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Supramolecular Nanocarrier of siRNA from PEG-Based Block Catiomer Carrying Diamine Side Chain with Distinctive pK_a Directed To Enhance Intracellular Gene Silencing

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The short double-stranded RNA species, called short interference RNA (siRNA), can be used to silence the gene expression in a sequence-specific manner in a process that is known as RNA interference (RNAi).[1] It has become a useful method for the analysis of gene functions and holds the significant possibility of therapeutic application. However, to promote an efficient gene knockdown, especially in an in vivo situation, two substantial issues must be considered: tolerability under physiological conditions and enhanced cellular uptake. Thus, the development of effective siRNA delivery systems is required.

Recently, a new delivery system of plasmid DNA and oligonucleotides has been developed, based on the micellar assembly of the poly-ion complex (PIC) of these compounds with block copolymers consisting of poly(ethylene glycol) (PEG) and polycation segments, leading to the self-assembled structure with a core−shell architecture (PIC micelles).[2] Their excellent properties for in vivo DNA delivery have been confirmed so far:[3] a diameter around 100 nm with a PEG palisade which enables complexes to avoid recognition by reticuloendothelial systems, increased nuclease resistance, increased tolerance under physiological conditions, and the excellent gene expression in a serum-containing medium.[4]

We now describe the structural design of a novel block catiomer-based PIC particularly available for siRNA delivery. PEG−poly-(3-[(3-aminopropyl)amino]propylaspartamide (PEG-DPT; PEG, 12 000 g/mol, polymerization degree of DPT segment, 68), carrying a diamine side chain with distinctive pK_a, was newly synthesized by a side-chain aminolysis reaction of PEG−poly(β-benzyl-L-aspartate) block copolymer (PEG-PBLA) with dipropylene triamine (DPT) (Figure 1A and Figure S1 in the Supporting Information). A model compound of a DPT unit, *tert*-butoxycarbonyl-β-*N*-3-(3-aminopropyl)aminopropylamido-α-*N*-propyl-(L)-aspartamide (Boc-Asp(DPT)-Pr), was also synthesized (see Supporting Information) to determine the pK_a values of the amino groups.

Boc-Asp(DPT)-Pr clearly gave a two-stage pH−α curve (Figure 1B), from which the pK_a values of the primary and secondary amino groups were determined to be 9.9 and 6.4, respectively. Amino groups in the PIC of polyamine with polynucleotides including siRNA generally undergo facilitated protonation due to the zipper effect or the neighboring group effect during the complexation process, hampering the proton buffering or the proton sponge capacity. The unique feature of PEG-DPT is the regulated location of primary and secondary amino groups in the side chain: the former, with higher pK_a, settles at the distal end of the side chain to participate in the ion complex formation with phosphate groups in siRNA molecule, whereas the latter, with lower pK_a, located closer to the polymer backbone, is expected to leave a substantial

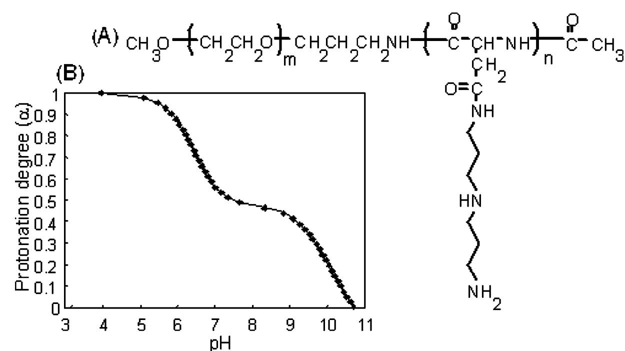

Figure 1. (A) Chemical structure of PEG-DPT. (B) Change in protonation degree (α) with pH for Boc-Asp(DPT)-Pr.

fraction of unprotonated form even in the complex, presumably due to the lower protonation power and the spatial restriction, directing to the enhanced intracellular activity of siRNA through the buffering capacity in the endosomal compartment.

The formation of the siRNA complex with the PEG-DPT was confirmed by polyacrylamide gel electrophoresis (PAGE) and the ethidium bromide (EtBr) exclusion assay (see Figure S2 in the Supporting Information). Note that intercalators such as EtBr bind the double-stranded (ds) RNA in the same fashion as dsDNA.[5] The free siRNA disappeared at the N/P ratio (= [total amines in cationic segment]/[siRNA phosphates]) >2, in line with a substantial fluorescence quenching of EtBr at N/P ≥ 2 due to the inaccessibility of EtBr to the complexed siRNA with PEG-DPT. Furthermore, the EtBr assay highlights the distinctive role of primary and secondary amino groups of the side chain in the complex. The PIC of the double-stranded oligo DNA, composed of sequences similar to the GL3 targeting siRNA, with PEG−poly(3-dimethylamino)propyl aspartamide (PEG-DMAPA; pK_a ≈ 7.9, see Figure S3 for chemical structure), revealed a lower degree of EtBr quenching compared to the PEG-DPT/ds-oligo DNA PIC, even in the region of excess N/P ratios (see Figure S4), suggesting that the presence of unprotonated amino groups in the former may hamper the tight association. PEG−poly(L-lysine) (PEG-PLL; pK_a ≈ 9.37, see Figure S3 for chemical structure) induced EtBr quenching as significantly as PEG-DPT upon complexation with the ds-oligo DNA, yet the quenching leveled off at the stoichiometric N/P ratio (N/P = 1.0) (Figure S4). This is in sharp contrast with the PEG-DPT/ds-oligo DNA complex, which showed leveling-off behavior of EtBr quenching at N/P ≈ 2.0, suggesting that secondary amines with the lower pK_a may be excluded from the ion complexation with oligonucleotides.

These distinctive features of the PEG-DPT, PEG-DMAPA, and PEG-PLL complexes indeed correlated with their gene knockdown abilities. For this evaluation, the GL3 luciferase gene was targeted

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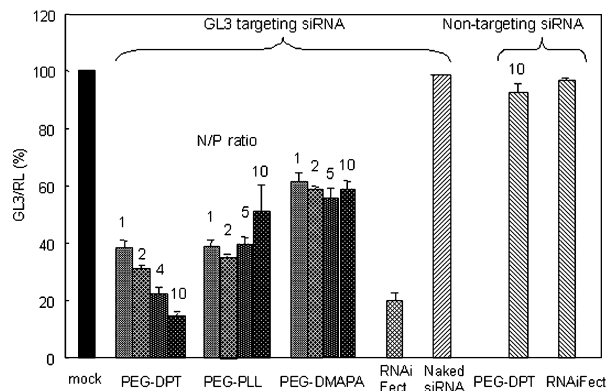

Figure 2. GL3 luciferase gene knockdown ($n = 4$; ±SD).

after transfecting two kinds of luciferase pDNAs (pGL3 and pRL; Promega) to HuH-7 cells. The expression ratio of GL3/RL was used as the knockdown marker.

Each complex system showed a sufficient knockdown of the GL3 luciferase, while neither the naked siRNA nor the nontargeting siRNA showed any knockdown (Figure 2). Thus, these results should be recognized as the veritable RNAi by the GL3-targeting siRNA delivered into the cytoplasm. Notably, the gene knockdown abilities of the siRNA/PEG-DPT complex were superior to those of the other two complexes, especially at higher N/P ratios. At N/P = 10, it showed more than an 80% knockdown, which exceeded the commercial RNAiFect. The cell viability evaluated by MTT assay was more than 75% of the mock cells, even after co-incubation with siRNA/PEG-DPT with N/P ≥ 10 (see Figure S5), suggesting the toxic effect to be eliminated. The siRNA/PEG-DMAPA complexes showed knockdown abilities to a lesser extent. Apparently, the loosely associated nature of siRNA, suggested by the EtBr exclusion assay, is unfavorable for facilitating an effective intracellular delivery of intact siRNA. PEG-PLL showed a considerable knockdown ability in the low N/P region, yet no particular enhancement with the increase in the N/P ratios. High efficacy of PEG-DPT may be characterized by the existence of additional secondary amines with a lower pK_a to promote the internalization of the siRNA molecules into the cytoplasm through buffering of the endosomal cavity, as is the case with the polyethylenimine-based polyplex that shows an enhanced tranfection efficiency at the higher N/P ratios.[6]

A serum incubation study was then performed to evaluate the complex stability under physiological conditions by incubating the complexes in 50% serum at 37 °C prior to transfection. The siRNA/PEG-DPT complexes showed comparable abilities of gene knockdown, even after co-incubation with serum for 30 min (Figure 3A). In contrast, the lipid-based RNAiFect system was significantly influenced by the serum incubation, probably due to the nonspecific association with serum proteins. Thus, these results highlighted the excellent feasibility of the PEG-DPT/siRNA complex, particularly under physiological conditions due to the segregation of siRNA into the PEG microenvironment.

The results of the endogenous gene knockdown were more fascinating. For this purpose, a cytoskeletal protein, Lamin A/C, was targeted.[1] The PEG-DPT system showed a significant gene knockdown of Lamin A/C mRNA, even after a 30-min preincubation in 50% serum, evaluated by the real-time RT-PCR analysis. Notably, in 293T cells, the expression was suppressed to the level of 20% of mock samples, which significantly exceeded the ability

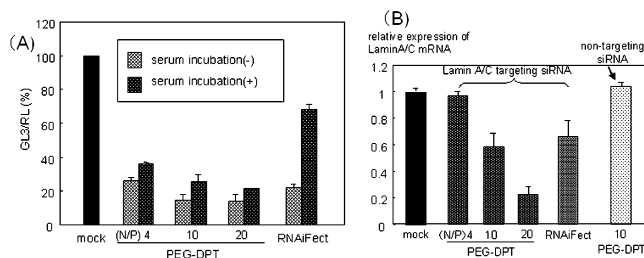

Figure 3. (A) GL3 knockdown by siRNA complexes after serum co-incubation with serum. (B) Endogenous gene (Lamin A/C) knockdown after co-incubation ($n = 4$; ±SD).

of the RNAiFect (Figure 3B). A similar trend was also observed in HuH-7 cells. However, neither the PEG-PLL nor PEG-DMAPA system showed any gene knockdown (data not shown). As Lamin A/C is assumed to abundantly express inside the cells, the threshold level of the siRNA's introduction that is necessary to show the inhibition of gene expression should be significantly higher than in the case of the luciferase cotransfection study. Thus, these results of PEG-DPT were very encouraging for the actual therapeutic knockdown of an endogenous gene by the siRNA delivering approach.

In conclusion, we reported here an effective siRNA nanocarrier system based on the self-assembly of the PEG-based block catiomer. The distinctive polymer design managed both a sufficient siRNA complexation and a buffering capacity of the endosomes. Notably, the siRNA/block catiomer complex revealed remarkable knockdown of the endogenous gene, even after the serum incubation. These results directed this newly designed system of block catiomer to have a promising feasibility for in vivo therapeutics.

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Supporting Information Available: Detailed Materials and Methods section; ^{1}H NMR spectrum of PEG-DPT block copolymer (Figure S1); results of PAGE and EtBr exclusion assay of PEG-DPT (Figure S2); chemical structures of PEG-DMAPA and PEG-PLL (Figure S3); summary of EtBr exclusion assay of these copolymers (Figure S4); and result of MTT assay (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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