Smart Polyion Complex Micelles for Targeted Intracellular Delivery of PEGylated Antisense Oligonucleotides Containing Acid-Labile Linkages

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A novel pH-sensitive and targetable antisense ODN delivery system based on multimolecular assembly into polyion complex (PIC) micelles of poly(L-lysine) (PLL) and a lactosylated poly(ethylene glycol)–antisense ODN conjugate (Lac-PEG–ODN) containing an acid-labile linkage (β-propionate) between the PEG and ODN segments has been developed. The PIC micelles thus prepared had clustered lactose moieties on their peripheries and achieved a significant antisense effect against luciferase gene expression in HuH-7 cells (hepatoma cells), far more efficiently than that produced by the nonmicelle systems (ODN and Lac-PEG–ODN) alone, as well as by the lactose-free PIC micelle. In line with this pronounced antisense effect, the lactosylated PIC micelles showed better uptake than the lactose-free PIC micelles into HuH-7 cells; this suggested the involvement of an asialoglycoprotein (ASGP) receptor-mediated endocytosis process. Furthermore, a significant decrease in the antisense effect (27% inhibition) was observed for a lactosylated PIC micelle without an acid-labile linkage (thiomaleimide linkage); this suggested the release of the active (free) antisense ODN molecules into the cellular interior in response to the pH decrease in the endosomal compartment is a key process in the antisense effect. Use of branched poly(ethylenimine) (B-PEI) instead of the PLL for PIC micellization led to a substantial decrease in the antisense effect, probably due to the buffer effect of the B-PEI in the endosome compartment, preventing the cleavage of the acid-labile linkage in the conjugate. The approach reported here is expected to be useful for the construction of smart intracellular delivery systems for antisense ODNs with therapeutic value.

Introduction

Antisense oligodeoxynucleotides (ODNs) have attracted much attention as a class of therapeutic agents that can be used to target mRNA for specific inhibition of gene expression.[1] Nevertheless, the therapeutic value of antisense ODNs under in vivo conditions has not been fully proven to be effective owing to several obstacles, including nonspecific interaction with plasma protein,[2] low stability against enzymatic degradation,[3] low permeability across the cell membrane,[4] and preferential liver and renal clearance.[3] Therefore, a high dose of the antisense ODN is generally required to achieve a significant antisense effect in vivo. To obtain the desired antisense effect, a variety of antisense ODN delivery systems such as cationic lipids (lipoplexes)[5] and cationic polymers (polyplexes)[6] have been developed, and some of these systems contribute substantially to ODN stability against enzymatic degradation and increased cellular uptake, at least under in vitro conditions. However, due to the nonspecific nature of interactions of cationic components with negatively charged biomacromolecules, they often tend to show nonspecific disposition characteristics and short circulating lifetimes in the blood after systemic injection.[7] Recently, a new class of antisense ODN delivery systems has emerged based on polyion complex (PIC) micelles composed of PEG–polycation block copolymers (PEG = poly(ethylene glycol)) and oppositely charged antisense ODNs, held together by electrostatic interactions.[8] The PIC micelles exhibited excellent solubility in aqueous media, high tolerance of entrapped ODNs against enzymatic degradation, and minimal interaction with negatively charged biomacromolecules and cell membrane, owing to the steric stabilization of the very dense PEG corona surrounding the PIC core. However, electrostatic

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interaction between PEG–polycation block copolymer and antisense ODN seems to be weak under extremely dilute conditions, due to the low molecular weight of the antisense ODN, with this often leading to the dissociation of the PIC micelles below the critical association concentration (cac). Therefore, the stability of the PIC micelles used for entrapping the antisense ODNs needs to be further improved for use in systemic ODN delivery. On the other hand, the smooth and efficient release of entrapped antisense ODNs from PIC micelles into the intracellular environment after their trafficking into the target cells is needed to achieve an antisense effect.

Recently, our group[9] and Park et al.[10] have independently reported novel PIC micelles composed of an alternative combination of an anionic PEG–ODN conjugate bearing an acid-labile linkage between the PEG and the antisense ODN segment and a polycation of appreciable molecular weight. Improved stability of the PIC micelles is to be expected from this approach, due to the increased association force of the entrapped polycations of appreciable molecular weight. In addition to showing resistance against enzymatic degradation and minimal interaction with negatively charged biomacromolecules, the PEG–ODN/polycation PIC micelles underwent cleavage of the acid-labile linkages between PEG and antisense ODN segments in response to the endosomal pH, which is known to be 1.4–2.4 units lower than that found under standard physiological conditions.[11] The detachment process of the PEG segment in response to the pH decrease in the endosomal compartment would be expected to correspond to the transport of the free (active) antisense ODN moiety from endosome to cytoplasm. Nevertheless, PEG–ODN/polycation PIC micelles may show reduced cellular uptake due to limited interaction between the PEG shell of the micelle and the cell membrane, so installation of specific ligand molecules on the surfaces of the PEG–ODN/polycation PIC micelles is indispensable in order to achieve specific and enhanced cellular uptake through receptor-mediated endocytosis, allowing the effective dose of antisense ODN to be reduced.

Here we wish to report the preparation and bioactivity of pH-sensitive lactosylated PIC micelles composed of a lactosylated PEG–ODN conjugate (Lac-PEG-ODN) bearing an acid-labile linkage between PEG and ODN segments and poly(L-lysine) (PLL), as shown in Figure 1. The lactose moieties on the surfaces of the PIC micelles act as a specific ligand for hepatocytes (liver cells), because hepatocytes are known to have an abundance of asialoglycoprotein (ASGP) receptors that recognize and internalize glycoproteins bearing terminal lactose moieties.[12] The lactosylated PIC micelles could potentially be expected to show enhanced antisense effects relative to PIC micelles without lactose moieties, due to the enhancement of cellular uptake through ASGP receptor-mediated endocytosis.

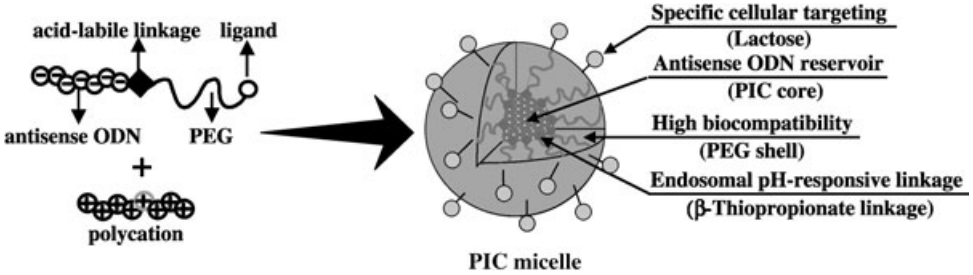
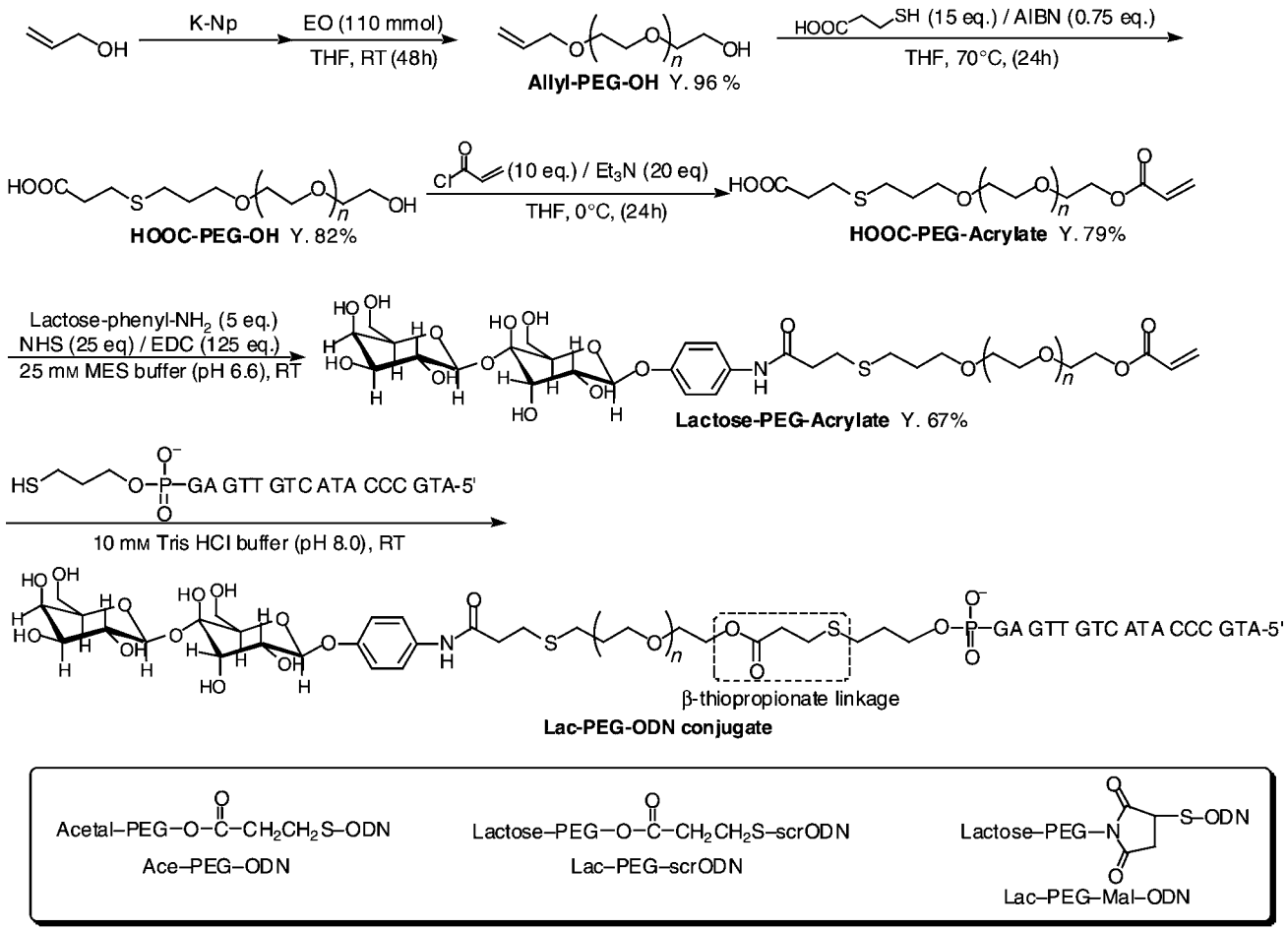


Figure 1. *Illustration of the PIC micelle.*

Results and Discussion

Synthesis of the lactosylated poly(ethylene glycol)–oligodeoxynucleotide (Lac-PEG-ODN) conjugate

A synthetic route to Lac-PEG-ODN conjugate is shown in Scheme 1. A heterobifunctional PEG bearing an allyl group at the α-end and a hydroxy group at the ω-end (Ally-PEG-OH) was synthesized by anionic ring-opening polymerization of ethylene oxide with use of the allyl alcohol/potassium/naphthalene initiator system.[13] The radical addition of 3-mercaptopropionic acid to the Allyl-PEG-OH in the presence of AIBN quantitatively afforded a carboxylic acid-PEG-OH (HOOC-PEG-OH), which was in turn converted into a HOOC-PEG-Acrylate by treatment with acryloyl chloride in the presence of triethylamine. According to SEC and ^{1}H NMR analyses (Figure 2), the molecular weight of the HOOC-PEG-Acrylate (SEC: $M_n = 4450$, $M_w/M_n = 1.04$ and ^{1}H NMR: $M_n = 4630$) agrees with the calculated molecular weight (calcd. $M_n = 5060$), and a carboxylic acid group and an acrylate group were shown to have been quantitatively introduced at the α-end and the ω-end of the PEG, respectively. The quantitative introduction of a lactose group at the carboxylic acid end of the HOOC-PEG-Acrylate was also performed by treatment with excess 4-aminophenyl β-D-lactopyranoside in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). A ^{1}H NMR spectrum of the Lac-PEG-Acrylate is shown in Figure 3 with assignments, the peaks of aromatic residue assignable to 4-aminophenyl β-D-lactopyranoside moiety being clearly observable at $\delta = 7.14$ and 7.34 ppm, along with the acrylate peaks at $\delta = 5.83$–6.54 ppm. From the integral ratios between aromatic peaks and acrylate peaks or PEG backbone peaks at 3.65 ppm, it was confirmed that the lactose moiety had been quantitatively introduced at the PEG end. To obtain a Lac-PEG-ODN conjugate bearing an acid-labile linkage (β-thiopropionate linkage), a Michael reaction between the 3′-thiol-modified antisense ODN (5′-ATG CCC ATA CTG TTG AG–$CH_2CH_2CH_2SH$, firefly luciferase, pGL3-control antisense sequence[14]) and excess Lac-PEG-Acrylate (10 equiv) was carried out according to our previous report.[9] For the controls, Ace-PEG-ODN conjugate bearing an acetal group at the PEG end and Lac-PEG-scrODN conjugate bearing a scrambled ODN sequence were also synthesized. Furthermore, a Lac-PEG-Mal-ODN conjugate with no acid-labile linkage between PEG and ODN segments was synthesized through a Michael reaction between Lac-PEG-Maleimide and the 3′-thiol-modified ODN, as shown in Scheme 1.



Scheme 1. *Synthetic route to Lac-PEG-ODN.*

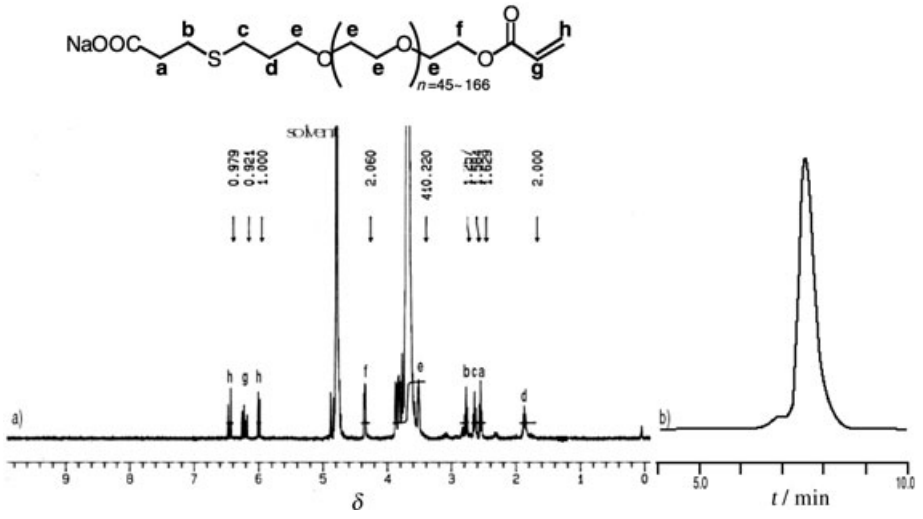


Figure 2. *a) ^{1}H NMR spectrum, and b) SEC chromatogram of HOOC-PEG–acrylate.*

Cellular association and internalization of the PIC micelles

The obtained Lac-PEG-ODN conjugate would be expected to form a PIC micelle through electrostatic interaction on mixing with the appropriate polycation, as illustrated in Figure 1. ω-FITC-labeled (FITC = fluorescein isothiocyanate) Lac-PEG-ODN conjugate and ω-FITC-labeled Ace-PEG-ODN conjugate were separately mixed with PLL (degree of polymerization (DP) = 460, M_w = 75 900) with an equal unit molar ratio of the phosphate group in the PEG–ODN conjugate and amino group in PLL (N/P = 1) to form the FITC-labeled PIC micelles. The fluorescence from the Lac-PEG-ODN-FITC/PLL PIC micelles and Ace-PEG-ODN-FITC/PLL PIC micelles was viewed under a fluorescence microscope at different time intervals (30 and 120 min) after their addition to cultured HuH-7 cells in the presence of 10% fetal bovine serum (FBS) (Figure 4). Note that HuH-7 cells express quite a few ASGP receptors that recognize and internalize compounds bearing terminal lactose moieties. The association of the Lac-PEG-ODN-FITC/PLL PIC micelles with the HuH-7 cells and their internalization were observed as early as after 30 min of incubation, and

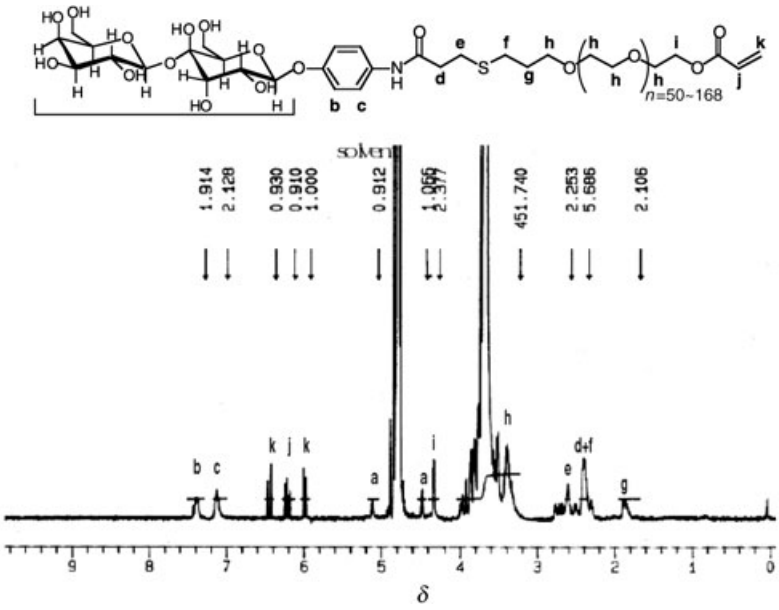


Figure 3. *^{1}H NMR spectrum of lactose-PEG–acrylate.*

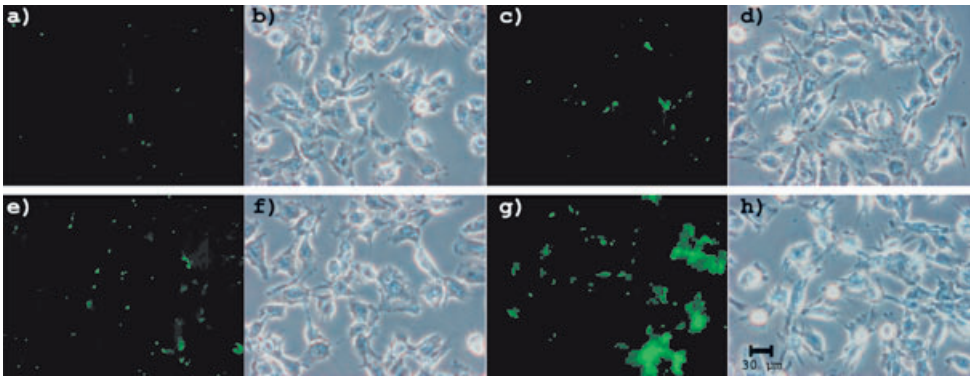


Figure 4. *Association and internalization of the Ace-PEG-ODN-FITC/PLL PIC micelles (N/P = 1) and Lac-PEG-ODN-FITC/PLL PIC micelles (N/P = 1) in HuH-7 cells after 30 or 120 min incubation. The PIC micelles used and the incubation time were as follows: a), b) Ace-PEG-ODN-FITC/PLL PIC micelle, 30 min. c), d) Ace-PEG-ODN-FITC/PLL PIC micelle, 120 min. e), f) Lac-PEG-ODN-FITC/PLL PIC micelle, 30 min. g), h) Lac-PEG-ODN-FITC/PLL PIC micelle, 120 min. a, c, e, and g are fluorescent images, and b, d, f, and h are phase-contrast images.*

furthermore, the number of fluorescence staining cells and the fluorescence intensity of each staining cell increased with prolongation of incubation time from 30 min to 120 min (26%→ 49% fluorescence positive cells). In contrast, low cellular association of Ace-PEG-ODN-FITC/PLL PIC micelles with HuH-7 cells and low internalization into them was observed even at 120 min (21% fluorescence-positive cells). This result strongly suggests that cellular association and internalization of a Lac-PEG-ODN-FITC/PLL PIC micelle may occur by an ASGP receptor-mediated process, whereas Ace-PEG-ODN-FITC/PLL PIC micelles are taken up into cells only by fluid-phase endocytosis, known to be a substantially slower process than receptor-mediated endocytosis.[15]

Antisense activity of the PIC micelles evaluated by dual luciferase reporter assay

To evaluate the antisense efficiency of the Lac-PEG-ODN in the PIC micelle delivery system, we carried out a dual luciferase reporter assay in HuH-7 cells. Open and closed bars in Figure 5 show the antisense effects of Ace-PEG-ODN/PLL and Lac-PEG-ODN/PLL PIC micelles (N/P = 1, PLL; DP = 460, M_w = 75900), respectively, in the presence of 10% FBS as a function of the concentration of the conjugate. As the conjugate concentration increases, firefly luciferase expression of the cells treated with Ace-PEG-ODN/PLL PIC micelles or Lac-PEG-ODN/PLL micelles was progressively reduced in dose-dependent manner ($^*P < 0.05$). It was also noticed that the antisense effect of the Lac-PEG-ODN/PLL PIC micelles was approximately 1.5 times higher than that of Ace-PEG-ODN/PLL micelles at 5 and 10 μM ($^*P < 0.05$). When asialofetuin (ASF), a natural glycoprotein ligand for the ASGP receptor, was preincubated with the cells (10 mg mL^{-1}) for 30 min before the addition of the Lac-PEG-ODN/PLL PIC micelle, the antisense effect was reduced significantly even at 10 μM: from 65% inhibition to 38% inhibition (hatched bar 1), the same level as achieved by Ace-PEG-ODN/PLL micelles. Since the ASF is known to act as an inhibitor of ASGP receptor-mediated endocytosis,[16] it is likely that an appreciable fraction of the Lac-PEG-ODN/PLL PIC micelle may be taken up into HuH-7 cells by an ASGP receptor-mediated endocytosis process to achieve pronounced antisense activity in relation to that produced by Ace-PEG-ODN/PLL micelles without any particular affinity toward cellular receptors.

It is worth noting that no reduction in firefly luciferase expression was observed even at 10 μM for Lac-PEG-scrODN/PLL PIC micelle (hatched bar 4), a micelle containing a conjugate of scrambled ODN sequence, indicating that inhibition of firefly luciferase expression by the Lac-PEG-ODN/PLL PIC micelles is indeed a sequence-specific event. Non-micelle systems—the 3′-unmodified antisense ODN alone (hatched bar 2) and Lac-PEG-ODN conjugate alone (hatched bar 3)—failed to inhibit the firefly luciferase expression even at high concentrations (up to 10 μM). This may be explained either by a much more rapid enzymatic degradation of antisense ODN and PEG–ODN conjugate in the medium, relative to the PIC micelle,[9] or by impaired diffusivity of naked antisense ODN and Lac-PEG-ODN conjugate, with their negatively charged and hydrophilic characters, through the nega-

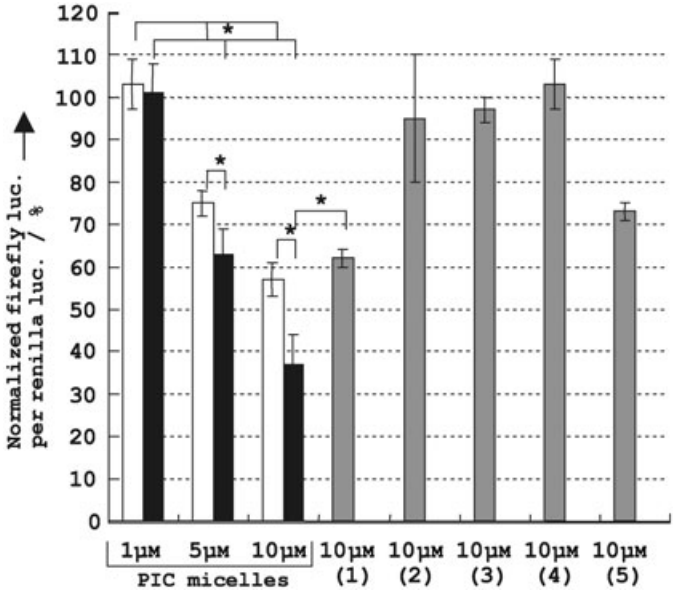


Figure 5. *Antisense effects against firefly luciferase gene expression in cultured HuH-7 cells. Open and closed bars show results for the Ace-PEG-ODN/PLL PIC micelles and the Lac-PEG-ODN/PLL PIC micelles, respectively, at varying concentrations. Hatched bars are results for: 1) Lac-PEG-ODN/PLL PIC micelle at 10 μM with ASF, 2) antisense ODN alone at 10 μM, 3) Lac-PEG-ODN conjugate alone at 10 μM, 4) Lac-PEG-scrODN/PLL PIC micelle at 10 μM, and 5) Lac-PEG-Mal-ODN/PLL PIC micelle at 10 μM. Normalized ratios between the firefly luciferase activity (firefly luc.) and the renilla luciferase activity (renilla luc.) are shown in the ordinate. The indicated concentrations of conjugate and antisense ODN were the final concentrations in the total transfection volume (250 μL). The data are shown as the averages from triplicate experiments ± SD.* $P^* < 0.05$.

tively charged cell membrane. It should also be noted that the Lac-PEG-Mal-ODN/PLL micelle, a micelle containing the conjugate with a non-acid-labile linkage,[17] was less effective than the Lac-PEG-ODN/PLL PIC micelle: the former showed 27% inhibition (hatched bar 5), whereas the latter achieved 65% inhibition at 10 μM (closed bar). The significant difference in antisense effects between Lac-PEG-ODN/PLL PIC micelle and Lac-PEG-Mal-ODN/PLL PIC micelle can be ascribed to the difference in the natures of their linkages between PEG and ODN segments. The Lac-PEG-ODN conjugate contains an acid-labile (β-thiopropionate) linkage that is cleavable in the low-pH endosome environment, provoking the release of hundreds of free PEG strands from the PIC micelle to increase the colloidal osmotic pressure within the endosomal compartment,[18] and eventually to induce the swelling and disruption of the endosome. This event may facilitate the transport of free antisense ODN into cytoplasm. On the other hand, the Lac-PEG-Mal-ODN conjugate, bearing a stable thiomaleimide linkage not cleavable in the endosomal compartment, may have no contribution in facilitating endosomal escape through osmotic pressure increase. Furthermore, the presence of PEG strands may restrict the interaction of ODN segments with target mRNA in cytoplasm through steric hindrance, as observed by Moulton et al. for conjugates of antisense phosphorodiamidate morpholino oligomers with peptide.[19] These results indicate that the design of the engineered linkage with programmed sensitivity toward intracellular environment ("smart" PEGylation) is of importance in the successful delivery of the PEG-antisense ODN conjugate.

The effect of the PLL length on the antisense activity of the PIC micelles

The effect of the PLL length (DP) on the antisense effect of the PIC micelles was then examined by comparing PLLs with varying DPs (40, 100, and 460). As can be seen in Figure 6a, a striking effect of PLL length on antisense efficacy of the PIC micelle was observed: micelles prepared from shorter PLL components (DP = 40, Mw = 8300) showed only limited efficacy at 5 μM antisense ODN with a 24 h incubation period ($^*P < 0.05$) relative to those with longer PLLs (DP = 100, Mw = 20900 and 460, Mw = 75900). Presumably the PIC micelles formed from PLL (DP = 40) may be unstable under the extremely dilute conditions ([ODN] ≈ 5 μM) due to the critical dissociation phenomenon, leading to the enzymatic degradation of the Lac-PEG-ODN conjugate. The effect of the incubation time was then further studied for the PIC micelles prepared from the PLLs of DP = 100 and 460. After incubation of HuH-7 cells with PIC micelles for 24 h, the medium was changed to a fresh one free of PIC micelles to continue the culture for a designated time period (24, 48, and 72 h). As can be seen in Figure 6b, both PIC micelles composed of PLL with DP = 100 and DP = 460 (N/P = 1) exhibited an appreciable time-dependent increase in antisense effect even at 1 μM. The antisense effect of the PIC micelle with PLL (DP = 460) reached a constant value after 48 h post-

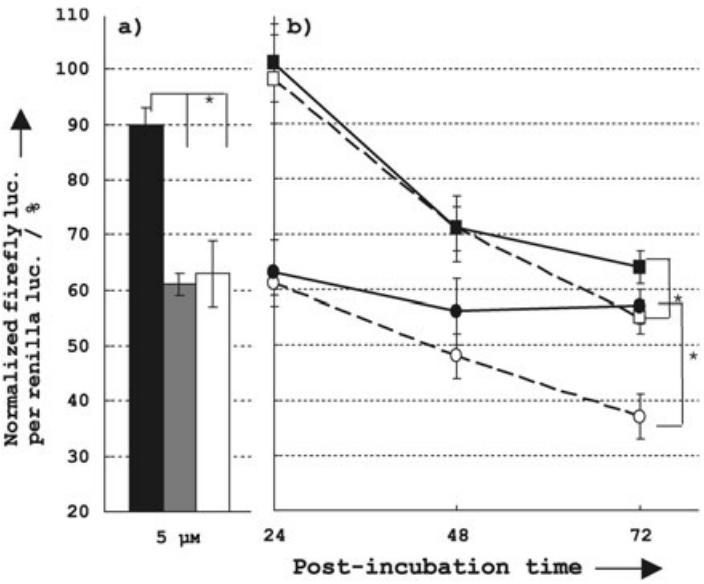


Figure 6. *Effect of: a) PLL length (DP = 40 ■, 100 ■, and 460 □), and b) post-incubation time (24, 48, and 72 h) on the antisense effect of the lactosylated PIC micelles. Normalized ratios between the firefly luc. and the renilla luc. are shown in the ordinate. The final concentrations of conjugate in the total transfection volume (250 μL) were 1 μM (squares) or 5 μM (circles) for DP = 100 (open symbol) or DP = 460 (filled symbol). The data are shown as the averages from triplicate experiments ± SD.* $P^* < 0.05$.

incubation, whereas that with PLL (DP = 100) exhibited an almost liner increase in antisense effect with the post-incubation time period. Notably, a significant antisense effect was achieved for the PIC micelles with PLL (DP = 100) (45% inhibition at 1 μM and 62% inhibition at 5 μM) after 72 h post-incubation relative to that with PLL (DP = 460) (35% inhibition at 1 μM and 42% inhibition at 5 μM). A lowered antisense effect for the systems with longer PLL (DP = 460) is presumably due to overstabilization of the PIC core, restricting the release of the antisense ODN into the cytoplasm.

The effect of the polycation structure in the PIC core on the antisense activity

To study the effect of the polycation structure in the PIC core on the antisense effects of PIC micelles, branched poly(ethylenimine) (B-PEI, DP = 580, M_w = 25 000) was chosen as the counter polycation, as it has often been used for gene delivery in the form of DNA/B-PEI complexes displaying high transfection efficiency due to the endosomal disruption property of B-PEI (so called "buffer" or "proton-sponge" effect).[20] Nevertheless, the antisense effect of Lac-PEG-ODN/B-PEI PIC micelles (N/P = 1, 9% inhibition at 5 μM and 42% inhibition at 10 μM) was lower than that of Lac-PEG-ODN/PLL micelles (N/P = 1) at 5 and 10 μM ($^*P < 0.05$), as shown in Figure 7. Presumably, the buffer effect of the B-PEI may prohibit the decrease in the endosomal pH,[21] and this should be unfavorable for the cleavage of the acid-labile linkage (β-thiopropionate) of the Lac-PEG-ODN conjugate in the endosome, leading to the release of intact and relatively less active Lac-PEG-ODN conjugate into the cytoplasm.

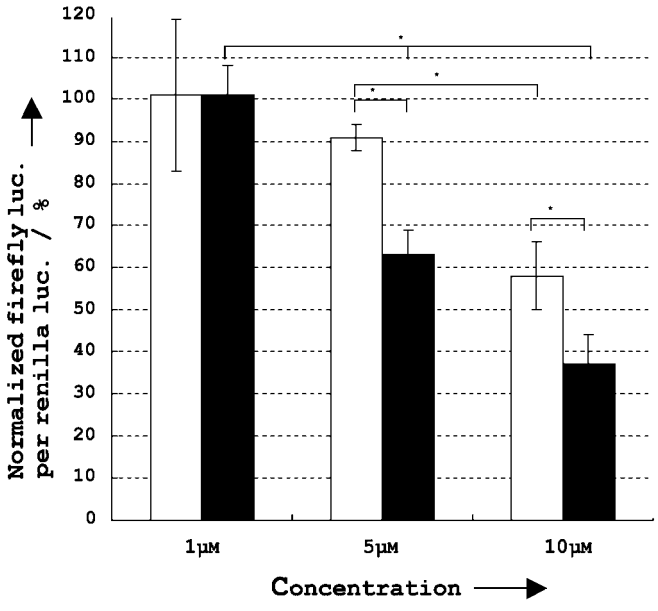


Figure 7. *Effect of PIC core polycation structure on the antisense effects of the lactosylated PIC micelles. Normalized ratios between the firefly luc. and the renilla luc. are shown in the ordinate. The indicated concentrations of conjugate were the final concentrations in the total transfection volume (250 μL): □ B-PEI PIC micelles, ■ PLL PIC micelles. The data are shown as the averages from triplicate experiments ± SD.* $P^* < 0.05$.

Conclusion

In conclusion, this study reports the preparation of a novel intracellular environment-responsive and targetable antisense ODN delivery system based on the PIC micelle composed of PLL and Lac-PEG-ODN conjugate bearing an acid-labile linkage (β-propionate) between PEG and ODN segments. The lactosylated PIC micelles thus prepared exhibited better association with HuH-7 cells than the lactose-free PIC micelles, as observed by fluorescence microscopy at different time intervals. Delivery of antisense ODN by lactosylated PIC micelle resulted in a significant antisense effect (65% inhibition) for firefly luciferase expression in HuH-7 cells, far more efficient than achieved by using the 3′-unmodified antisense ODN alone (3% inhibition), Lac-PEG-ODN alone (3% inhibition), or lactose-free PIC micelles (45% inhibition), as evaluated by dual luciferase reporter assay. This pronounced antisense effect of the lactosylated PIC micelles indicates that ASGP receptor-mediated endocytosis is considerably involved in the cellular uptake of the lactosylated PIC micelles. Furthermore, a decrease in antisense effect was observed for the lactosylated PIC micelles without any acid-labile linkage (65→27% inhibition), suggesting that cleavage of the acid-labile linkage may occur in response to the lower pH in the endosomal compartment, inducing the efficient release of the active (free) antisense ODN from the PIC core. Such structural parameters—length and type of counter polycation used to make PIC micelles—substantially affected their antisense effects. Significant antisense effects of the PIC micelles (63% inhibition at 5 μM and 45% inhibition at 1 μM) were achieved at 72 h post-incubation time by using PLL with DP = 100, presumably due to the efficient release of antisense ODN from the PIC core through the polyanion exchange reaction in cytoplasm after the detachment of the PEG shell from the PIC micelle in the endosome. All of these results indicate that the system reported here is highly feasible as a smart intracellular delivery system for antisense ODN and related nucleotide compounds for diverse therapeutics.

Experimental Section

Materials: Tetrahydrofuran, ethylene oxide (Sumitomo Seika), allyl alcohol (Wako), triethylamine (Wako), and acryloyl chloride (Wako) were purified by conventional methods. Propan-2-ol, 3-mercaptopropionic acid, 2,2′-azobisisobutyronitrile (AIBN), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Wako and were used without further purification. 4-Aminophenyl β-D-lactopyranoside was purchased from Toronto Research Chemicals, Inc. Potassium naphthalene was used as a THF solution, the concentration of which was determined by titration. Water was purified with a Milli-Q apparatus (Millipore). Plasmid DNAs (pDNA) encoding firefly luciferase (pGL3-Control, Promega; 5256 bpa) and renilla luciferase (pRL-TK, Promega; 4045 bpa) were amplified by use of EndoFree Plasmid Maxi or Mega Kits (QIAGEN). The DNA concentration was determined by reading of the absorbance at 260 nm. PLL (DP = 40, M_w = 8300; DP = 100, M_w = 20 900; DP = 460, M_w = 75 900) and B-PEI (DP = 580, M_w = 25 000) were purchased from Sigma and Aldrich, respectively. 3′-Thiol-modified ODNs (5′-ATG CCC ATA CTG TTG AG-$CH_2CH_2CH_2SH$, firefly luciferase, pGL3-control antisense sequence,[14]

and 5′-TCC GTC TAA TGA CGA GT-$CH_2CH_2CH_2SH$, scrambling sequence) were synthesized as in our previous report[9] with a DNA synthesizer (94DNA/RNA Synthesizer, Applied Biosystems).

Polymer analysis: ^{1}H NMR (400 MHz) spectra were obtained in D_2O with a JEOL EX400 spectrometer. Chemical shifts are reported in ppm relative to D_2O ($\delta = 4.79$, ^{1}H). Size exclusion chromatography (SEC) in an organic solvent was performed with a TOSO HLC-8020 apparatus equipped with an internal refractive index (RI) detector (RID-6 A) with a combination of TSK $G4000_{HR}$ and $G3000_{HR}$ columns and THF as the eluent.

Synthesis of HOOC-PEG-OH: Allyl-PEG-OH ($M_n = 4340$, $M_w/M_n = 1.03$) was prepared as in the previous report.[13] Allyl-PEG-OH (4.34 g, 1.0 mmol) was dissolved in anhydrous THF (30 mL), together with 3-mercaptopropionic acid (1.6 g, 15 mmol, 15 equiv) and AIBN (0.123 g, 0.75 mmol, 0.75 equiv), and the resulting mixture was degassed by three freeze–pump–thaw cycles. The radical addition was carried out at 70 °C for 24 h. The polymer was recovered by precipitation in cold propan-2-ol (−15 °C, 2 L) and centrifuged for 45 min at 6000 rpm. Further purification was carried out by dialysis against distilled, deionized water (M_W cutoff 3500), and the product was then freeze-dried to give HOOC-PEG-OH (3.64 g, 82% yield). SEC $M_n = 4330$, $M_w/M_n = 1.04$ (calcd. $M_n = 5010$); ^{1}H NMR (D_2O): $\delta = 1.83$–1.96 (m, 2H; $NaOOCCH_2CH_2$–S–$CH_2CH_2CH_2O$–), 2.57 (t, $J = 7.6$ Hz, 2H; $NaOOCCH_2CH_2$–S–), 2.63 (t, $J = 7.2$ Hz, 2H; $NaOOCCH_2CH_2$–S–$CH_2CH_2CH_2O$–), 2.82 (t, $J = 7.6$ Hz, 2H; $NaOOCCH_2CH_2$–S–), 3.63 ppm (s, 457H; PEG-backbone)

Synthesis of HOOC-PEG-acrylate: A solution of the HOOC-PEG-OH (2.0 g, 0.45 mmol) and Et_3N (0.911 g, 9.0 mmol, 20 equiv) in THF (15 mL) was added dropwise over 1 h at 0 °C to a mixture of acryloyl chloride (0.400 g, 4.5 mmol, 10 equiv) in THF (5.0 mL). The reaction was allowed to proceed at 0 °C for 24 h in the dark. The polymer was recovered by precipitation in cold propan-2-ol (−15 °C, 1 L) and centrifuged for 45 min at 6000 rpm. Further purification was carried out by dialysis against distilled, deionized water (M_W cutoff 3500) and the product was then freeze-dried to give HOOC-PEG-acrylate (1.60 g, 79% yield). SEC $M_n = 4450$, $M_w/M_n = 1.04$ (calcd. $M_n = 5060$); ^{1}H NMR (D_2O, in Figure 2): $\delta = 1.81$–1.94 (m, 2H; $-H_d$), 2.55 (t, $J = 7.6$ Hz, 2H; $-H_a$), 2.63 (t, $J = 7.2$ Hz, 2H; $-H_c$), 2.80 (t, $J = 7.6$ Hz, 2H; $-H_b$), 3.63 (s, 410H; PEG-backbone, $-H_e$), 4.36 (t, $J = 6.4$ Hz, 2H; $-H_f$), 5.98 (dd, $J = 1.8$, 12.8 Hz, 1H; $-H_h$), 6.23 (dd, $J = 12.8$, 25.4 Hz, 1H; $-H_g$), 6.44 (dd, $J = 1.8$, 12.8 Hz, 1H; $-H_h$).

Lactosylation of HOOC-PEG-acrylate: The HOOC-PEG-acrylate (100 mg, 22 μmol) and 4-aminophenyl β-D-lactopyranoside (48 mg, 111 μmol) were dissolved in MES buffer (25 mM, pH 6.6, 5.0 mL) together with EDC (532 mg, 2.78 mmol, 125 equiv) and NHS (64 mg, 555 μmol, 25 equiv). The reaction mixture was stirred at room temperature for 24 h. The polymer was recovered by precipitation in cold propan-2-ol (−15 °C, 200 mL) and centrifuged for 45 min at 6000 rpm. Further purification was carried out by dialysis against distilled, deionized water (M_W cutoff 3500) and the product was then freeze-dried to give lactose-PEG-acrylate (69 mg, 67% yield). SEC $M_n = 4630$, $M_w/M_n = 1.04$ (calcd. $M_n = 5490$); ^{1}H NMR (D_2O, in Figure 3): $\delta = 1.79$–1.95 (m, 2H; $-H_d$), 2.37 ~ 2.41 (m, 4H; $-H_d$, $-H_f$), 2.63 (t, $J = 7.2$ Hz, 2H; $-H_e$), 3.65 (s, 452H; PEG-backbone, $-H_h$), 4.37 (t, $J = 6.4$ Hz, 2H; $-H_i$), 4,46 ~ 4,54 (m, 1H; $-H_a$), 5.13 ~ 5.19 (m 1H; $-H_a$), 5.98 (dd, $J = 1.8$, 12.8 Hz, 1H; $-H_k$), 6.21 (dd, $J = 12.8$, 25.4 Hz, 1H; $-H_j$), 6.43 (dd, $J = 1.8$, 12.8 Hz, 1H; $-H_k$), 7.14 (d, $J = 7.7$ Hz, 2H; $-H_c$), 7.39 ppm (d, $J = 7.7$ Hz, 2H; $-H_b$).

Synthesis of PEG–ODN conjugates: To obtain a Lac-PEG-ODN conjugate bearing an acid-labile linkage, a Michael reaction of the 3′-thiol-modified ODN with excess lactose-PEG-acrylate (10 equiv) was carried out according to our previous report (79% yield).[9] In addition, three types of PEG–ODN conjugate—possessing an acetal group at the PEG end (Ace-PEG-ODN, 84% yield), a scrambled ODN-sequence (Lac-PEG-scrODN, 88% yield), and a non-acid-labile linkage (Lac-PEG-Mal-ODN, 67% yield)—were also synthesized in the same manner. ^{1}H NMR (for Ace-PEG-ODN conjugate, D_2O): $\delta = 1.19$ (t, $J = 9.4$ Hz, 6H; $-OCH_2CH_3$), 1.73 (t, $J = 7.3$ Hz, 2H; $-SCH_2CH_2COO$–), 1.81–2.33 (m, 36H; 2′-methylene + ODN–$CH_2CH_2CH_2S$–), 2.43 (t, $J = 7.3$ Hz, 2H; $-CH_2CH(OEt)_2$), 3.02–3.16 (m, 4H; $-CH_2SCH_2$–) 3.58 (s, 432H; PEG-backbone + ODN–$CH_2CH_2CH_2S$– + $-OCH_2CH_3$), 3.88–3.94 (m, 36H; 4′-methine + $-COOCH_2$–), 4.16 (s, 34H; 5′-methylene), 4.48 ppm (t, $J = 7.3$ Hz, 1H; $-CH_2CH(OEt)_2$). 1′-Methine protons and 3′-methine protons were overlapped by the peak of H_2O (4.79 ppm).

Preparation of PIC micelle: Specific amounts of the PEG–ODN conjugates and polycations were dissolved in Tris-HCl buffer (10 mM, pH 7.4) to prepare the stock solutions. The solutions were filtered through a 0.1 μm filter to remove the dust. The PEG–ODN conjugate stock solution was mixed with polycation stock solution at an equal unit molar ratio of phosphate group in the PEG–ODN conjugate and amino group in the polycation ($N/P = 1$), followed by the addition of Tris-HCl buffer (10 mM, pH 7.4) including NaCl (0.3 M) to adjust the ionic strength of the solution to physiological conditions (0.15 M NaCl). The size and size distribution of the PIC micelle was elucidated by DLS measurements(DLS-7000, Photal, Otsuka Electronics).[9]

Cell culture: HuH-7 human cancer cells derived from a hepatocarcinoma cell line were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified 5% CO_2 atmosphere.

Fluorescence microscopy: FITC-labeled (FITC = fluorescein isothiocyanate) Lac-PEG-ODN and Ace-PEG-ODN conjugates were prepared from 5′-FITC- and 3′-thiol-modified ODN and lactose-PEG-acrylate. HuH-7 cells were seeded at a density of 5×10^5 cells per dish in a 35 mm glass-bottomed dish (Iwaki, Japan) and kept overnight at 37 °C in a 5% CO_2 atmosphere. The Lac-PEG-ODN-FITC/PLL and Ace-PEG-ODN-FITC/PLL PIC micelles ($N/P = 1$, PLL; DP = 460) were added at a conjugate concentration of 1 μM and incubated at 37 °C in a 5% CO_2 atmosphere for the designated time (30 and 120 min). The cells were washed three times with phosphate buffered saline (PBS) and imaged directly in the cell culture medium with an Olympus IX70 and an appropriate filter.

Dual luciferase reporter assay: HuH-7 cells were plated in a 24-well plate (5×10^4 cells per well) to reach about 50% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPIMEM I. The cells were co-transfected with two luciferase plasmids (firefly luciferase, pGL3-control and renilla luciferase, pRL-TK) in the presence of LipofectAMINE (Invitrogen). For each well, pGL3 (0.0835 μg) and pRL (0.75 μg) were applied; the final volume was 250 μL per well. The cells were incubated for 4 h, and the transfection medium was then changed to DMEM with FBS (10%, 225 μL per well). The PIC micelle ($N/P = 1$) (25 μL per well) was added to make up a prescribed concentration. After 24 h incubation, the transfection medium was changed to fresh DMEM with FBS (10%), and the cells were further incubated for the designated time (24, 48, and 72 h). The luciferase expression was monitored with the dual luciferase assay kit (Promega) and ARVOSX-1 (Perkin–Elmer).

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