siRNA Conjugates

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Acidic pH-Responsive siRNA Conjugate for Reversible Carrier Stability and Accelerated Endosomal Escape with Reduced IFNα-Associated Immune Response**

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Small interfering RNA (siRNA) has garnered much interest as a potential drug because of its strong gene-silencing activity.[1] Toward the success in siRNA therapeutics, many strategies have been developed for efficient siRNA delivery into the cytosol of target cells.[2] Among them, siRNA conjugates have arisen as one of the promising strategies in siRNA delivery, as siRNA can be readily conjugated to a functional molecule to acquire the ability of "programmed transfer" to the target sites.[3] Indeed, several ligand molecules, such as lactose and RGD peptide, were conjugated with siRNA for site- (or cell)-specific delivery.[3] Furthermore, multimolecular siRNA conjugates enable stable polyion complex (PIC) formation because of the increased electrostatic interactions with polycations, leading to facilitated cellular uptake through charge neutralization of siRNA and also protection of siRNA from enzymatic degradations.[4] However, those siRNA conjugates potentially stimulate immune responses through the activation of toll-like receptor 3 and/or protein kinase R,[4,5] and thus they are desired to disintegrate into monomeric siRNAs (mono-siRNAs) in the cell for reduced immune responses.[4] Meanwhile, considering that macromolecular drugs, including siRNA and its conjugates, would be taken up by cells through endocytosis and then delivered to the late endosome toward lysosomal degradation, siRNA needs to escape from the endosome into the cytosol for efficient gene silencing.[6] Therefore, design of a smart siRNA conjugate for programmed endosomal escape and release of mono-siRNA is a great challenge for successful siRNA delivery.

Herein, we developed a smart siRNA conjugate to fulfill the multifunctionality desired for enhanced siRNA delivery with reduced immunogenicity; that is, reversible PIC stability, endosomal escapability, and mono-siRNA releasability, based on a single chemical process. It is known that maleic acid amide (MAA) is relatively stable at extracellular neutral pH, while rapidly hydrolyzed at endosomal acidic pH.[7] Thus, we utilized this MAA chemistry as an acid-labile anionic moiety for linking siRNA to an endosome-disrupting polycation and concurrently converting the cationic sites into a biologically inert anionic derivative.[8] In design, the MAA-based conjugate is expected to improve the PIC stability through increased electrostatic interaction, while degrading the MAA moieties in the endosome for triggering three actions: 1) complex destabilization through unbalanced charges within PICs; 2) endosome disruption with the regenerated parent polycation; and 3) mono-siRNA release by MAA cleavage (Figure 1a). Figure 1b shows the chemical structure of siRNA-releasable/endosome-disrupting conjugate (REC), in which several siRNA molecules are grafted into the endosome-disrupting polymer side chains by the MAA linkage. The parent polycation is a polyaspartamide derivative with two repeating units of aminoethylene in each side chain (termed PAsp(DET)), which destabilizes the endosomal membrane integrity with the cationic diprotonated side chains to accelerate endosomal escape of the payload.[9]

A precursor polyanion was synthesized from PAsp(DET) to have a dibenzyl cyclooctyne (DBCO) group by MAA linkage as a conjugation site for siRNA. Then, an azide-modified siRNA (azide-siRNA) was reacted with the DBCO group in the polyanion side chains. Notably, the size exclusion chromatography (Supporting Information, Figure S5) confirmed that more than 95% of azide-siRNAs were conjugated to the polymer backbone utilizing a freeze–thaw treatment for the generation of a highly concentrated reactant phase.[10] This successful conjugation at the quite high rate allows the use of the obtained conjugate without further purification. As a result, about 30% of DBCO groups in the polymer side chains reacted with azide-siRNA; that is, about 5 siRNAs contained in the conjugate (Figure 1b). To investigate the

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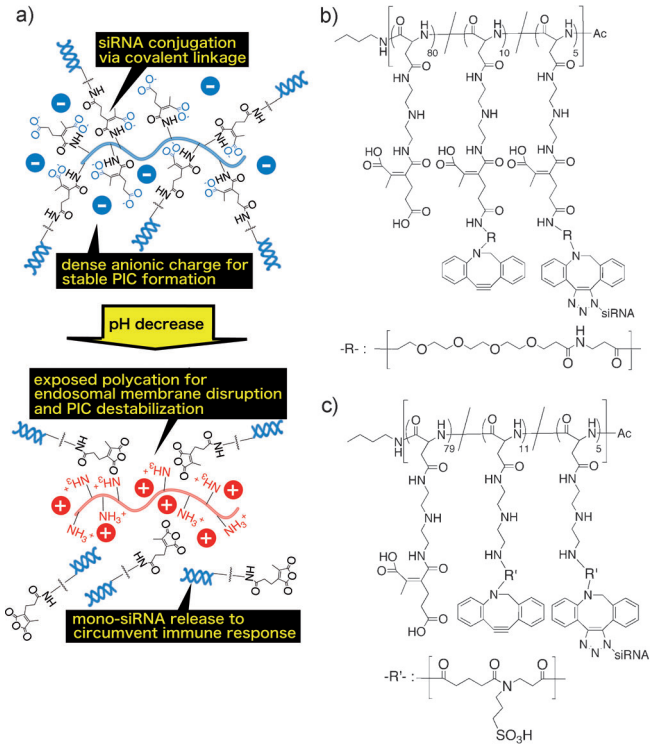

Figure 1. a) Illustration of releasable/enzyme-disrupting conjugate (REC) with the multifunctionality toward endosomal escape and release of mono-siRNA. b) Chemical structure of REC. c) Chemical structure of uREC. The PAsp derivative in this study has the mixed sequence of α and β isomers. Only α isomers are depicted in (b) and (c) for simplicity.

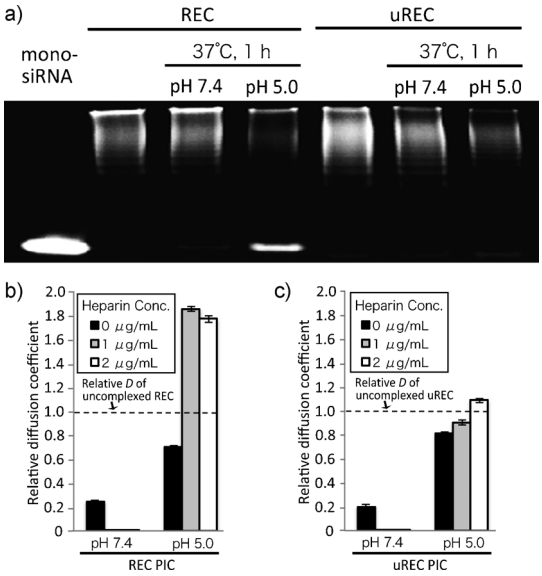

Figure 2. a) PAGE analysis of REC and uREC before and after 1 h incubation at 37 °C and at pH 7.4 or pH 5.0. b),c) Relative *D* values of siRNA conjugate PICs after a 30 min incubation at 37 °C with various heparin concentrations at pH 7.4 or pH 5.0. Relative *D* values are calculated by normalization of *D* to that of uncomplexed siRNA conjugates; REC PIC (b) and uREC PIC (c). Results were shown as mean and standard deviation obtained from 10 measurements.

effect of MAA linkage on the siRNA releasability, another siRNA conjugate, in which the DBCO group was directly conjugated to primary amines in PAsp(DET) without MAA linkage, was also synthesized as an siRNA-unreleasable but endosome-disrupting control (uREC; Figure 1c). The obtained siRNA conjugates were analyzed for their pH-sensitivity by polyacrylamide gel electrophoresis (PAGE) analysis (Figure 2a). The retarded bands in siRNA conjugates, compared to mono-siRNA, indicate that both siRNA conjugates had significantly higher molecular weight than mono-siRNA. A 1 h incubation of REC at pH 5.0 resulted in the band appearance at the same position as mono-siRNA, whereas such band was not observed at pH 7.4, indicating that mono-siRNA release was triggered selectively at the acidic pH. In contrast, the band corresponding to mono-siRNA was not observed for uREC after a 1 h incubation at both pHs of 5.0 and 7.4, indicating the essential role of MAA linkage for mono-siRNA release from REC.

Next, siRNA conjugates were mixed with a polycation PAsp(DET) to form PICs at N/P 10 (residual molar ratio of amines of PAsp(DET) to phosphates of siRNA) for their facilitated cellular uptake. PIC formation with siRNA conjugates as well as mono-siRNA was confirmed by fluorescence correlation spectroscopy (FCS) using Cy3-labeled siRNA (Cy3-siRNA) and its conjugates (Supporting Information, Table S2) as well as agarose gel electrophoresis (Supporting Information, Figure S6). The diffusion coefficients *D* in 10 mM HEPES buffer (pH 7.4) were determined to be 66.2 $\mu m^2 s^{-1}$ for mono-siRNA PIC and 2.9 $\mu m^2 s^{-1}$ for both siRNA conjugate PICs. These values were significantly smaller than those of the uncomplexed controls; that is, mono-siRNA (94.5 $\mu m^2 s^{-1}$) and siRNA conjugates (15.5 $\mu m^2 s^{-1}$ for REC and 18.8 $\mu m^2 s^{-1}$ for uREC). Considering that the *D* value of nanoparticles is inversely correlated with their size,[11] the smaller *D* values in the presence of polycation indicate successful PIC formation with the siRNA conjugates as well as mono-siRNA in the aqueous condition (siRNA concentration: 100 nM). The substantially smaller *D* values of the conjugate PICs, compared to the mono-siRNA PIC, indicate a larger association number of siRNA in the conjugate PICs, which is presumably due to increased anionic charges in the conjugate. Then, the acidic pH-sensitive PIC stability was further evaluated by FCS after a 30 min incubation of PICs at 37 °C in 10 mM HEPES (pH 7.4) and 10 mM MES (pH 5.0) containing heparin. Heparin is a major component of extracellular matrices on cellular surface and probably serves as a strong polyanionic counterpart to induce PIC dissociation.[12] The obtained *D* values of each sample were normalized to that of the corresponding uncomplexed siRNA control; that is, uncomplexed REC for REC PIC, uncomplexed uREC for uREC PIC, and uncomplexed mono-siRNA for mono-siRNA PIC (Figure 2b,c; Supporting Information, Figure S7, respectively). After incubation with heparin, a relative *D* of mono-siRNA PICs progressively increased with the increase in heparin concentration similarly at both pH values of 7.4 and 5.0, indicating that mono-siRNA PICs gradually dissociated with the increased counter polyanion, regardless of the environmental pH (Supporting Information, Figure S7). In contrast, relative *D* values of

REC and uREC PICs decreased after incubation with heparin at pH 7.4, suggesting that the conjugated siRNA is more stably encapsulated within PICs, compared to mono-siRNA, even after binding of heparin onto PIC surface. Notably, the incubation of REC and uREC PICs at pH 5.0 dramatically increased their relative *D* values, and furthermore, the increase in the relative *D* values was facilitated in the presence of heparin, indicating the acidic pH-responsive destabilization of the siRNA conjugate PICs (Figure 2b,c). Considering the fact that the MAA linkage contained in both siRNA conjugates can degrade at pH 5.0 to generate the polycations in PIC, the destabilization of siRNA conjugate PICs at pH 5.0 is presumably due to the electrostatic repulsion between the generated polycations and the originally incorporated polycations in PIC. Furthermore, the increased relative *D* values of REC PICs in the presence of heparin, beyond that of uncomplexed REC, strongly suggest the mono-siRNA release triggered by the cleavage of MAA linkage. These results demonstrate that the acidic pH-sensitivity of the MAA-based conjugates can be maintained even after PIC formation, and also they provide siRNA PICs with a reversible stability in response to the intracellular environment.

Delivery functionalities of REC PICs, namely cellular uptake efficiency and intracellular trafficking profile, were evaluated with cultured human ovarian cancer cells stably expressing luciferase (SKOV3-Luc). Cellular uptake of siRNA was estimated using Cy3-siRNA with a fluorescence microscopy (Supporting Information, Figure S8). REC and uREC PICs (N/P 10) allowed 30% increase in Cy3 fluorescence from cells compared to mono-siRNA PICs (N/P 10, $p < 0.005$), indicating that the conjugate formulation significantly enhanced the cellular uptake of siRNA is probably due to the higher stability, as suggested by the FCS result at pH 7.4 (Figure 2b,c; Supporting Information, Figure S7). Next, confocal laser scanning microscopic (CLSM) observation was performed to examine subcellular distribution of siRNA PICs (N/P 10), especially focusing on the colocalization of siRNA with the late endosome/lysosome as an indicator for endosomal entrapment (Figure 3a–c).[13] In the cells treated with mono-siRNA PICs, the colocalization (yellow) ratio of Cy3-siRNA (red) with a late endosome/lysosome marker LysoSensor Green (green) was increased up to 70% for the initial 12 h and then kept constant for subsequent 36 h (Figure 3d). In contrast, the cells treated with REC and uREC PICs showed that the colocalization ratio was progressively decreased over incubation period and reached about 30% after a 48 h incubation. The significantly lower colocalization ratios (or less endosomal entrapment) of REC/uREC PICs strongly suggest more efficient endosomal escape of siRNA compared to mono-siRNA PICs (Figure 3d). This enhanced endosomal escape with REC and uREC is consistent with the endosome-disrupting functionality of the backbone polymer, which should be converted into the parent polycation PAsp(DET) in the acidic late endosome/lysosome for the membrane disruption, as suggested by a membrane disruption assay at pH 7.4 and 5.0 (Supporting Information, Figure S9).[8,9]

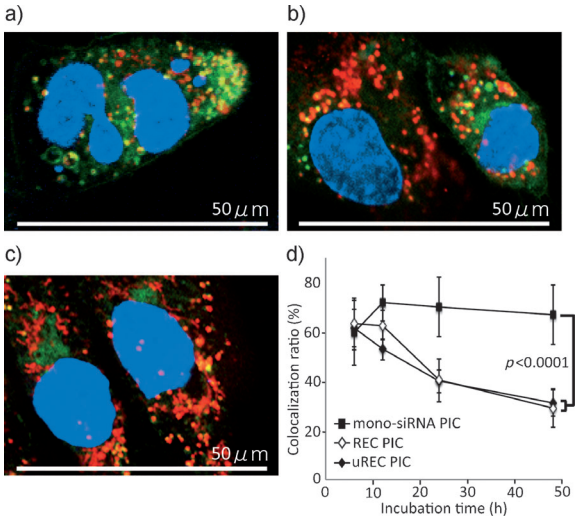

Figure 3. a–c) CLSM images 48 h after treatment of SKOV3-Luc cells with mono-siRNA PIC (a), REC PIC (b), and uREC PIC (c). Red Cy3-siRNA, green late endosome/lysosome (LysoSensor Green), blue nucleus (Hoechst 33342). A yellow pixel indicates colocalization between a red pixel and green pixel. d) Time-dependent change in the colocalization ratio between Cy3-siRNA and late endosome/lysosome. The colocalization ratio was shown as mean and standard deviation obtained from 10 cells. The *p* value was calculated according to Student's *t* test.

Next, the gene silencing ability of REC PICs was compared with mono-siRNA and uREC PICs by luciferase assay with cultured SKOV3-Luc cells (Figure 4a). Obviously, REC and uREC PICs achieved more efficient sequence-specific gene silencing in the cells than mono-siRNA PICs, which is presumably due to the enhanced endosomal escape of siRNA conjugate PICs (Figure 3) as well as facilitated cellular uptake of siRNA (Supporting Information, Figure S8). Interestingly, REC PICs induced significantly stronger gene silencing than uREC PICs ($p < 0.005$), demonstrating the positive effect of siRNA releasability by the MAA linkage on the siRNA delivery functionality. Mono-siRNA

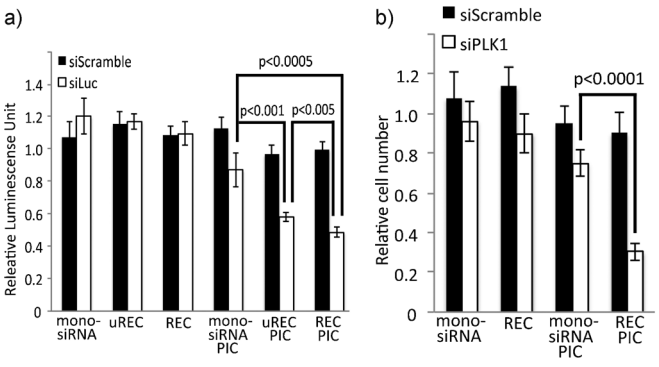

Figure 4. a) Luciferase gene expression in cultured SKOV3-Luc cells after PIC treatment at 100 nM Luc siRNA (siLuc) or scramble siRNA (siScramble) for 48 h. b) Cell viability in cultured A549 cells after PIC treatment at 100 nM PLK1 siRNA (siPLK1) or siScramble for 72 h. In both figures, results were shown as mean and standard deviation obtained from 6 samples. The *p* values were calculated according to Student's *t* test.

releasates from REC might be more readily associated with the gene silencing pathway owing to compromised steric hindrance compared to the conjugated structure. Also, no cytotoxicity was observed for all the tested PIC formulations under the same conditions as the gene-silencing assay (Supporting Information, Figure S10). Significantly stronger luciferase gene silencing of REC PICs was also confirmed in comparison with mono-siRNA PICs prepared with PAsp-(DET)/PAsp(DET-CDM) (a non-covalent control) and a commercially available reagent ExGen500 (linear polyethylenimine; Supporting Information, Figure S11), demonstrating the advantage of REC formulation, including covalent conjugation between siRNA and the backbone polymer. The effect of the siRNA-releasability of REC was further examined from the standpoint of immune responses; IFNα response was determined as an indicator of immune response by enzyme-linked immunosorbent assay (ELISA). REC, uREC, and their PICs did not induce a detectable level of IFNα production for SKOV3-Luc cells ($< 10\ \mathrm{pg\,mL^{-1}}$, data not shown). Thus, the similar ELISA experiment was further challenged for murine macrophage cells (Raw264.7), which are known to be highly sensitive to immunogen.[14] As a result, REC PICs induced a significantly lower level of IFNα production ($24.3 \pm 3.5\ \mathrm{pg\,mL^{-1}}$) compared to uREC PICs ($60.8 \pm 12.9\ \mathrm{pg\,mL^{-1}}$, $p < 0.005$), indicating that the siRNA-releasability based on MAA linkage successfully decreased the immune response for siRNA conjugates. Uncomplexed REC and uREC without polycation did not induce a detectable level of IFNα production, suggesting that they should not stimulate IFNα response at least on the cellular surface. Finally, the utility of REC PICs was verified for other cell lines, using a therapeutic siRNA targeting polo-like kinase 1 (PLK1). PLK1 is known to be a cell cycle regulator, and thus its silencing can arrest the cell cycle toward the apoptosis.[15] REC PICs with PLK1 siRNA (N/P 20) sequence-specifically suppressed the growth of human lung carcinoma cells (A549) and human hepatocarcinoma cells (Huh-7; Figure 4b; Supporting Information, Figure S12, respectively), demonstrating a strong potential of the REC formulation bearing the MAA linkage for siRNA-based cancer therapy.

In summary, an acidic pH-responsive siRNA conjugate was developed for enhanced siRNA delivery with reduced immunogenicity. A single chemical process based on the MAA linkage successfully provided the multifunctionality required for successful siRNA delivery; that is, reversible carrier stability, endosomal escapability, and mono-siRNA releasability. Ultimately, the siRNA conjugate sequence-specifically achieved the significant growth inhibition of cancerous cells. The programmed siRNA delivery based on the smart conjugate will be further investigated for the success in siRNA therapeutics.

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