siRNA Delivery

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A Phenylboronate-Functionalized Polyion Complex Micelle for ATP-Triggered Release of siRNA**

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Therapeutics based on small interfering RNA (siRNA) offer an attractive clinical option because of its ability to silence genes in a highly sequence-specific manner. [1] A key challenge lies in developing a delivery system that helps protect the siRNAs from endogenous RNase degradation while allowing for controlled pharmacokinetics. One promising approach is a formulation of polyion complex (PIC) micelles that spontaneously form in an aqueous environment simply through electrostatic interactions between the anionic siRNA and cationic polymers.[2] With versatile designs of the counterpart cationic polymers, representative poly(ethylene glycol) based block co-polymers, many creative PIC-based strategies have emerged, some of which have shown encouraging in vitro gene silencing abilities.[3–5] However, in general, these PIC-based carriers suffer from instability under physiological conditions, primarily because of the relatively short chain length of the siRNA, that is 20–25 nucleotides, which results in poor thermodynamic stability. Therefore, stabilization of the PIC-based carriers so that programmed destabilization upon arrival at the site of intracellular targets (to release siRNA) has been of interest. Current efforts have focused on either one or combinations of the following three representative approaches: covalent conjugation of siRNAs to a homing polymer,[3,6–8] introduction of hydrophobic moieties to reinforce the core-aggregation,[9–11] and cross-linking the core aggregate by disulfide bridging.[5,12] As such, the combination of these approaches often results in a highly complex structure and method of preparation.

Herein, we describe a sophisticated solution that can remarkably simplify the synthesis of PICs. It uses a phenylboronate functionality, which incorporates all of the aforementioned methods of stabilization (Scheme 1) while maintaining a wide window of control for environmental sensitivity. Phenylboronic acid (PBA) is a synthetic molecule capable of forming reversible covalent esters with 1,2- or 1,3-*cis*-diols including on a ribose ring,[13–15] a structure which is present at the 3′ end of RNAs and several kinds of ribonucleotides. Because of this property, PBA has historically been used as a ligand for RNA in affinity chromatography.[16] Therefore, this binding property offers a facile route for chemical conjugation of siRNAs to the pendant PBA groups. Once electrostatically condensed into the PIC, the chances of equilibrium binding are increased, in which intermolecular cross-links could also form because of the bis-bidentate ribose arrangement at the 3′ end of the double-stranded siRNA, thereby further stabilizing the complex. Furthermore, PBA is unique in that it undergoes a dramatic inversion in its level of hydrophobicity depending on the degree of acid disassociation;[17] it is strongly hydrophobic when uncharged but it becomes hydrophilic when negatively charged at pH values above its pK_a. As shown in Figure 1, the binding between PBA and siRNAs is essentially a reversible equilibrium process dependent on the concentrations of each species. These features can be used to fine-tune or switch the stability of the complex, which is relevant to creating a system that is sensitive to the inter- and intracellular environments. Herein, we demonstrate that the PBA-assisted PIC micelles can be tailored to exhibit a dramatic disruption accompanied by the release of siRNAs in response to a change in the ribose concentration (which parallels events in the intracellular environment).

A platform cationic polymer poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLys) was first prepared, the lysine residues of which were quantitatively modified with 3-fluoro-4-carboxyphenylboronic acid (FPBA) to different extents. The weight-average molecular weight (M_w) of PEG and the mean degree of polymerization of PLys were determined to be 12000 Da and 42, respectively (Supporting Information, Table S1). The synthesized polymers are referred to as PEG-*b*-P(Lys/FPBA$_X$)$_{42}$, where X denotes number of FPBA units introduced per polymer chain. According to the scattered light intensity of the polymer solutions, polymers were soluble (at 5 mg mL^{-1}) in HEPES buffered solution (HBS, pH 7.3) up to 55% FPBA modification, that is (PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$, however, those with 66% (that is (PEG-*b*-P(Lys/FPBA$_{28}$)$_{42}$) or higher degrees of FPBA modification were partially insoluble because of the strong hydrophobicity of FPBA (data not shown). The HBS-soluble series of polymers, that is PEG-*b*-P(Lys/FPBA$_{0,10,19,23}$)$_{42}$, were allowed to complex with siRNA at various N/P ratios, which is defined as the molar

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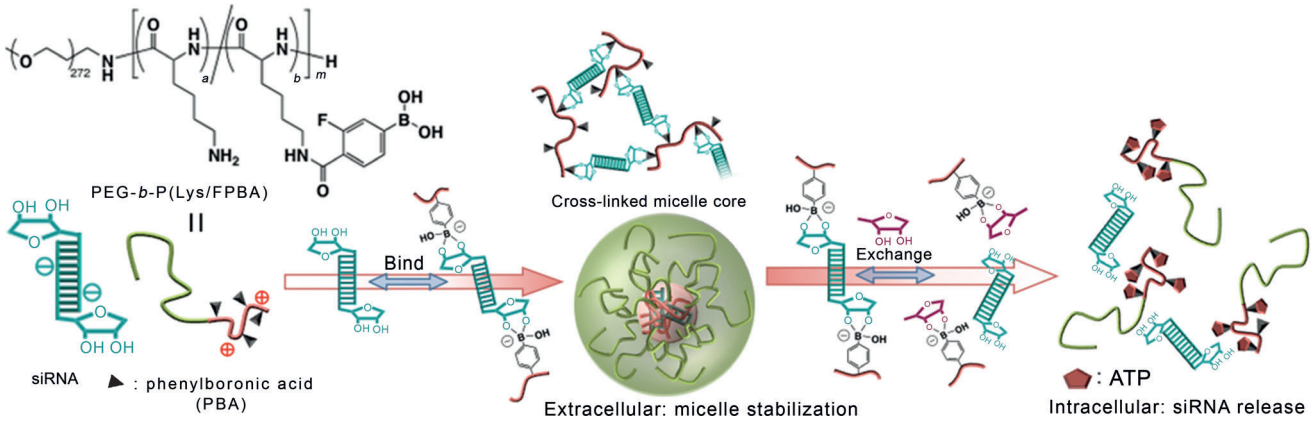

Scheme 1. Schematic representation of the phenylboronic acid based strategy for siRNA delivery; the chemical formula of the polymer, enhanced stability of the micelle, and the mechanism of selective intracellular release are shown.

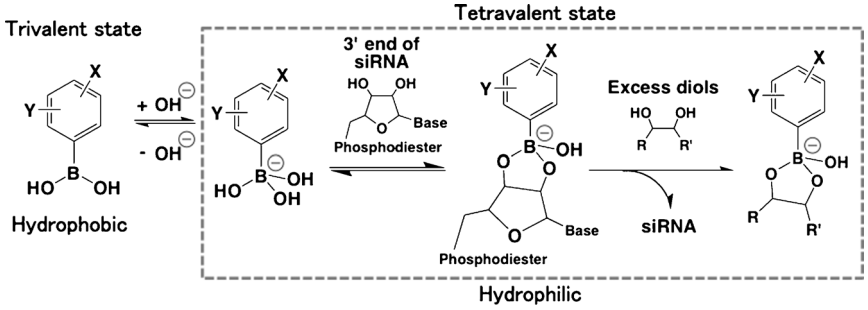

Figure 1. Equilibrium between a PBA derivative binding to siRNAs and other diols in aqueous solution.

ratio of the primary amino groups in the PLys segments to the phosphate groups in the siRNAs. These polymers were assessed by fluorescence correlation spectroscopy (FCS) with regard to the stability of the complex (Figure 2a). From measured values of the diffusion coefficient (D) relative to that for the free siRNA (72.0 $\mu m^2 s^{-1}$), we concluded that PEG-*b*-$PLys_{42}$ with no FPBA modification and PEG-*b*-P(Lys/$FPBA_{10}$)$_{42}$ (the lowest FPBA content of the series) could not form complexes with siRNAs under physiological salt condition in the presence of 10% fetal bovine serum (FBS), although the latter was found to form a complex in the absence of FBS (see Figure S1). In contrast, complexes of PEG-*b*-P(Lys/$FPBA_{19}$)$_{42}$ and PEG-*b*-P(Lys/$FPBA_{23}$)$_{42}$, with increased FPBA content, do tolerate such conditions. Indeed, PEG-*b*-P(Lys/$FPBA_{23}$)$_{42}$, with the highest FPBA content of the series, showed no significant change in its diffusion coefficient regardless of the presence of FBS (Figure 2a, see also Figure S1c). PEG-*b*-P(Lys/$FPBA_{19}$)$_{42}$ and PEG-*b*-P(Lys/$FPBA_{23}$)$_{42}$ were further compared by agarose gel electrophoresis at a fixed N/P ratio of 4 (above which no more change in the diffusion coefficient was observed in Figure 2a) in the presence of a complex-destabilizer polyanionic dextran sodium sulfate (DSS, $M_w = 5000$ Da; Figure 2b). In this experiment, bands of Cy3-labeled siRNA, which are indicative of partial or whole collapse of the complex, could be seen at the bottom of each lane. In both cases, the bands became more intense with increasing concentrations of DSS (or increasing A/P ratio in Figure 2b), which destabilizes the complex because its polyanionic charges replace the PIC-engaged siRNAs. Even so, in comparison, the complex of PEG-*b*-P(Lys/$FPBA_{23}$)$_{42}$ shows relatively high resistance to DSS. Thus, these results establish that the stability of the complex has been increased by the PBA functionality, presumably because of its multiple modes of stabilization, as described above.

To gain more clear insight into the siRNA release, three distinct types of double-stranded siRNAs were prepared (Figure 3), that is a normal one terminated with ribose at both 3′ ends (R-siRNA-R) and those modified with deoxyribose at either one (R-siRNA-dR) or both 3′ ends (dR-siRNA-dR), so that each represents bi-, mono-, or non-functionality when binding with PBA (Figure 3). Each siRNA was complexed with PEG-*b*-P(Lys/$FPBA_{23}$)$_{42}$, with various N/P ratios, and analyzed by gel electrophoresis (Figure 3). In this experiment, bands in the gel can be attributed to free siRNA that did not participate in complex formation under each condition. Therefore, the intensity of the bands decreases with an increasing N/P ratio for all cases, which is consistent with Figure 2a. By comparing the three systems, it can be observed that the trend is in accord with the number of the ribose functionalities, R-siRNA-R > R-siRNA-dR > dR-siRNA-dR, indicating the order of binding efficiency. Accordingly, this result supports an important role for the PBA–siRNA binding in the stabilization of the complex.

Further evidence for ribose-specific stabilization and its sensitivity to the surrounding environment was determined by analysis of complexes subjected to competitive ribose or non-ribose environments. For competitors, adenosine triphosphate (ATP), uridine monophosphate (UMP), deoxythymidine monophosphate (dTMP), and glucose were each assessed at their in vivo concentrations. Note that among

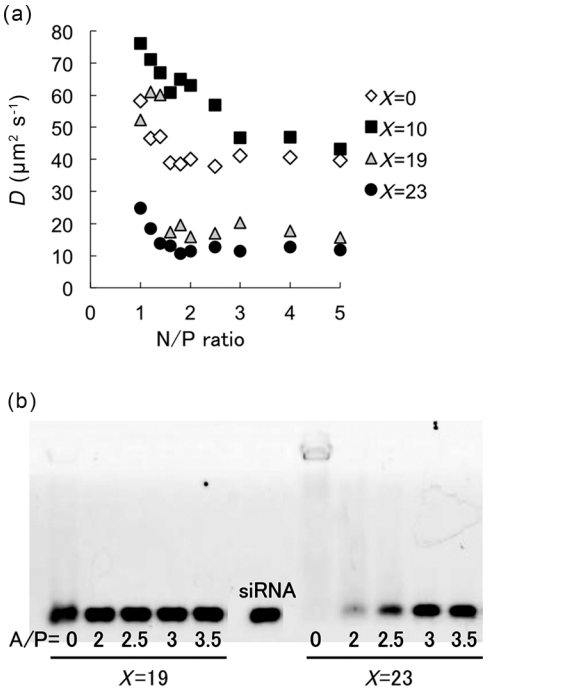

Figure 2. Stability of the complex between R-siRNA-R and PEG-*b*-P(Lys/FPBA$_X$)$_{42}$. a) Diffusion coefficients of Cy3-labeled siRNA in various polymer-complexes as a function of N/P ratio, as determined by FCS, after a 1 h incubation in HBS containing 10% FBS. b) Agarose gel electrophoresis of the complex for $X=19$ (left) and $X=23$ (right) at N/P = 4 after a 1 h incubation in HBS containing DSS. A/P denotes the molar ratio of the anionic charges in DSS to the phosphate groups in the siRNA.

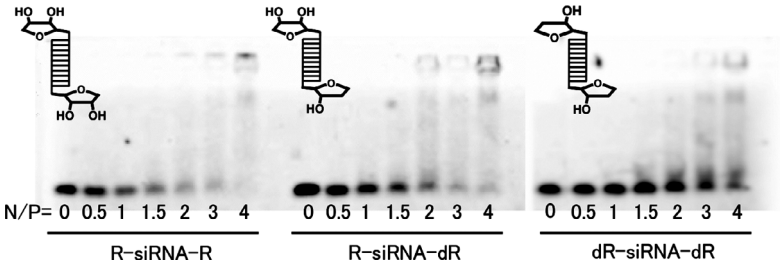

Figure 3. Agarose gel electrophoresis of the complex formed between PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ and different types of double-stranded siRNAs; R-siRNA-R (left), R-siRNA-dR (middle) and dR-siRNA-dR (right), for various N/P ratios, after a 4 h incubation in HBS.

these additives only ATP and UMP have ribose moieties, which have the potential to compete with siRNA for binding to PBA. Figure 4a shows changes in the diffusion coefficient of the siRNA (R-siRNA-R) complexed with PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ as a function of the concentration of the additive. As expected, the complex is markedly destabilized, as seen by increasing diffusion coefficients, upon addition of ATP and UMP. On the contrary, neither dTMP, incapable of binding to PBA, nor glucose, the most abundant polyol in the blood (normally approximately 10 mM) with relatively weak binding affinity to PBA, caused any significant destabilization even when high concentrations were used. Interestingly, the complex is more sensitive to the triphosphate ATP than it is to a monophosphate UMP. This difference may be due to the different number of the phosphate anions of each molecule. That is to say, the higher anionic density of ATP, upon binding with the pendant PBA, should aid in both electrostatic repulsion against the siRNA and also weaken the core hydrophobic interaction of PBA more than UMP. Figure 4b compares the stability of complexes consisting of di-, mono- and non-ribose functionalized siRNAs when exposed to ATP. In accordance with Figure 3, the complex stability (that is resistance to ATP) strikingly depends on the number of ribose functionalities. Such graded control of the complex stability, depending on the type of siRNA, should be of benefit to fine-tuning the release of siRNAs. Significantly lower, but still appreciable ATP resistance, was shown by the dR-siRNA-dR complex; this confirmed that the contribution of the hydrophobic PBA interaction was significant to the resultant stability. However, as illustrated in Figure 1, the fraction of trivalent hydrophobic PBA, which is responsible for such a hydrophobic interaction, can dramatically decrease with increased ribose concentration. Also the most abundant ribonucleotide in vivo, ATP, is present in the extracellular environment at about 0.4 mM, but is dramatically higher (up to 3 mM) within the intracellular matrix.[18–20] Results shown in Figure 4 demonstrate that the PIC will respond to ATP concentrations of exactly this range, thus offering the potential for the selective release of siRNAs upon entry into the cell. Preliminary studies of the R-siRNA-R engaged complex of PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ have shown an encouraging dose-dependent silencing of the polo-like kinase 1 (PLK-1) gene, a well-known proto-oncogene, in a human renal carcinoma cell line (OSRC-2) with minimal cytotoxicity (Figure S3).

In summary, our results demonstrate that a PBA-functionalized PIC micelle can be used for intracellular ATP-triggered release of siRNAs. The window of concentrations over which complex stability can be controlled is ideal for further fine-tuning. The strength of the ribose–PBA binding and the hydrophobicity of PBA, both critical determinants of complex stability, as well as ATP sensitivity, are further controllable on the basis of the substituent group structures.[13,14,17] By varying the type and length of the counterpart polycations, improved biocompatibility and endosomal escape[21–23] could be acheived. Further efforts are being directed toward these possibilities, along with in vitro and in vivo studies of gene silencing.

Experimental Section

PEG-*b*-PLys was synthesized using a previously described method.[24] FPBA modification of lysine residue in the PEG-*b*-PLys$_{42}$ with different numbers of FPBA was performed by aqueous phase condensation reaction using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMT-MM). The resulting polymers (5 mg mL^{-1}) and siRNA (15 μM) were dissolved in HEPES buffered solution (HBS; 10 mM, pH 7.3). These were mixed in various N/P ratios to prepare the complexes. The cumulant diameter and zeta

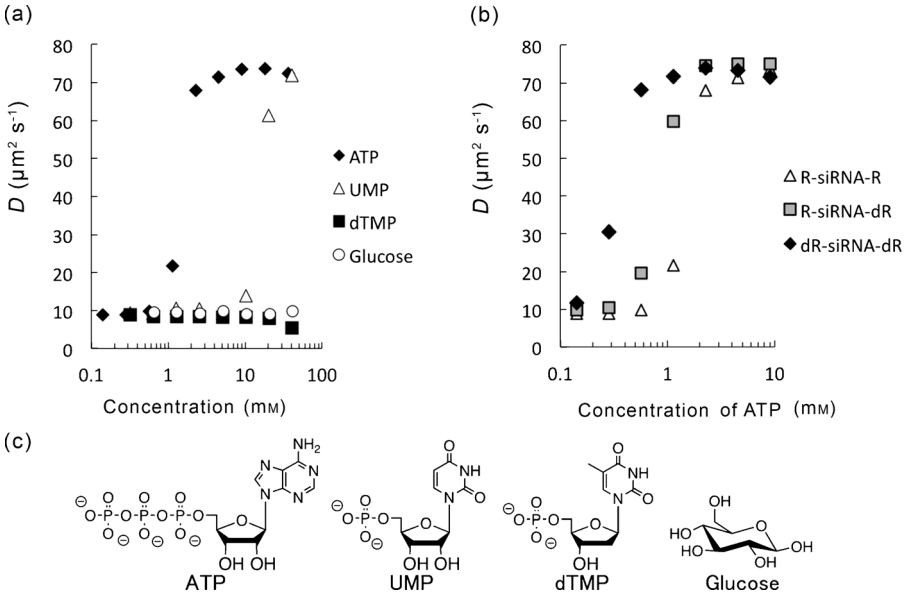

Figure 4. Ribose-specific stabilization of the complex and its sensitivity to a changing environment. a) Changes in the diffusion coefficient of Cy3-labeled siRNAs complexed with PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ as a function of various additive concentrations after a 1 h incubation. b) Changes in the diffusion coefficient of siRNAs with different numbers of ribose functionalities when complexed with PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ as a function of ATP concentration. c) Chemical formulas of the additives.

potential of the complexes were measured using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK) after dilution with HBS to give 4 μM siRNA. To compare the siRNA loading ability of the FPBA modified polymers, siRNAs that were labeled with Cy3 at the 5′ end of the anti-sense strand were subjected to 1% agarose gel electrophoresis at 100 V for 20 min. The complexes with PEG-*b*-P(Lys/FPBA$_{19,23}$)$_{42}$ at N/P = 4 were diluted with HBS containing DSS at various A/P ratios to a final siRNA concentration of 50 nM and allowed to stand for 1 h at room temperature. To demonstrate the importance of ribose functionality at the 3′ end(s) of the siRNA, three types of siRNA-loaded micelles (R-siRNA-R, R-siRNA-dR, dR-siRNA-dR) were prepared from PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ at various N/P ratios, diluted with HBS to be a final siRNA concentration of 50 nM, and allowed to stand for 1 h at room temperature before gel electrophoresis was performed. To demonstrate the stability of the micelles in serum, or with competitive monosaccharides or ribose, FCS analyses were conducted using an LSM-510-META equipped with FCS setup (ConfoCor3, Carl Zeiss Microscopy, Ltd.). For the stability test, the Cy3-labeled siRNA was complexed with PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ at various N/P ratios, diluted with HBS or HBS containing 10% FBS to be a final siRNA concentration of 50 nM, and allowed to stand for 1 h at room temperature. For the ribose-resistance test, a complex of Cy3-labeled siRNA and PEG-*b*-P(Lys/FPBA$_{23}$)$_{42}$ at N/P = 4 was diluted with HBS containing various amount of ATP, UMP, dTMP, or glucose to be a final siRNA concentration of 50 nM and allowed to stand for 1 h at room temperature. FCS measurements were performed with a sampling time of 20 s at room temperature. The autocorrelation curves obtained from ten repeated measurements were converted to the diffusion time according to the manufacturer's instructions. Finally, the diffusion time was converted to the diffusion coefficient based on calibration using Rhodamine 6G.

More detailed information on materials, instruments, polymer synthesis, methods of complex characterization, complex stabilities in the absence of FBS, and an in vitro study of RNA interference is provided in the Supporting Information.

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