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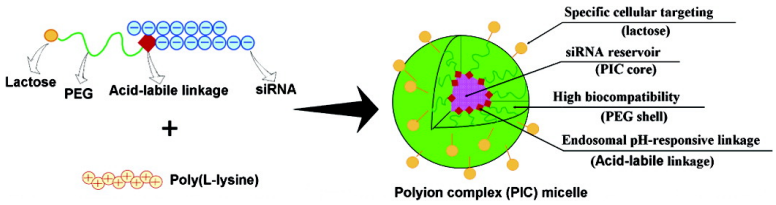
J. Am. Chem. Soc., **2005**, 127 (6), 1624-1625• DOI: 10.1021/ja044941d • Publication Date (Web): 22 January 2005



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Lactosylated Poly(ethylene glycol)-siRNA Conjugate through Acid-Labile β-Thiopropionate Linkage to Construct pH-Sensitive Polyion Complex Micelles Achieving Enhanced Gene Silencing in Hepatoma Cells

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Nucleic acid medicines such as antisense DNAs[1] and small, interfering RNAs (siRNAs)[2] have attracted much attention as a new class of therapeutic agents. In particular, siRNAs are recently recognized as the most powerful tools for sequence-specific gene silencing via naturally occurring RNA interference (RNAi) process.[3] Nevertheless, the therapeutic value of siRNAs under in vivo conditions is still controversial due to their low stability against enzymatic degradation, low permeability across cell membrane, and preferential liver and renal clearance.[4] A major key to the therapeutic success of siRNA is believed to be the development of carrier systems achieving the modulated disposition in the body through the intravenous route as well as the smooth transport of intact siRNA into the interior of the target cell. Worth noticing in this regard is a new class of nanometric-scaled carriers (nanocarriers) of oligonucleotides formulated through the self-assembly of PEG-based block ionomers (polyion complex (PIC) micelles).[5] Both combinations of PEG-*block*-polycation/oligonucleotide and PEG-*block*-oligonucleotide (or PEG-oligonucleotide conjugate)/polycation are feasible for PIC micelle formulation with a segregated PIC core surrounded by a palisade of flexible and hydrophilic PEG layers to increase biocompatibility and enzymatic tolerability. Ligands may be installed on the periphery of the PEG palisade of the PIC micelles to increase the uptake into the target cells through a receptor-mediated endocytotic pathway.[6] A unique finding, which we would like to communicate here, is the remarkably enhanced RNAi in cultured hepatoma cells through the assembly of siRNA into smart lactosylated-PIC micelles with pH-sensitive dissolution properties, thus achieving an appreciable silencing of the target gene at an extremely low siRNA concentration.

Our strategy of formulating pH-sensitive and targetable PIC micelles of siRNA is based on the novel conjugation of siRNA with lactosylated PEG through acid-labile linkage of β-thiopropionate (Lac-PEG-siRNA; Figure 1), followed by the complexation with poly(L-lysine). Note that β-thiopropionate linkage (3-sulfanylpropionyl linkage)[7] is readily cleaved at the pH corresponding to that of the intracellular endosomal compartment (pH = 5.5).[5a] Michael addition of the 5′-thiol-modified sense RNA (firefly luciferase, pGL3-control sense sequence) toward the ω-acrylate group of the α-lactosyl-ω-acryl-PEG gave a conjugate of Lac-PEG with single-stranded RNA (Lac-PEG-ssRNA), which revealed a retarded migration in gel electrophoretic assay (Figure 2, lane 3) compared to free-sense RNA (Figure 2, lane 1) in line with

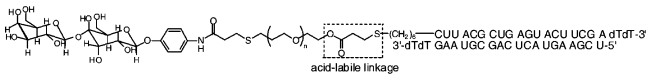

Figure 1. Chemical structure of the Lac-PEG-siRNA conjugate.

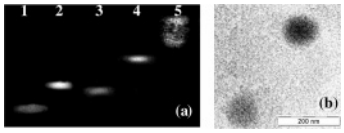

Figure 2. (a) Polyacrylamide gel retardation assay: lane 1, sense RNA; lane 2, siRNA; lane 3, Lac-PEG-ssRNA; lane 4, Lac-PEG-siRNA; and lane 5, PIC micelle. (b) Transmission electron micrograph of the disulfide cross-linked PIC micelle.

PEGylation. Then, the Lac-PEG-ssRNA was annealed with antisense RNA to undergo hybridization, preparing the Lac-PEG conjugate with siRNA (Lac-PEG-siRNA). The Lac-PEG-siRNA thus prepared gave a single band in gel electrophoresis (lane 4) and had further retarded migration compared to Lac-PEG-ssRNA (lane 3) and free siRNA (lane 2). All of these results are consistent with the successful preparation of the Lac-PEG-siRNA with negligible contamination with unreacted and intermediate compounds.

The PIC micelles from the Lac-PEG-siRNA conjugate and PLL (degree of polymerization = 40) were then prepared at the charge ratio of 1 (N/P = 1), where no free Lac-PEG-siRNA conjugate and almost complete retardation were observed in a polyacrylamide gel electrophoresis (Figure 2a, lane 5), suggesting that polyion complexation between the siRNA segment and the PLL quantitatively took place. The PIC micelle with disulfide cross-linked core was also prepared by using thiolated PLL (see Supporting Information) tolerable for the transmission electron microscopy (TEM) observation. As seen in Figure 2b, the disulfide cross-linked PIC micelles have spherical shapes with an average size (n = 36) of 117 ± 26 nm, consistent with the formation of multimolecular micellization of the Lac-PEG-siRNA with PLL.

The dual luciferase reporter assay was done in HuH-7 cells (human hepatoma cells) possessing asialoglycoprotein (ASGP) receptors, which recognize compounds bearing terminal galactose moieties,[8] to evaluate the gene silencing ability of the conjugate and the PIC micelle system (Figure 3). Both the Lac-PEG-siRNA conjugate and the PIC micelle (N/P = 1) revealed RNAi activities with a dose-dependent manner even in the presence of 10% FBS, and in particular, the PIC micelles achieved far more effective RNAi activity than the Lac-PEG-siRNA conjugate alone, viz., 50% inhibitory concentration (IC_{50}) was found to be 1.3 nM and 91.4 nM for the PIC micelle and Lac-PEG-siRNA conjugate, respec-

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10.1021/ja044941d CCC: $30.25

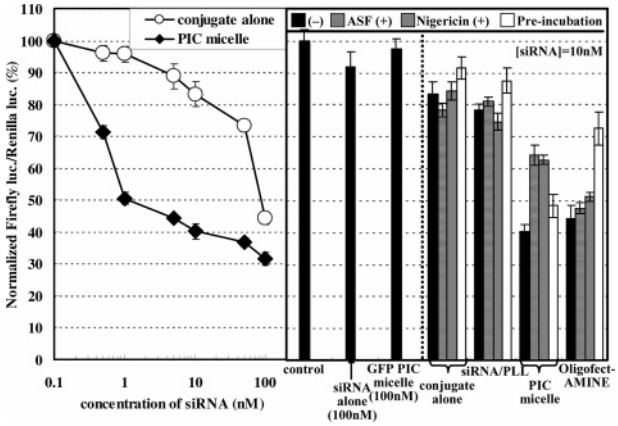

Figure 3. RNAi activities against the firefly luciferase gene generated in cultured HuH-7 cells. Normalized ratios between the firefly luciferase activity (firefly luc.) and the renilla luciferase activity (renilla luc.) are shown on the ordinate. The indicated concentrations of siRNA were the final concentrations in the total transfection volume (250 μL). The plotted data are averages of triplicate experiments ± SD. $P^* < 0.05$ (vs Lac-PEG-siRNA conjugate).

tively. This almost 100 times increase in RNAi activity by PIC micelle is remarkable.

On the other hand, no RNAi activity was observed for free siRNA even at 100 nM of siRNA concentration. The lack of RNAi activity for free siRNA may be ascribed to the low tolerability against enzymatic attack[5a] and/or the restricted uptake into the cellular interior due to the elcetrostatic repulsion with the negatively charged plasma membranes. Note that the PIC micelle including a GFP sequence induced no RNAi, strongly suggesting that an inhibition of firefly luciferase expression observed here indeed occurred through the sequence-specific RNAi effect. In addition, siRNA/PLL (polyplex) showed significantly lower RNAi activity compared to the PIC micelle probably owing to the aggregation at charge-neutralized conditions (N/P = 1) and nonspecific interaction with serum proteins. Although the RNAi activity for the PIC micelle at 10 nM of conjugate concentrations was the same level compared to the commercially available oligofectAMINE (cationic liposome), the RNAi activity for the oligofectAMINE after preincubation with 50% serum for 30 min was significantly reduced (56 → 27% inhibition, $P < 0.05$) due to the nonspecifically interacting nature of the cationic carriers with negatively charged serum proteins. In sharp contrast, the PIC micelle still retained the RNAi activity even after preincubation for 30 min with 50% serum due to the segregation of the siRNA into the PEG environment.[9] To confirm the cellular uptake pathway, asialofetuin (ASF) as the inhibitor for the ASGP receptor-mediated endocytosis[10] was added to the culture medium (4 mg/mL). As a consequence, RNAi activities were reduced significantly for the PIC micelles (60 → 36% inhibition, $P < 0.05$), whereas there was negligible effect of ASF on RNAi activities for Lac-PEG-siRNA conjugate, siRNA/PLL, and oligifectAMINE in HuH-7 cells (Figure 3). Note that no effect of ASF was observed for ASGP receptor-negative NIH 3T3 cells (mouse fibroblast) even for the PIC micelles (see Supporting Information). Obviously, these results indicate that the lactose moieties clustering on the surface of PIC micelle appreciably facilitates ASGP receptor-mediated endocytosis to direct a remarkable RNAi efficacy. Then, nigericin as the inhibitor for the endosomal acidification[11] was added to the culture medium (5 μM) to confirm that the acid-labile linkage in the conjugate contributes RNAi activity. Consequently, the RNAi activity was significantly reduced for the PIC micelle (60 → 37% inhibition, $P < 0.05$), whereas no effect was observed for the Lac-PEG-siRNA conjugate, siRNA/PLL, and the oligofectAMINE. This result suggests that after the endocytotic internalization the cleavage of the acid-labile linkage of the micelles occurred in the manner synchronized with the pH decrease in the endosomal compartment, releasing hundreds of free PEG strands to increase the colloidal osmotic pressure. This may induce the swelling and disruption of the endosome,[12] facilitating the transport of free siRNA into the cytoplasm. Several important factors are likely to be synergistically involved in the pronounced RNAi activity of the PIC micelles, such as the improvement of the stability against enzymatic degradation, minimal interaction with serum proteins, enhancement of the cellular uptake through the ASGP receptor-mediated endocytosis, and the effective transport of free siRNA from endosome into cytoplasm. It should be noted that the PIC micelles entrapping the Lac-PEG-siRNA conjugate reported here showed about 5800 times higher gene-silencing effect compared to that entrapping the Lac-PEG-antisense DNA conjugate targeting the same gene sequence (IC_{50} = 7.6 μM).[13]

In conclusion, the pH-responsive and targetable PIC micelle composed of PLL and Lac-PEG-siRNA conjugate bearing an acid-labile linkage exhibited significant gene silencing for firefly luciferase expression in HuH-7 cells. Therefore, this approach of PIC micellization of PEG-siRNA conjugate with an appropriate polycation has promise as a targetable siRNA delivery system used in a practical context. Further study on gene silencing against endogenous genes as well as in vivo performance is now in progress in our laboratories.

Acknowledgment. This work was supported by the Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Agency [JST]. We appreciate Mr. Teisaku Nakamura for taking the TEM image.

Supporting Information Available: Experimental details, materials, and the dual luciferase reporter assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA044941D