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PEG-siRNA Conjugate Bearing 27 bp siRNA to Form Novel PEGylated Polyplexes with Improved Stability

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Methoxy-PEG-*b*-siRNAs possessing 27 base pairs siRNA (27 bp-siRNA) and 21 base pairs siRNA (21 bp-siRNA) were successfully synthesized via the Michael addition reaction of 5'-thiol modified siRNA and poly(ethylene glycol) (PEG) bearing an acrylate group at ω-end. PEGylated polyplexes were prepared through the self-assembly of poly(*N*-3,6-diazahexyl aspartic acid amide) (P[Asp(DET)]) and each of PEG-siRNA conjugates bearing an endosomal pH-responsive linkage between PEG chain and siRNA. The stability of the PEGylated polyplexes containing 27 bp-siRNA segment with various ratios of the number of protonated amino group units per nucleotide (N^+/P ratios) was compared with that of the PEGylated polyplexes containing 21 bp-siRNA segment by means of polyacrylamide gel electrophoresis. Worth noticing that complete retardation of the conjugate was observed at an N^+/P ratio ≥ 3 for PEGylated polyplex composed of PEG-27 bp-siRNA conjugate, whereas the PEGylated polyplex composed of PEG-21 bp-siRNA showed the complete retardation of the conjugate at N^+/P ratio ≥ 5. This result indicate that longer siRNA segment in conjugate contributes to the improvement of the stability of PEGylated polyplexes due to the increase in the electrostatic interactions.

Key words: RNAi, PEGylated polyplex, PEG-siRNA conjugate, 27 base pairs siRNA, 21 base pairs siRNA, stability

1. INTRODUCTION

Small interfering RNAs (siRNAs)[1] are recently recognized as one of the most powerful tools for sequence-specific gene silencing via naturally occurring RNA interference (RNAi) process. To use clinical applications of siRNAs, however, the development of siRNA delivery system is strongly desired due to their low stability against enzymatic degradation, low permeability across cell membrane, and preferential liver and renal clearance. Worth noticing in this regard is the combination of PEGylation and carrier system, *viz.* a "smart" siRNA carrier (PEGylated polyplex) formulated through the supramolecular assembly (electrostatic interactions) of poly(L-lysine) (PLL) and lactosylated poly(ethylene glycol)-siRNA conjugate (Lac-PEG-siRNA). These smart PEGylated polyplexes, which have a size of approximately 100 nm, showed the high biocompatibility and enzymatic tolerability due to their segregated polyion complex core surrounded by a palisade of flexible and hydrophilic PEG layers. In particular, PEGylated polyplexes with clustered lactose moieties on their periphery were successfully transported into the HuH-7 cells (human hepatocarcinoma cell lines) by mediation of the asialoglycoprotein (ASGP) receptor, inducing significant gene silencing in monolayer-cultured HuH-7 cells [2] and growth inhibition of multicellular HuH-7 spheroids for up to 21 days [3]. Nevertheless, the stability needs to be further improved for use in *in vivo* injection due to the dissociation of the PEGylated polyplex in the blood stream. The electrostatic interaction between siRNA segment with 21 base pairs (21 bp-siRNA) and PLL seems to be insufficiently low to overcome this dissociation problem of the PEGylated polyplex.

Recently, siRNA with 27 base pairs (27 bp-siRNA) has been reported to be 100 times higher RNAi activity than that of conventional siRNA (21 bp), keeping low interferon response [4]. The PEGylated polyplex constructed from PEG-27 bp-siRNA conjugate and polycation strategy is effective in the successful delivery of siRNA *in vivo*, *viz.*, longer 27 bp-siRNA segment that settled in the core of the PEGylated polyplex, acquired higher stability and activity compared with the PEGylated polyplex containing 21 bp-siRNA segment. We described here the preparation and stability of novel

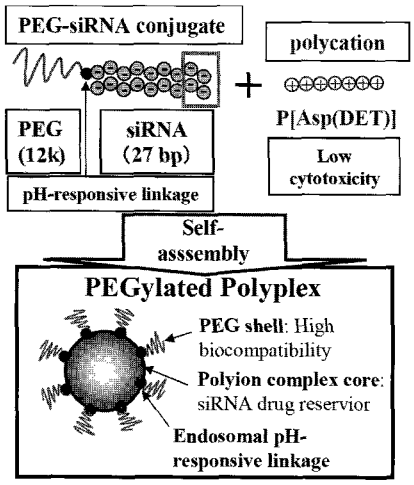


Fig. 1 Schematic illustaration of novel PEGylated polyplex composed of PEG-27 bp-siRNA conjugate andP[Asp(DET)].

PEGylated polyplex composed of PEG-27 bp-siRNA conjugate and poly(*N*-3,6-diazahexyl aspartic acid amide) (P[Asp(DET)]). Note that P[Asp(DET)] showed low cytotoxicity and high buffering efficiency based on the distinctive two-step protonation behavior of the flanking ethylenediamine moiety [5]. Furthermore, the character of the siRNA length (27 bp vs. 21 bp) on the stability of the PEGylated polyplexes was compared in this study to demonstrate the applicability of this type of molecular design in diverse circumstances.

2. EXPERIMENTAL PROCEDURE

General

Tetrahydrofuran (THF), acryloyl chloride, and triethylamine were purified by conventional methods. D,L-dithiothreitol and acetonitrile were purchased from Wako (JAPAN) without further purification. Trizma base and 1 M Tris-HCl solution (pH 8.0) were purchased from Sigma-Aldrich (US) without further purification. Water was purified using a Milli-Q instrument (MILLIPORE, US). α-methoxy-poly(ethylene glycol) (M_n = 11,700, M_w/M_n = 1.02) was purchased from Nichiyu (JAPAN). 5'-thiol modified 27 bp RNA(5'-HS(CH_2)$_6$-ACA AGU GAA GUC AAC AUG CCU GCC CCA-3', B-cell lymphoma, anti-apototic gene), complementary antisense 27 bp RNA (3'-UGU UCA CUU CAG UUG UAC GGA CGG GGU-5'), unmodified siRNA (27 bp, annealed), 5'-thiol-modified sense 21 bp RNA (5'-HS(CH_2)$_6$-GUG AAG UCA ACA UGC CUG CdTdT-3'), and complementary antisense 21 bp RNA (3'-dTdTC ACU UCA GUU GUA CGG ACG-5') were purchased from Eurogentec (Belgium). Poly(*N*-3,6-diazahexyl aspartic acid amide) (M_w = 27,000, degree of polymerization (DP) = 98) was synthesized according to the previous paper [5]. ^{1}H NMR (270 MHz) spectra were obtained in $CDCl_3$ with a EX270 spectrometer (JEOL, JAPAN). Chemical shifts are reported in ppm relative to $CDCl_3$ (δ = 7.26, 1H). Size exclusion chromatography (SEC) was performed with a HLC-8020 apparatus (TOSOH, JAPAN) equipped with an internal refractive index (RI) detector (RID-6 A) with a combination of TSK G4000HR and G3000HR columns and THF as the eluent. The RNA concentration was determined by reading the absorbance at 260 nm using NanoDrop ND-1000 (NanoDrop Technologies, US). Reversed phase chromatography was performed using the JASCO HPLC system equipped with an ultraviolet detector (UV-2075) and a TOSOH OligoDNA-RP column, and mixture of 100 mM ammonium acetate buffer (pH 7.0) and acetonitrile (gradient ranging from 5%(v/v) to 70%(v/v)) as the eluent.

Synthesis of poly(ethylene glycol) bearing an acryloyl group at the ω-end

Synthesis of α-methoxy-ω-acryloyloxy-poly(ethylene glycol) was carried out according to our previous paper [6]; To a mixture of poly(ethylene glycol) bearing a methoxy group at α-end and a hydroxyl group at ω-end (4.68 g, 0.4 mmol) and triethylamine (1.67 mL) in THF (60 mL), acryloyl chloride (0.485 mL, 6.0 mmol) was added, and the reaction mixture was stirred at room temperature under argon atmosphere in the dark for 2 days. The polymer was recovered by precipitation into cold 2-propanol (-15 °C, 2 L) and centrifuged for 30 min at 5,000 rpm. Further purification was carried out by dialysis against distilled, deionized water (MW cutoff 3,500), and the product was freeze-dried to give poly(ethylene glycol) bearing an acryloyl group at the ω-end (4.30 g, 91% yield). SEC M_n = 11,200, M_w/M_n=1.06 (calcd. M_n = 11,740); ^{1}H NMR ($CDCl_3$): δ = 3.40 (s, 3H, CH_3O), 3.66 (s, 1008H, PEG-backbone + $COOCH_2CH_2$), 4.33 (t, J=8.1 Hz, 2H, $COOCH_2$), 5.84 (d, J=10.8 Hz, 1H, CH_2=CH), 6.15 (dd, J=10.8, 18.1 Hz, 1H, CH_2=CH), 6.43 (d, J=18.1 Hz, 1H, CH_2=CH).

Synthesis of PEG-siRNA conjugates

Synthesis of PEG-siRNA conjugates (27 and 21 bps) was carried out via Michael addition reaction of acrylated poly(ethylene glycol) (M_n = 11,200) and siRNA bearing a 5'-thiol-modified sense RNA, according to our previous papers [2, 6]. To a solution of 27 bp-siRNA (232 μg, 13.3 nmol) in 200 mM Tris-HCl buffer pH 8.0 (1000 μL), excess acrylated poly(ethylene glycol) (7.35 mg, 1930 nmol, 145 eq.) was added, and the reaction mixture was stirred at room temperature for 48 h in the dark. Purification of PEG-27 bp-siRNA conjugate was carried out by means of reversed phase HPLC, followed by removal of acetonitrile by centrifugation concentration at room temperature for 1 h, and then dialyzed by ultrafiltration against 10 mM Tris-HCl buffer pH 7.4 (MW cutoff 20,000). Then PEG-27 bp-siRNA conjugate was obtained (18% yield). PEG-21 bp-siRNA conjugate was also synthesized with the same procedure described above (21% yield).

Preparation of PEGylated polyplexes and their polyacrylamide gel electrophoresis for retardation assay

Specific amounts of PEG-siRNA conjugate (27 and 21 bps) were dissolved in 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) to prepare the 500 nM stock solution. P[Asp(DET)] was added to 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) to prepare the 5 μM stock solutions. The PEG-siRNA conjugate (27 and 21 bps) in 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) was mixed with

P[Asp(DET)] in 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) at various molar ratio of phosphate groups in the siRNA segment to protonated amino groups in P[Asp(DET)] (N^+/P ratios = 0.5, 1, 1.25, 1.5, 2, 3, 5, and 8). Aliquots (10 μL) of the PEGylated polyplex in 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) at various N^+/P ratios were loaded onto a 12 % acrylamide gel. The amount of PEG-siRNA conjugate was adjusted to 250 nM of PEG-siRNA/lane. The PEGylated polyplexes were subjected at 100 V for 1 h in 10 mM TBE buffer (pH 7.4). After EtBr (0.5 mg/mL) staining for 1 h, retardation of PEG-siRNA conjugates were visualized under UV irradiation.

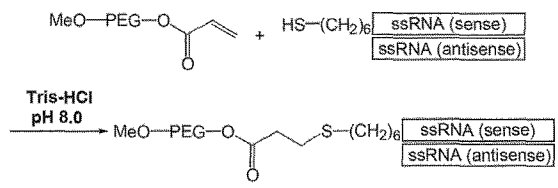
Dynamic Light Scattering Measurement

Dynamic light scattering (DLS) measurement of PEGylated polyplex prepared from PEG-27 bp-siRNA and P[Asp(DET)](98) at N^+/P ratio = 3 was performed using the Zetasizer nano ZS (Malvern Instruments Ltd., UK) at 37 °C. The data obtained from the rate of decay in the photon correlation function were analyzed by means of the cumulant method, and the corresponding hydrodynamic average diameter and polydipersity index (PDI; μ_2/Γ^2) of PEGylated polyplex were calculated by the Stokes–Einstein equation.

3. RESULTS AND DISCUSSION

Synthesis of PEG-siRNA conjugate

A synthetic route of PEG-siRNA conjugates is shown in **Scheme 1**.



Scheme 1

Michael addition reaction of the siRNA (27 and 21 bps) bearing a 5'-thiol-modified sense RNA was carried out in the presence of excess amount of the acrylated PEG, according to the previous papers [2, 6]. The PEGylation profile was monitored by RP-HPLC equipped with a UV detector (wave length: 260 nm). Figure 2 shows the RP-HPLC chromatograms of (a) the SH-27 bp-siRNA, (b) PEG-acrylate, (c) the reaction mixture after 2 h, and (d) the purified PEG-27 bp-siRNA conjugate. Obviously, a new peak was observed at 26.5 min after 2 h in the reaction mixture (**Fig. 2c**), indicating the formation of PEG-27 bp-siRNA conjugate. Furthermore, the purified PEGsiRNA conjugates showed a single band in polyacrylamide gel electrophoresis (**Fig. 3**, lanes c and f) with a retarded migration compared with the free siRNA and ssRNA (**Fig. 3**, lanes a, d and b, e, respectively), visualized under UV irradiation (302 nm). All of these results are consistent with the successful preparation of the PEG-siRNA conjugates without contamination.

Stability of PEGylated polyplexes: polyacrylamide gel retardation assay

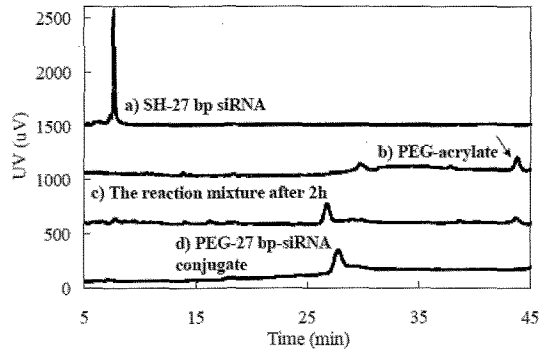


Fig. 2 RP-HPLC chromatograms of (a) SH-27 bp-siRNA, (b) PEG-acrylate, (c) the reaction mixture after 2 h, and (d) the purified PEG-27 bp-siRNA conjugate. (wave length, 260 nm; ambient temperature)

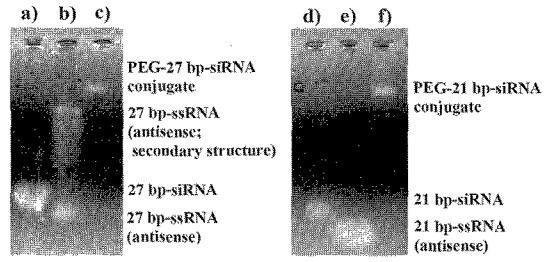


Fig. 3 Results of polyacrylamide gel retardation assay of PEG-siRNA conjugates were visualized under UV irradiation (302 nm). (left) (a) 27 bp-siRNA, (b) 27 bp ssRNA (antisense), and (c) PEG-27 bp-siRNA conjugate. (right) (d) 21 bp-siRNA, (e) 21 bp-ssRNA (antisense), and (f) PEG-21 bp-siRNA conjugate. Final concentration of PEG-siRNA conjugates were adjusted to 250 nM.

PEGylated polyplexes were prepared through the mixing with PEG-siRNA conjugates and P[Asp(DET)] (DP = 98) at various N^+/P ratios based on electrostatic interaction. The final concentration of PEG-siRNA conjugate for each samples were unity (250 nM). To evaluate the stability of the PEGylated polyplexes (27 and 21 bps), polyacrylamide gel retardation assay was carried out, the results of which are shown in **Fig. 4**. EtBr-intercalated free PEG-siRNA conjugate was visualized under UV irradiation (wave length: 302 nm). Figure 5 shows the relative fluorescence intensity from the intercalated EtBr as a function of N^+/P ratio of PEGylated polyplexes. Relative intensity of free PEG-siRNA conjugate band was plotted. In the cases of PEGylated polyplexes composed PEG-21 bp-siRNA conjugate, complete retardation of the conjugate was observed at an N^+/P ratio > 5 (**Figs. 4b** and **5**). In sharp contrast, the PEGylated polyplex composed of PEG-27 bp-siRNA showed the complete retardation of the conjugate at N^+/P ratio > 3 (**Figs. 4a** and **5**). Thus, PEGylated polyplex composed of longer siRNA segment showed the improvement of the stability of PEGylated polyplexes due to the increase in the electrostatic interactions. According to the result of lactose-PEG5k-17mer ODN / PLL (DP = 46, 100, and 460) complex, larger polycation made its complex more stable than smaller one, however, lowered antisense effect for the systems with longer PLL (DP = 460) due

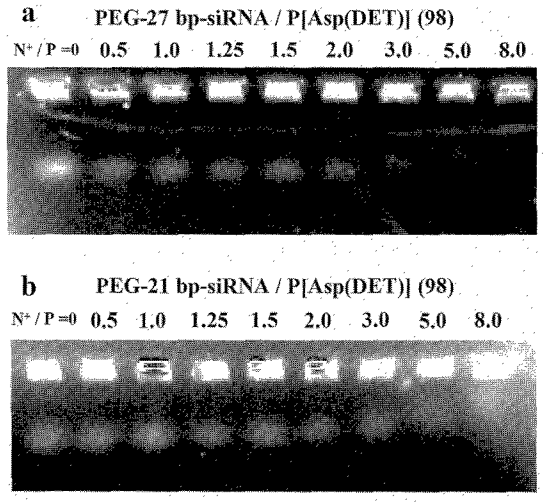


Fig. 4 Results of polyacrylamide gel retardation assay of PEGylated polyplexes with various N^+/P ratios; (a) PEG-27 bp-siRNA / P[Asp(DET)] (98), (b) PEG-21 bp-siRNA / P[Asp(DET)] (98). EtBr-intercalated free PEG-siRNA conjugate was visualized under UV irradiation (302 nm). Final concentration of PEG-siRNA conjugates were 250 nM.

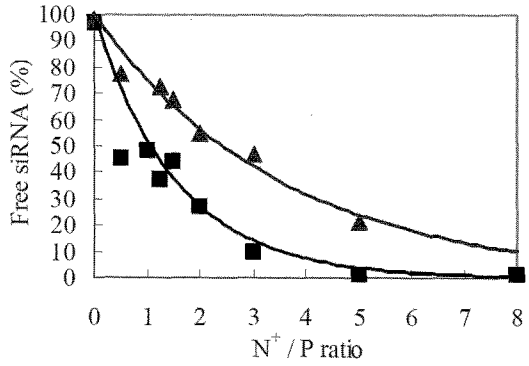


Fig. 5 Relative fluorescence intensity from the intercalated EtBr as a function of N^+/P ratio of PEGylated polyplexes. Relative intensity of free PEG-siRNA conjugate band was plotted. Closed square, PEG-27 bp-siRNA / P[Asp(DET)] (98); closed triangle, PEG-21 bp-siRNA / P[Asp(DET)] (98).

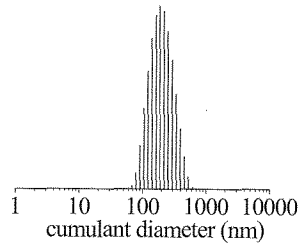


Fig. 6 Size distribution of PEG-27 bp-siRNA / P[Asp(DET)] (98) (N^+/P ratio = 3) analyzed by DLS. Final concentration of PEG-siRNA was 100 nM.

to overstabillization of polyplex core, restricting the release of the antisense ODN into cytoplasm [7], thus the most suitable polylysine length of three PLLs in this case was DP = 100. From the result of PEGylated polyplexes in this work, it is possible that larger siRNA (27 bp) make its complex more stable. It is expected that the stability of PEGylated polyplex is increased with larger siRNA, however, RNA duplex longer than 30 bp showed progressive loss of functional RNAi activity [4], thus, 27 bp should be the most suitable siRNA length. It is considered that combination of PEG-27 bp-siRNA conjugate and polycation with DP around 100 must be the best for preparing PEGylated polyplex with potent RNAi activity through our works.

Dynamic Light Scattering Measurement of PEGylated polyplex

The size of the PEGylated polyplex was evaluated by dynamic light scattering (DLS). **Fig. 6** shows the size (gamma) distribution of PEG-27 bp siRNA / P[Asp(DET)](98) (N^+/P ratio = 3), where the free PEG-27 bp-siRNA was absence as observed by PAGE (**Fig. 4**). The average diameter and polydispersity index (PDI; μ_2/Γ^2) of the PEGylated polyplex were found to be 184 nm and 0.057, respectively, indicating the formation of nano-sized PEGylated polyplex through the electrostatic interaction between the siRNA segment and P[Asp(DET)](98), as described in **Fig. 1**.

4. CONCLUSION

We have succeeded to isolate PEGylated nucleic acid composed of 27 bp-siRNA and revealed that PEGylated polyplex containing 27 bp-siRNA segment was more stable nanoparticle compared to that containing 21 bp-siRNA segment, due to the increase in the electrostatic interactions. It is expected that the stability of PEGylated polyplex containing 27 bp-siRNA segment in the blood stream should be better than that containing 21 bp-siRNA segment, in addition, the PEGylated polyplex containing 27 bp-siRNA segment is expected to facilitate the practical utility as siRNA therapeutics.

5. ACKNOWLEDGMENT

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6. REFERENCES

[1] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, *Nature* **411**, 494-498 (2001).
[2] M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama, and K. Kataoka, *J. Am. Chem. Soc.* **127**, 1624-1625 (2005).
[3] M. Oishi, Y. Nagasaki, N. Nishiyama, K. Itaka, M. Takagi, A. Shimamoto, Y. Furuuchi, and K. Kataoka, *ChemMedChem* **2**, 1290-1297 (2007).
[4] D.-H. Kim, M. A. Behlke, S. D. Rose, M.-S. Chang, S. Choi, and J. J. Rossi, *Nat. Biotec.* **23**, 222-226 (2005).
[5] M. Han, Y. Bae, N. Nishiyama, K. Miyata, M. Oba, and K. Kataoka, *J. Control. Release* **121**, 38-48 (2007).
[6] M. Oishi, S. Sasaki, Y. Nagasaki, and K. Kataoka, *Biomacromolecules* **4**, 1426-1432 (2003).
[7] M. Oishi, F. Nagatsugi, S. Sasaki, Y. Nagasaki, and K. Kataoka, *ChemBioChem* **6**, 718-725 (2005).

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