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Deoxyribozymes, or DNAzymes, are DNA molecules with enzymatic activity. DNAzymes with ribonuclease activity have various potential applications in biomedical and bioanalytical fields; however, most constructs have limited turnover despite optimization of reaction conditions and DNAzyme structure. Cationic comb-type copolymer accelerates DNA hybridization and strand exchange rates, and we hypothesized that the copolymer would enhance deoxyribozyme activity by promoting turnover. The copolymer did not change DNAzyme activity under single-turnover condition, suggesting that the copolymer affects neither the folding structure of DNAzyme nor the association of a divalent cation, a catalytic cofactor, to DNAzyme. The copolymer enhanced activity of the evaluated DNAzyme over a wide temperature range under multiple-turnover conditions. The copolymer increased the DNAzyme k_{cat}/K_m by fifty fold at 50ºC, the optimal temperature for the DNAzyme in the absence of the copolymer. The acceleration effect was most significant when reaction temperature was slightly higher than the melting temperature of the enzyme/substrate complex; acceleration of two orders of magnitude was observed. We concluded that the copolymer accelerated the turnover step without influencing the chemical cleavage step. In contrast to the copolymer, a cationic surfactant, CTAB, strongly inhibited the DNAzyme activity under either single- and multiple-turnover conditions.

Introduction

Deoxyribozymes (DNAzymes) are DNA sequences that possess enzymatic activity. Compared with protein enzymes and ribozymes, the DNAzymes are easier to synthesize, active under a wider range of temperature and buffer conditions, and more readily modified. The first DNAzyme was demonstrated by Breaker and Joyce in 1994 [1]. Since then DNAzymes with diverse enzyme activities have been reported [2,3]. DNAzymes with RNA cleaving (ribonuclease) activity have been most extensively studied. RNA-cleaving DNAzymes have been demonstrated to inactivate target RNAs [4], and detect divalent metal ions [5] and have been used in construction of computational device elements [6]. Sensitive detection of DNA/RNA sequences has also been reported [7].

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RNA-cleaving DNAzymes are composed of catalytic core and substrate-recognizing domains. DNAzyme-catalyzed reactions involve several steps. The first is the formation of substrate/DNAzyme (ES) complex. The next step is the chemical cleavage of the substrate with the aid of a divalent cation. This step is followed by the release of the cleavage products. This generates a DNAzyme ready to load a fresh substrate for turnover. DNAzyme activity has been enhanced by screening core sequences under different selection pressures [8–13], use of modified nucleotides [14–17], conjugation with intercalators [18], and optimization of divalent metal cations [19–21]. Both the chemically cleaving step and the turnover step should be improved for enhancing overall activity of DNAzyme. DNAzymes designed to form stable complex with substrates showed higher catalytic activity under single-turnover condition, but occasionally resulted in low turnover efficacy owing to a slower product release from the enzymes [17, 21]. Similarly, at lower reaction temperatures release rate of the product is limiting, but at high temperature slow substrate/DNAzyme association limits the reaction rate. Conditions that increase dissociation and association dynamics of nucleotide hybrids are considered to improve turnover efficacy.

We have explored interactions between DNA strands with cationic comb-type copolymer composed of a polycationic backbone and water soluble graft chains [22, 23]. The copolymer with more than 80 weight % graft chains forms a soluble inter-polyelectrolyte complex with DNA without inducing the coil-globule transition of the DNA [24–26]. The copolymer considerably stabilizes double-stranded (dsDNA) [23, 27] and triple-stranded DNA structures [22, 28]. Interestingly, the copolymer stabilized these structures by increasing the association rates rather than decreasing dissociation rates [29, 30]. This kinetic effect of the copolymer is unique because decrease in the dissociation rate was generally accounted for hybrid stabilization by other methods including uses of chemically modified nucleotides [31, 32]. More notably, the copolymer facilitates a strand exchange between dsDNA and a homologous strand [33, 34]. The copolymer likely increases dissociation and association dynamics of nucleotide hybrids. The activity of the copolymer was utilized to refine a DNA analytical method [35] and nanodevices [36, 37].

We hypothesized that the turnover efficacy of DNAzyme can be improved by the copolymer, if the copolymer would satisfy the following requirements: the copolymer disorders neither 1) association of a divalent cation to DNAzyme nor 2) active structure of DNAzymes. In the present study, we examined the effect of the copolymer on activity of the 10-23 DNAzyme [8, 38], one of most active RNA-cleaving DNAzymes, under single- and multiple-turnover conditions.

Experiment

Materials

Sodium poly(vinyl sulfonate) (PVS, 25 wt % aq.) and poly(L-lysine hydrobromide) (PLL·HBr, $M_W = 7.5 \times 10^3$) were obtained from Sigma-Aldrich Co. LLC (St. Louis, USA), and dextran (Dex, $M_W = 8 \times 10^3 - 1.2 \times 10^4$) was obtained from Funakoshi Co., (Tokyo, Japan). Sodium hydroxide, sodium chloride, magnesium sulfate, manganese (II) chloride tetrahydrate, and acetic acid were purchased from Wako Pure

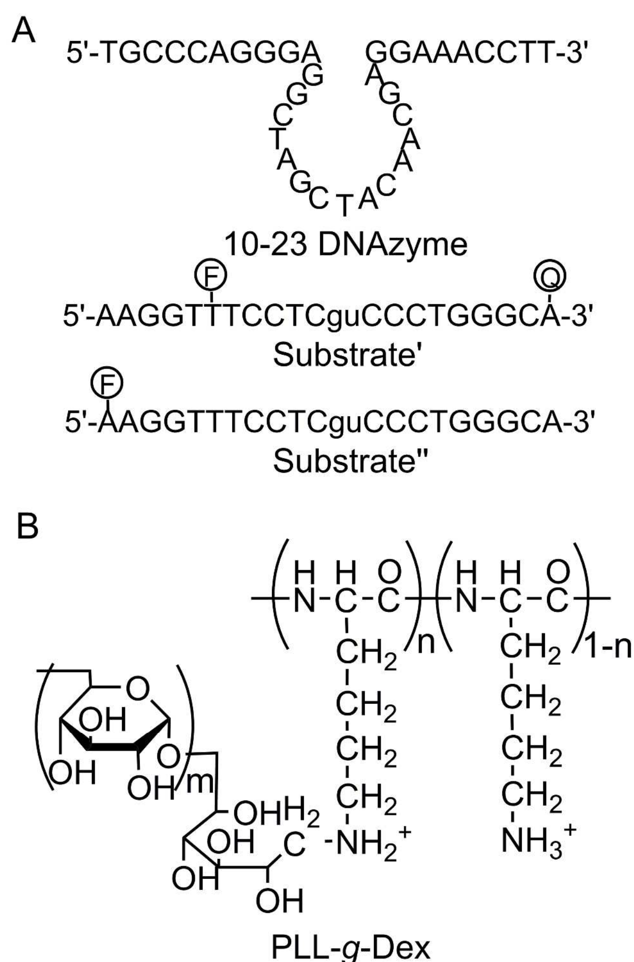

Fig. 1. (A) Sequences of 10-23 DNAzyme and substrates. Substrate I was labeled with both fluorophore (F, FITC) and quencher (Q, BHQ-1), and substrate II was labeled with only a fluorophore. (B) Structural formula of cationic comb-type copolymer PLL-*g*-Dex. M_w of PLL backbone: 7.5×10^3, M_w of dextran grafts: 1.0×10^4, grafting degree of dextran: 11.5 mol%.

Chemical Industries (Osaka, Japan). Sodium tetraborate decahydrate, urea, (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and ethylenediaminetetraacetic acid tetrasodium salt (EDTA·4Na) were purchased from Nacali Tesque, Inc. (Kyoto, Japan). HPLC-grade oligonucleotides with the sequences summarized in Fig. 1 were purchased from Fasmac Co., Ltd. (Atsugi, Japan) and used without further purification. Cationic comb-type copolymer poly(L-lysine)-*graft*-Dextran (PLL-*g*-Dex) was prepared according to the previously published procedure [22, 23]. Briefly, PLL-*g*-Dex was prepared by a reductive amination reaction of dextran with PLL. The resulting copolymer was isolated through an ion exchange column, dialyzed, and lyophilized. The product was characterized by ^{1}H NMR and GPC. PLL-*g*-Dex of 90 wt% dextran (11.5 mole % of lysine units of PLL were modified with dextran) was used in this study.

FRET analysis of the DNAzyme cleavage reaction

Substrate I (final concentration 200 nM) was dissolved in a reaction buffer consisting of 50 mM HEPES (pH 7.3), 150 mM

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NaCl, and 5.0 mM Mg^{2+} or Mn^{2+} in the absence or presence of PLL-*g*-Dex. Unless otherwise noted, the ratio of [positively charged amino groups]$_{copolymer}$/[negatively charged phosphate groups]$_{DNA}$ (N/P ratio) was 2. The substrate was pre-incubated with PLL-*g*-Dex at reaction temperature for 5 min in a quartz cell. After the pre-incubation, DNAzyme solution (final concentration 6.7 nM) was injected into the cell to initiate the reaction. The fluorescence intensity was measured using a FP-6500 spectrofluorometer (Jasco, Tokyo, Japan) at an excitation wavelength, λ_{ex}, of 494 nm and an emission wavelength, λ_{em}, of 520 nm. The fluorescence intensity curve over time was used to fit the equation $I_t = I_0 + (I_\infty - I_0)(1 - e^{-kobst})$, where I_t was fluorescence intensity at any reaction time t, I_∞ was the fluorescence intensity of the synthesized product, I_0 was the initial fluorescence intensity (background). The k_{obs} was obtained by fitting the initial 20% of cleavage reaction curve.

Gel electrophoresis analysis of DNAzyme reaction progress

Substrate II (500 nM) was mixed with DNAzyme (750 nM for single-turnover or 16.7 nM for multiple-turnover reactions) in 50 mM HEPES buffer (pH = 7.3) containing 0.5 mM Mg^{2+} and 150 mM NaCl with or without PLL-*g*-Dex (N/P = 2). The reactions were incubated at 25ºC. Aliquots (3 μL) were taken at various reaction times, and 0.5 μL of 0.1 M EDTA was added to stop reaction. After addition of 3 μL formamide and 0.5 μL 5 mg/mL PVS, reaction products were separated on a denaturing polyacrylamide gel (16%) run at 25ºC for 75 min. The fluorescence was monitored using a Bio-Rad molecular imager pharosFX at λ_{ex} 388 nm and λ_{em} 532 nm.

Determination of DNAzyme kinetic constants

The Michaelis-Menten kinetic parameters, k_{cat} and K_m, were determined from the y-intercept and negative slope, respectively, of the best-fit line to the Eadie-Hofstee plot of V_0 versus V_0/[S] [21]. Each plot consisted of five data points for values of [S] that ranged from K_m to 10-fold K_m, with [S] always in ≥10-fold excess over [E]. V_0 was determined from the initial 10% of the curve. All the reactions were carried out in the presence of 5 mM Mn^{2+} in the absence or presence (N/P = 2) of PLL-*g*-Dex in 50 mM HEPES (pH 7.3), 150 mM NaCl and were initiated by injecting DNAzyme solution

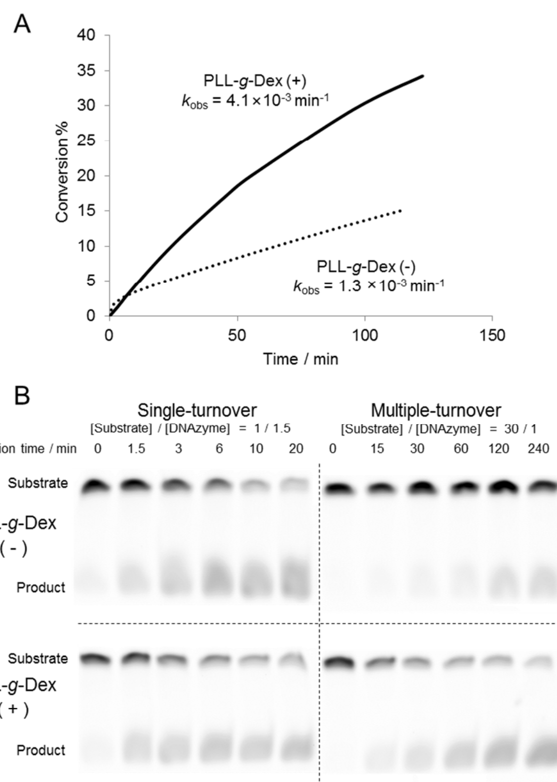

Fig. 2. Influence of PLL-*g*-Dex on single- and multiple-turnover reaction of DNAzyme at 0.5 mM Mg^{2+} and 25 ºC. (A) DNAzyme multiple-turnover reactions in the absence and presence of PLL-*g*-Dex (N/P = 2) were monitored by analysis of labeled substrate I. ([substrate I]$_0$ = 200 nM, [DNAzyme] = 6.7 nM). (B) DNAzyme reactions under single- and multiple-turnover conditions in the absence and presence (N/P = 2) of PLL-*g*-Dex were monitored by analysis of substrate II. The samples contained 500 nM substrate II and either 750 nM (single-turnover) or 16.7 nM (multiple-turnover) DNAzyme.

Results

Influence of cationic comb-type copolymer on multiple- and single-turnover reactions

Firstly, we investigated the effect of the copolymer PLL-*g*-Dex on ribonuclease activity of the 10-23 DNAzyme [38,39] on substrate I (Fig. 1) with 0.5 mM Mg^{2+} as a cofactor under multiple turnover conditions, [S]/[E] = 30 (Fig. 2A). Whereas in the absence of the copolymer k_{obs} was 1.3×10^{-3}, in the presence of the copolymer at an N/P ratio of 2, k_{obs} was 3 fold greater.

To estimate the role of the copolymer in the observed acceleration, the DNAzyme reactions were carried out under either single- or multiple-turnover conditions. Substrate I (500 nM) was mixed with 750 nM or 16.7 nM of DNAzyme in a buffer containing 0.5 mM $MgSO_4$ with or without PLL-*g*-Dex. After incubation at 25°C the reaction mixtures were separated by denatured gel electrophoresis (Fig. 2B). As the reaction time increased the intensity of the upper band, which corresponds to uncleaved substrate, decreased and the intensity of the lower band, which corresponds to products, increased. In the reaction under the single-turnover condition no difference in cleavage rates was observed regardless of the presence of PLL-*g*-Dex. The results indicated that the copolymer did not significantly influence chemically cleavage activity of the DNAzyme. The active folding structure of the DNAzyme and the association of a divalent metal ion to DNAzyme were likely not disordered by the copolymer. In contrast, under multiple-turnover reaction condition faster cleavage was observed in the presence of PLL-*g*-Dex, implying that the copolymer promoted turnover. Calculated thermal melting temperatures (T_ms) at 0.5 mM Mg^{2+} were 39.8ºC for the enzyme complex with the 5' product and 54.0ºC for the enzyme complex with the 3' product when product concentration is 500 nM and DNAzyme concentration is 16.7 nM [39]. This calculation suggests that dissociation of the products from the DNAzyme should be slow at 25ºC. The copolymer likely facilitates strand exchange of EP for ES at 25ºC.

The effect of the copolymer was compared with that of cethyltrimethyl ammonium bromide (CTAB), a cationic surfactant known to promote DNA hybridization [40]. CTAB significantly decreased the rate of the DNAzyme reaction under both single- and multiple-turnover conditions (Fig. 3A and B). We observed turbidity in the reaction mixture in the presence of CTAB (Fig. 3C). CTAB likely inhibited DNAzyme activity by DNA compaction and aggregation [41]

Rate-determining step shifted by the copolymer

To investigate further the effect of the copolymer on the DNAzyme multiple-turnover reaction, the temperature dependence of the reaction was assessed. Cleavage activity with or without the copolymer increased as the reaction temperature was increased from 25ºC to 60ºC (Fig. 4 and Fig. 5D). When the DNAzyme reaction was carried out at 60ºC, significant loss in the activity was observed in the absence of the copolymer. As the calculated value of *T*m [40] of DNAzyme/substrate hybrid is about 53ºC, this loss in the DNAzyme reactivity likely resulted from a decrease in association rate of the substrate with the DNAzyme. In the presence of the copolymer, no loss in activity was observed until 60ºC (Fig. 5D), indicating that the copolymer increased the association rate of a substrate with a DNAzyme. At 60ºC, the copolymer enhanced the DNAzyme activity by 30 fold relative to activity in the absence of copolymer.

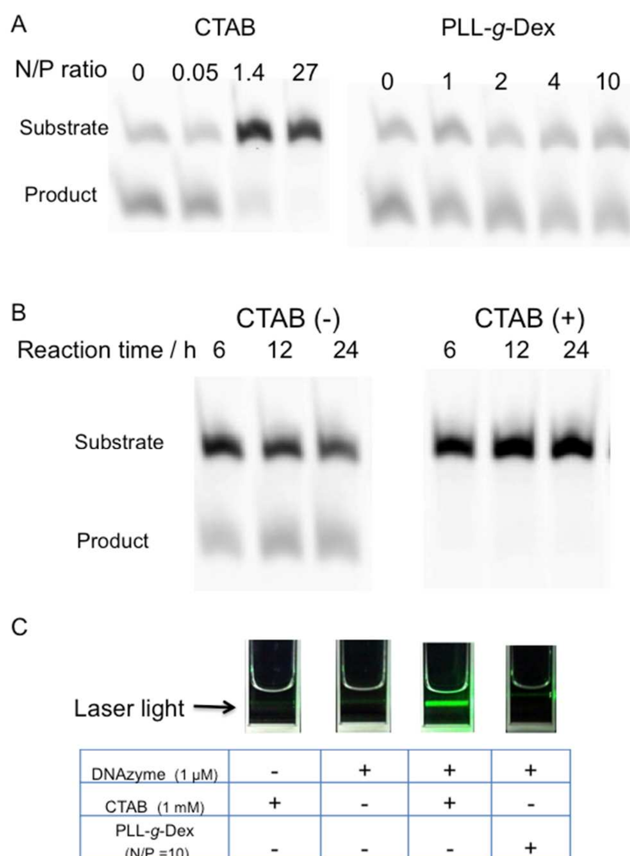

Fig. 3. (A) Comparison of PLL-*g*-Dex and CTAB on DNAzyme reactions under single-turnover reaction condition (20 min reaction at 25 ºC, 0.5 mM $MgSO_4$, 0.5 μM Substrate II, and 0.75 μM DNAzyme). (B) Influence of CTAB (N/P = 24, 1 mM) on multiple-turnover DNAzyme reaction. (reaction at 25ºC, 0.5 mM $MgSO_4$, 0.5 μM Substrate II, and 0.017 μM DNAzyme). (C) Solubility of DNA complex with either CTAB or PLL-*g*-Dex.

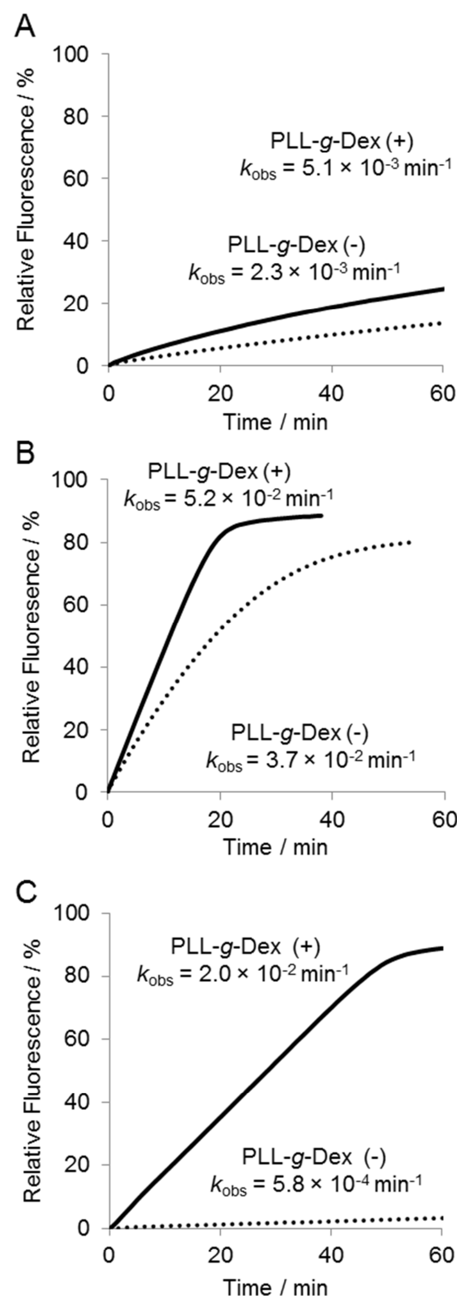

Fig. 4. Temperature dependence of DNAzyme multiple-turnover reactions in 5 mM Mg^{2+} with or without PLL-*g*-Dex. Samples containing 200 nM substrate I and 6.7 nM DNAzyme were incubated in the absence or presence of PLL-*g*-Dex (N/P = 2) at (A) 25 ºC, (B) 50 ºC, and (C) 60 ºC.

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It should be noted that the reaction in the presence of the copolymer at 50ºC or higher obeyed the zero-order reaction kinetics (Fig. 4B and C). This result indicated that the reaction rate was limited by the chemical cleavage process and not by turnover process involving product release and substrate binding. This supports our hypothesis that the copolymer facilitates the turnover process. Moreover, this observation suggested that further acceleration of the DNAzyme reaction should be possible by replacing Mg^{2+} with a more active metal ion.

The multiple-turnover reaction accelerated in the presence of Mn^{2+} and copolymer

Various divalent metal ions have been evaluated as cofactors of the 10-23 DNAzyme. Among the metal ions investigated, Mn^{2+} is one of the most active cofactors [21]. Hence, we carried out the reaction with Mn^{2+} either in the absence or presence of the copolymer (Fig. 5). At temperatures below 30ºC only slight differences were observed in DNAzyme activity in Mn^{2+} and Mg^{2+} regardless of the absence or presence of the copolymer (Fig. 4A vs. Fig. 5A, Fig. 5D). This is reasonable because under 30ºC the rate-determining step is not the chemical cleavage process but the turnover process. At higher temperatures a more significant enhancement by Mn^{2+} was observed in the presence of the copolymer than the absence. The DNAzyme activity at 50ºC was increased nearly 60 fold by replacing Mg^{2+} for Mn^{2+} in the presence of the copolymer, whereas it was increased only 10 fold in the absence of the copolymer. As described above, the DNAzyme reaction with the copolymer in Mg^{2+} at higher temperature was limited by the chemical cleavage step; Mn^{2+} ion effectively increases the overall reaction rate by facilitating this step. At 60–65ºC the DNAzyme was two orders of magnitude more active in the presence of copolymer than without. The observed rate constants were higher over the range of temperature from 35ºC to 65ºC in the presence than in the absence of copolymer (Fig. 5D). To confirm the role of the copolymer in the 10-23

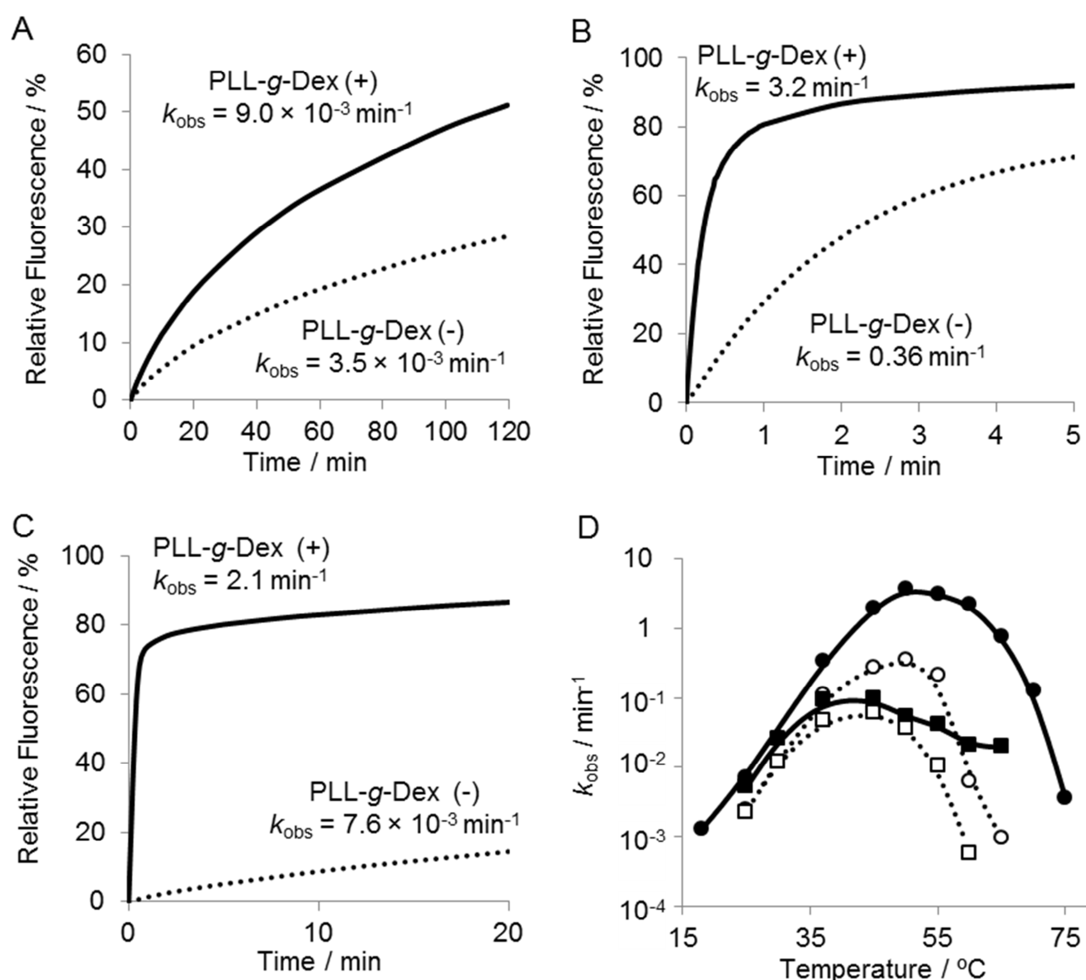

Fig. 5. Temperature dependence of DNAzyme multiple-turnover reactions at 5 mM Mn^{2+} with or without PLL-*g*-Dex. Samples containing 200 nM substrate I and 6.7 nM DNAzyme were incubated in the absence or presence of PLL-*g*-Dex (N/P = 2) at (A) 25 ºC, (B) 50 ºC, and (C) 60 ºC. (D) Temperature dependence of rate constants, k_{obs}, estimated in 5 mM Mg^{2+} (square) and in Mn^{2+} (circle) in the absence (dotted lines) and presence (solid lines) of copolymer.

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Table 1 Effect of PLL-*g*-Dex[a] to DNAzyme catalytic activity at 5 mM Mn^{2+}.

Temperature	PLL-*g*-Dex	V_{max} (nM·min^{-1})	k_{cat} (min^{-1})	K_M (M)	k_{cat}/K_M (M^{-1}·min^{-1})
37ºC	-	6.6×10^1	6.6	1.5×10^{-7}	4.4×10^7
	+	5.8×10^{-1}	5.8	4.5×10^{-9}	1.3×10^9
50ºC	-	1.0×10^2	5.2×10	8.5×10^{-7}	6.1×10^7
	+	1.5×10^2	7.8×10	2.4×10^{-8}	3.2×10^9

a) N/P = 2

DNAzyme reactions, the catalytic parameters were determined under multiple-turn over conditions and are summarized in Table 1. The copolymer did not affect k_{cat} but considerably decreased K_m. Hence, the copolymer did not affect the chemical cleavage step but facilitated ES complex formation. As a result the copolymer increased k_{cat}/K_m, a measure of the overall enzymatic activity, by 50 fold at 50ºC.

Influence of the copolymer on ES complex

To determine the effect of the copolymer on stability of the ES complex UV melting experiments were performed in the absence of divalent metal ions. As shown in Fig. 6, the T_m of the complex was 47ºC in the absence of the copolymer. The T_m was increased to 60ºC in the presence of the copolymer, indicating that the copolymer stabilized the ES complex. The effect of the copolymer on ES complex was also evaluated by circular dichroismic (CD) spectroscopy using substrates in which ribonucleotides were replaced with deoxyribonucleotides to prevent cleavage during the CD measurement. As shown in Fig. 7A, the CD profile characteristic of B-DNA with 240-nm negative and 260-nm positive bands was obtained for a mixture of DNAzyme and substrate. A slight difference in CD signal was observed with Mn^{2+}. The copolymer did not change the CD profiles regardless of the presence of Mn^{2+}. Though CD spectroscopy is not sensitive enough for us to be able to discuss local structures of the complex, the result implied that

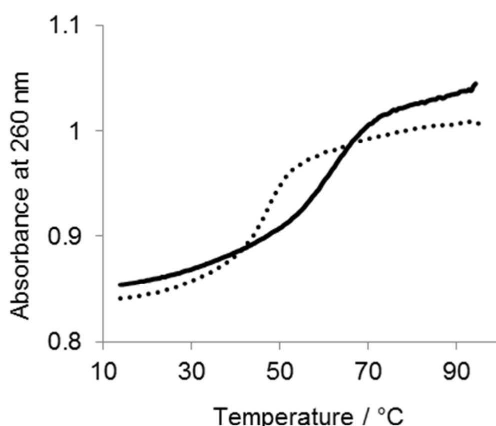

Fig. 6. UV melting profile of DNAzyme/substrate complex in the presence or absence of PLL-*g*-Dex copolymer. Samples contained 1.5 μM substrate I and 1.5 μM DNAzyme in the absence (dotted line) or presence (solid line, N/P = 2) of PLL-*g*-Dex in 50 mM HEPES (pH = 7.3), 150 mM NaCl. Temperature was increased 1 K/min, and spectra were recorded on a V-630 spectrophotometer (Jasco).

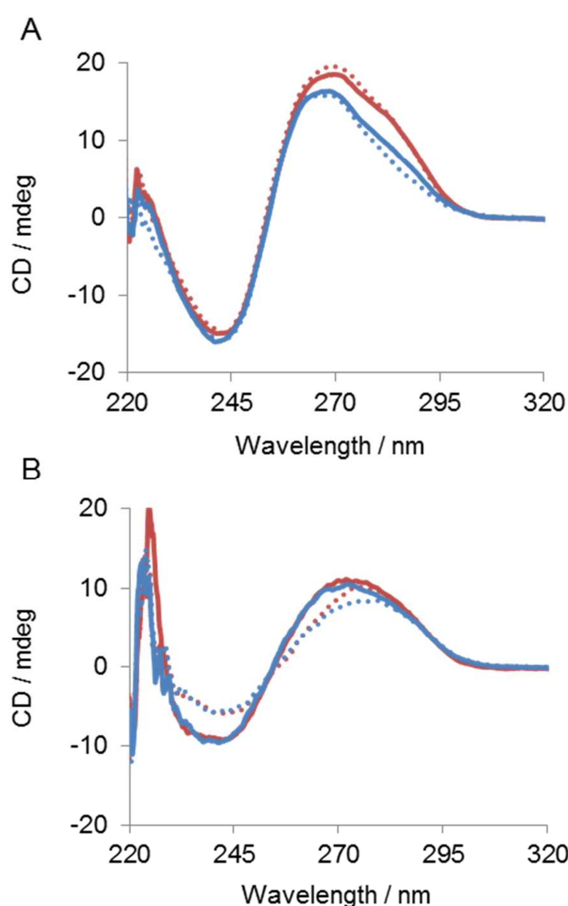

Fig. 7. CD spectra of complexes between DNAzyme and DNA-substituted substrate (ribo gu nucleotides of the substrate were replaced with deoxy GT nucleotides to avoid cleavage) at (A) 25 ºC and (B) 65 ºC in the absence or presence of divalent ion and PLL-*g*-Dex. Samples contained DNAzyme (2.5 μM) was mixed with DNA substrate (2.5 μM) in 50 mM HEPES (pH = 7.3), 150 mM NaCl in the absence (dotted line) or presence (N/P = 2, solid line) of PLL-*g*-Dex and in the absence (blue line) or presence (red line) of 5 mM Mn^{2+}.

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interactions of the copolymer did not significantly influence the conformation of the ES complex. Also, the copolymer did not influence Mn^{2+}-induced conformational change of the complex, indicative of inertness of the copolymer to Mn^{2+}/DNA interactions. The stabilizing effect of the copolymer on the ES complex was also shown by this CD analysis. At 65ºC the ES complex was dissociated in the absence of the copolymer but was stable in the presence of the copolymer (Fig. 7B).

The dependence of the DNAzyme reaction on the concentration of the copolymer was estimated in the presence of Mn^{2+} at 50°C. As shown in Fig. 8, the cleavage rate increased with an increase in polymer concentration until a plateau at an N/P ratio of about 0.7. This indicates that an electrostatically equivalent amount of the copolymer was sufficient for maximum rate enhancement.

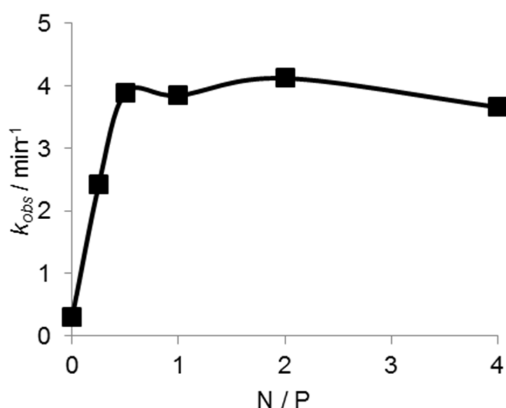

Fig. 8. PLL-*g*-Dex concentration dependence of DNAzyme multiple-turnover reaction rate. Reactions were performed at 50 ºC, 5 mM Mn^{2+}, 6.7 nM DNAzyme, and 200 nM substrate I with various concentrations of PLL-*g*-Dex.

Discussion

We have shown that cationic comb-type copolymers accelerate DNA hybridization and stabilize DNA structures through inter-polyelectrolyte complex formation. In this study we examined how PLL-*g*-Dex influenced ribonuclease activity of the 10-23 DNAzyme. As shown in Fig. 2B, the copolymer did not influence the DNAzyme activity under single-turnover conditions. This result indicated that association with the copolymer did not affect chemical cleavage activity of the DNAzyme. This was also supported by the fact that the copolymer did not influence k_{cat} (Table 1). The association of the copolymer did not alter the shape of the CD spectrum of the ES complex suggesting that the copolymer does not significantly alter the secondary structure of the ES complex (Fig. 7). These results also implied that the association of polycationic copolymer did not markedly influence interaction of a divalent metal ion to the ES complex. This inertness of the copolymer to the interaction between divalent metal ion and DNA is interesting. We previously observed that the copolymer did not interfered with K^+-dependent DNA quadruplex formation [42].

Under multiple-turnover reaction condition the copolymer enhanced activity of the DNAzyme (Fig. 2 and Fig. 4). The enhancement was not large when Mg^{2+} was used as a cofactor. The zero-order reaction kinetics observed in the presence of the copolymer (Fig. 4B and 4C) implied that the rate-determining step was shifted to the chemical cleavage step by the presence of the copolymer. The result clearly revealed that the copolymer increased turnover rate. Indeed, in the presence of Mn^{2+}, which results in higher cleavage activity than Mg^{2+}, the copolymer more significantly enhanced the DNAzyme activity (Fig. 3 and 4).

In addition to the increase in the DNAzyme reaction rate, the copolymer also increased the effective temperature range of the DNAzyme reaction. In the absence of the copolymer, the DNAzyme activity began to decrease at 55°C and the enzyme was almost completely inactive at 60°C (Fig. 5D). In the presence of the copolymer, substantial activity was retained up to 60°C. At 60°C the copolymer accelerated the reaction by 250 fold relative to the reaction in the absence of copolymer (Fig. 5C) by stabilizing the ES complex. At the optimal temperature, 50°C, for the DNAzyme in the absence of the copolymer, the copolymer increased $k$cat/Km value by 50 times (Table 1). The increase in $k$cat/Km value was largely caused by the decrease in Km value by the presence of the copolymer. The copolymer facilitated association of DNAzyme to its substrate.

Interestingly the copolymer enhanced the DNAzyme reaction even at lower temperatures, 20–27°C, where product release is the rate-determining step, though the magnitude of the enhancement was not as large as at 50°C. As previously reported, the copolymer accelerates a strand exchange reaction between DNA duplex and its complementary single strand [33, 34]. The copolymer likely facilitates exchange of product with substrate on the DNAzyme by a similar mechanism. This enhancement of DNAzyme activity was also observed as decrease in Km value in Michaelis-Menten parameters (Table 1). Michaelis-Menten kinetics assumes that the enzyme is regenerated soon after the product is produced. But this is not the case because scission products are bound to binding arms and inhibit the reaction. Accelerated turnover can be evaluated as increase in dissociation constant Ki of the enzyme-inhibitor complex. According to this inhibition model, $k$cat does not change but apparent Km decreases, suggesting that the copolymer increased the turnover efficacy.

As shown in Fig. 8, an electrostatically equivalent amount (N/P = 1) of the copolymer was sufficient to produce the maximum efficacy. The copolymer at N/P = 2 increased k_{cat}/K_m values by 50 fold compared to the reaction in the absence of copolymer (Table 1). The copolymer concentration was only 150 nM (in strand) (or 8.9 μM in ionic unit) at this N/P ratio, suggesting stable association of the copolymer to DNA. Regardless of the strong association of the copolymer, inertness of the copolymer to DNAzyme conformation and its interaction with a divalent cation should be noted. Abundant dextran grafts of the copolymer play a pivotal role on this strong but gentle interaction. The copolymer may be used to refine DNAzyme-based biomedical and nanomechanical devices.

Conclusions

The cationic comb-type copolymer, PLL-*g*-Dex, was shown to enhance the DNAzyme cleavage reaction. The copolymer accelerated the turnover process without affecting the chemical cleavage step and increased the active temperature window of DNAzyme reaction.

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Notes and References

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†Electronic Supplementary Information (ESI) available: % Cleavage vs. reaction time plots for Fig. 2B. See DOI: 10.1039/c000000x/

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