm²/sec after 1 h incubation in 10% fetal bovine serum-containing medium. This value was substantially lower than that of naked siRNA (101 ± 7 μm²/sec), indicating that PAsp(TET)-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.

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-T) 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.

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