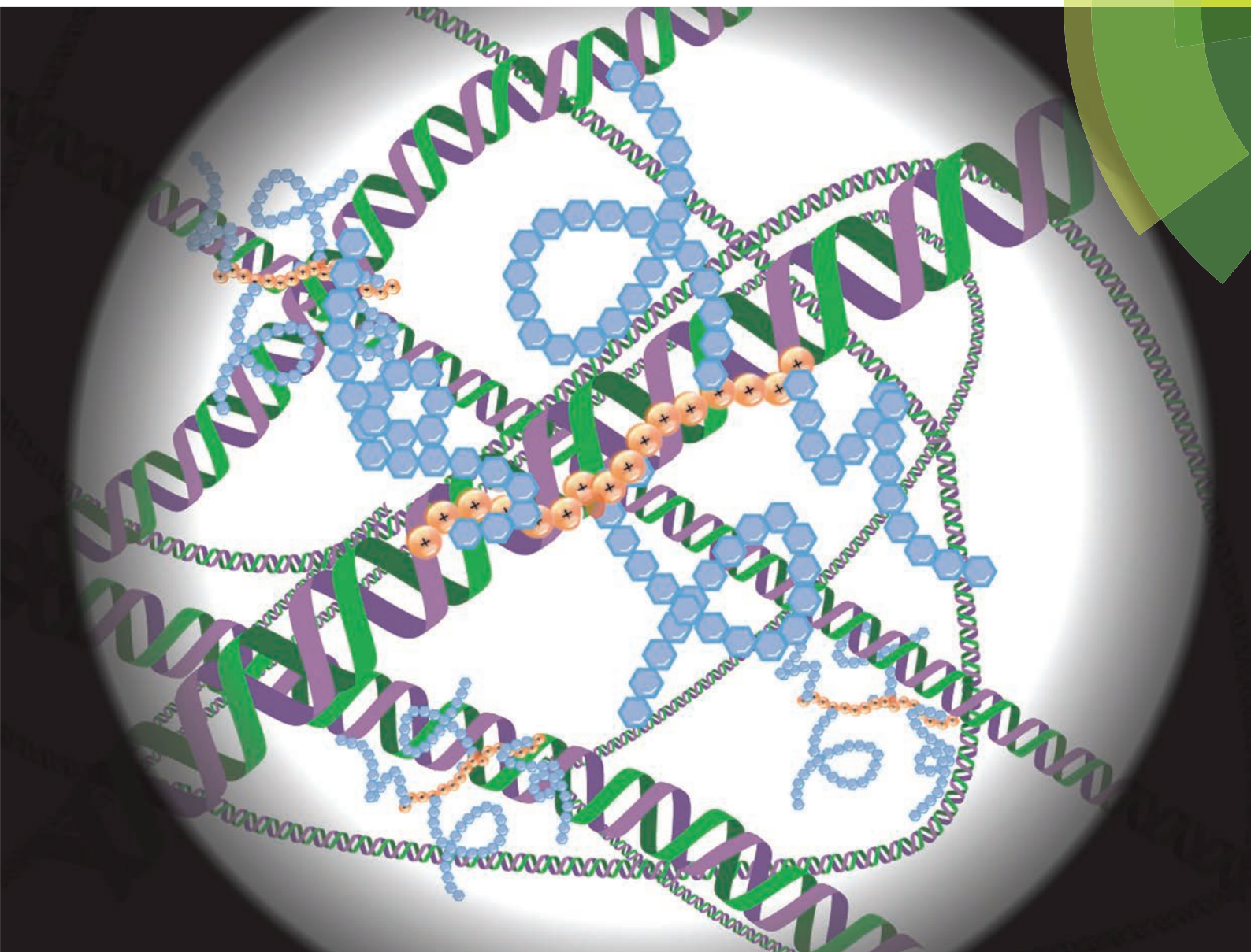


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# MNAzyme-catalyzed nucleic acid detection enhanced by a cationic copolymer†

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Multi-component nucleotide acid enzymes (MNAzymes) derived from RNase-mimic DNAzymes have potential as simple and accurate DNA detectors. To enhance the MNAzyme activity under multiple-turnover conditions, a cationic comb-type copolymer, PLL-*g*-Dex, that facilitates hybridization and strand exchange reactions of DNA was utilized. The copolymer increased the MNAzyme reaction rate by 200 times, allowing target DNA detection at picomolar concentrations at physiological temperature.

## Introduction

The emergence of life-threatening infections such as severe acute respiratory syndrome (SARS) and viral haemorrhagic fevers caused by the Ebola and Marburg viruses highlights the urgent need for efficient infection control practices. High-speed, accurate, and simple methods to detect pathogens that can be used in field hospitals are required. Such methods are also important for establishing point-of-care diagnostic capabilities. Isothermal amplification methods,<sup>1,2</sup> such as rolling circle amplification (RCA),<sup>3,4</sup> loop-mediated isothermal amplification (LAMP),<sup>5</sup> and helicase-dependent amplification (HAD),<sup>6</sup> to detect pathogen genetic material or disease markers have advantages over current PCR-based methods because of their simple protocols and because they could be conducted at ambient temperature. However, these isothermal amplification methods still have complicated setups and require multiple enzymes and/or primers.

Multi-component nucleic acid enzyme (MNAzyme)-based assays<sup>7,8</sup> are another type of isothermal, signal amplifying DNA-detection method. MNAzymes<sup>9,10</sup> are composed of short oligonucleotides that function as partial enzymes or partzymes. Each partzyme includes a region of a catalytic core, a substrate-binding arm, and a sensor arm. The sensor arms recognize and bind to a target nucleic acid. When the part-

zymes are assembled in the presence of a target nucleic acid, they form an active MNAzyme assembly that catalytically produces signals. The signal results from substrate cleavage, generally upon release of a fluorophore from proximity to a quencher. As multiple signals can be generated from a single target molecule, isothermal signal amplification is possible. The MNAzyme integrated with various detection methods has been widely investigated.<sup>11–16</sup>

We previously reported that a cationic comb-type copolymer consisting of a polycationic backbone and hydrophilic graft chains of dextran (Fig. 1A) promoted hybridization of a pair of complementary DNAs.<sup>17–19</sup> The copolymer also facilitates the strand exchange reaction between a double-stranded DNA and homologous single-stranded DNA.<sup>20–22</sup> Recently, we showed that the copolymer considerably enhanced ribonuclease activity of the previously described 10–23 DNAzymes.<sup>23</sup> The copolymer facilitated turnover of the DNAzyme and stabilized the DNAzyme over a wide range of temperature.<sup>23</sup>

Here, we show that the cationic copolymer also enhances activity of an MNAzyme derived from the 10–23 DNAzyme and increased MNAzyme sensitivity. Furthermore, the copolymer enabled us to shorten the substrate-binding arms of the MNAzyme, decreasing the optimum temperature of the MNA assay from 50 °C to physiological temperature.

## Experiment

### Materials

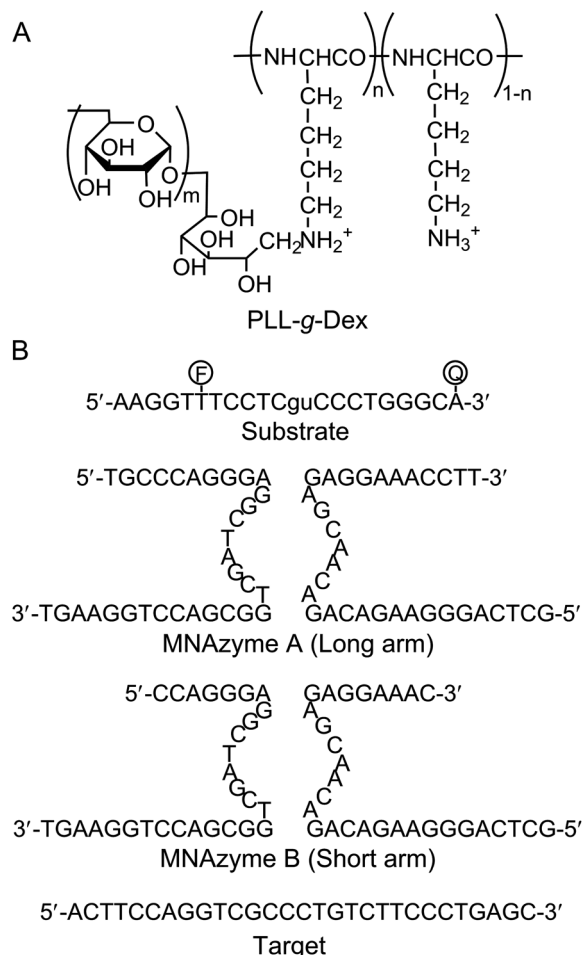
Poly(L-lysine hydrobromide) (PLL-HBr,  $M_w = 7.5 \times 10^3$ ) was obtained from Sigma-Aldrich Co. LLC (St Louis, MO, USA), and dextran (Dex,  $M_w = 8 \times 10^3$ – $1.2 \times 10^4$ ) was obtained from Funakoshi Co. (Tokyo, Japan). Sodium hydroxide, sodium chloride, magnesium sulfate, and manganese(II) chloride tetrahydrate were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and ethylene diamine tetraacetic acid

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†Electronic supplementary information (ESI) available: Comparison of effect of cofactors Mn and Mg ions to the MNAzyme reaction in the absence of PLL-*g*-Dex. Sequences and multiple-turnover reaction rates for the parent DNAzymes, Fig. 5B plotted with the reduced y-axis, and predicted melting temperatures ( $T_m$ ) of duplexes having sequences of substrate- or target-binding domains of MNAzymes A and B. MNAzyme LoD determination. See DOI: 10.1039/c4bm00449c





**Fig. 1** (A) Structural formula of the cationic comb-type copolymer PLL-g-Dex, (B) nucleotide sequences of the substrate and MNAzymes used in this study. The substrate was labeled with a fluorophore (F, Fluorescein) and a quencher (Q, BHQ-1). In the MNAzymes, the upper portions bind the substrate and the lower portions are complementary to the target DNA.

tetrasodium salt (EDTA-4Na) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). HPLC-grade oligonucleotides with sequences shown in Fig. 1 were purchased from Fasmac Co., Ltd (Atsugi, Japan) and used without further purification. A cationic comb-type copolymer poly(L-lysine)-*graft*-dextran (PLL-g-Dex) was prepared according to the previously published procedure.<sup>18</sup> 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 <sup>1</sup>H NMR and GPC. PLL-g-Dex of 90 wt% dextran (11.5 mol% of lysine units of PLL were modified with dextran) was used in this study.

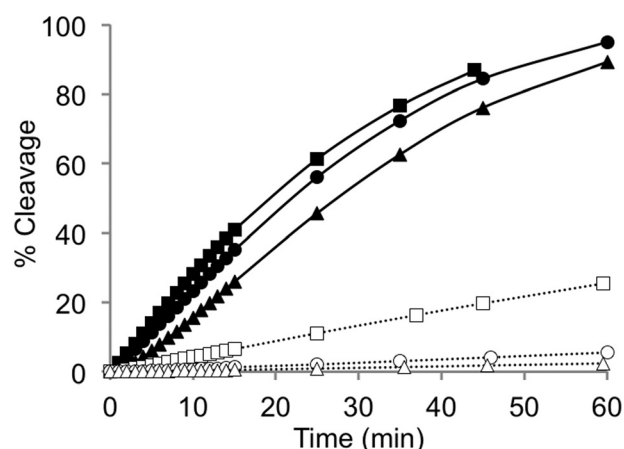
## Methods

For FRET analyses, substrate (final concentration: 200 nM) and MNAzyme parts (final concentrations: 200 nM, 20 nM, 6.7 nM, or 2 nM) were dissolved in a reaction buffer, 50 mM

HEPES (pH 7.3), 150 mM NaCl, 25 mM Mg<sup>2+</sup> or 5 mM Mn<sup>2+</sup>. The mixture of substrate and MNAzyme parts was pre-incubated with or without PLL-g-Dex at reaction temperature for 15 min in a quartz cell. The ratio of positively charged amino groups of the copolymer to negatively charged phosphate groups of DNA (N/P ratio) was 2. After pre-incubation, 20  $\mu$ L target solution (final concentrations: 2 pM–50 nM) was injected into the cell (final volume of reaction solution was 1800  $\mu$ L) to initiate the reaction. The fluorescence intensity was measured using a FP-6500 spectrofluorometer (Jasco, Tokyo, Japan) at an excitation wavelength,  $\lambda_{ex}$ , of 494 nm and an emission wavelength,  $\lambda_{em}$ , of 520 nm. The percent cleavage ( $P\%$ ) as a function of time was obtained from the equation  $P\% = (I_t - I_0)/(I_\infty - I_0)$ , where  $I_t$  is the fluorescence intensity at any reaction time  $t$ ,  $I_\infty$  is the fluorescence intensity of the synthesized product, and  $I_0$  is the initial fluorescence intensity (background). The values of  $k_{obs}$  were obtained by fitting the reaction curve with the equation  $I_t = I_0 + (I_\infty - I_0)(1 - e^{-k_{obs}t})$ .

## Results and discussion

Firstly, we examined MNAzyme A, which has longer substrate-binding arms than MNAzyme B (Fig. 1B), activity. In initial experiments, the substrate and partzyme concentrations were 200 nM and the target was present at 2 nM. Buffer contained 25 mM Mg<sup>2+</sup>. Considerably higher cleavage activity was observed in the presence than in the absence of the PLL-g-Dex (Fig. 2), suggesting that the copolymer promoted turnover of the target. We then decreased the MNAzyme concentration while holding initial substrate and target concentrations constant. At lower MNAzyme concentrations we observed a significant decrease in MNAzyme activity in the absence of the copolymer, but only a slight decrease in the activity was



**Fig. 2** MNAzyme A reactions at different MNAzyme concentrations in the presence (filled symbols and solid lines) and absence (open symbols and dotted lines) of PLL-g-Dex. Squares indicate 200 nM MNAzyme; circles indicate 20 nM; triangles indicate 2 nM. Reactions were performed at 50 °C in 25 mM MgSO<sub>4</sub> with 200 nM substrate and 2 nM target.





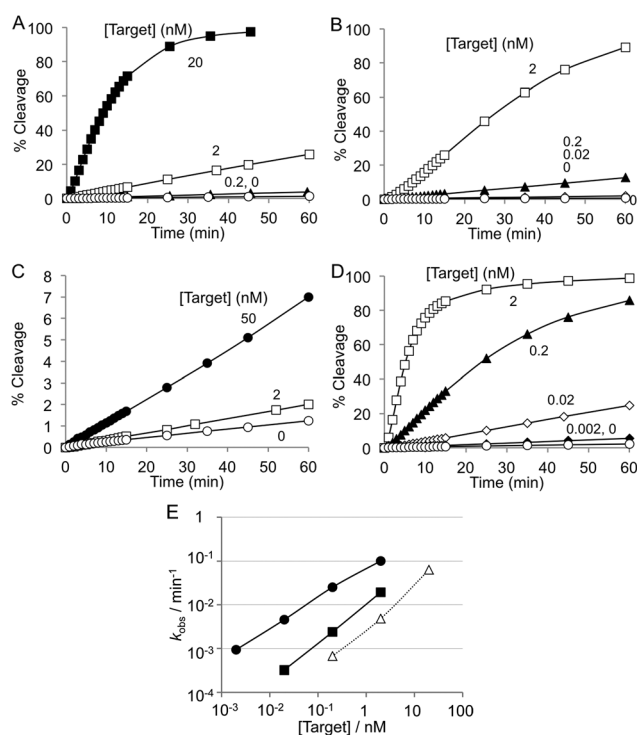
observed in the presence of a copolymer even at 2 nM MNzyme concentration (Fig. 2). This result indicated that the copolymer promoted target-induced assembly of an active MNzyme complex at low MNzyme concentrations. At 2 nM of each of the partzymes, the copolymer increased the cleavage rate by 50 fold relative to cleavage in the absence of copolymer.

To estimate the target sensitivity of the MNzyme, the target concentration dependence of the MNzyme reaction was investigated. Fig. 3A shows target concentration dependence observed at substrate and MNzyme concentrations of 200 nM in the absence of PLL-g-Dex. Under these conditions, the sensitivity was 2 orders of magnitude lower than that described previously,<sup>8</sup> presumably owing to difference in length of the sensor arms that significantly influenced the stability of MNzyme assembly. The sensor arms of MNzyme in this study were 14 and 15 nucleotides (nt) whereas those in the previous study were 20 and 21 nt. In the presence of PLL-g-Dex, the sensitivity was enhanced approximately 5 times at an MNzyme concentration of 2 nM (Fig. 3B), indicating that the copolymer significantly improved the MNzyme reactivity under multiple-turnover conditions. This suggests that the use of the copolymer could enable use of the MNzyme assay for

detection of short nucleotide targets such as miRNA and siRNA.

In order to improve the MNzyme sensitivity further, we investigated the effect of the metal ion cofactors. MNzyme activity did not significantly change in 25 mM  $Mg^{2+}$  and 5 mM  $Mn^{2+}$  in the absence of the copolymer (Fig. S1†). Considerably higher MNzyme activity was observed in 5 mM  $Mn^{2+}$  than in 25 mM  $Mg^{2+}$  when the copolymer was present (Fig. 3B and D). This is owing to the difference in the rate determining step of the MNzyme reaction. While the rate determining step in the absence of the copolymer was the turnover process it was the chemical cleavage step in the presence of the copolymer.<sup>23</sup> The target concentration dependences in the presence of 5 mM  $Mn^{2+}$  were determined in the presence and absence of PLL-g-Dex. The MNzyme can detect the target at the nanomolar level without the copolymer (Fig. 3C), and it was at the picomolar level in the presence of PLL-g-Dex (Fig. 3D). As the reactions were initiated by the addition of the target to a mixture of MNzymes and substrate, there was an initial lag during which the MNzyme assembled. This lag was obvious in the absence of PLL-g-Dex (Fig. 3C) but was not observed in the presence (Fig. 3D), indicative of significant acceleration of MNzyme assembly due to PLL-g-Dex. Fig. 3E summarizes the target concentration dependences of  $k_{obs}$ . Compared with the sensitivity without the copolymer at 200 nM MNzyme, that with the copolymer was improved more than two orders of magnitude. The sensitivity was in the pM range even at a 2 nM MNzyme concentration when the copolymer was added to the reaction at an N/P of 2.

We previously reported that PLL-g-Dex enlarged the temperature window in which a DNzyme was active.<sup>23</sup> The requirement that temperature be cycled limits utility of the PCR-based assay in non-laboratory settings. If MNzyme reactions are active over a wide range of temperature, these systems could be adapted to formats such as chromatography, nanoparticle- and microfluidics-based detection. We analyzed the temperature dependence of the multiple-turnover MNzyme reaction at 200 nM substrate, 6.7 nM MNzyme, and 2 nM target in a buffer containing  $Mn^{2+}$  in the absence and presence of the copolymer. As shown in Fig. 4, the optimum temperature of the MNzyme reactions was increased by the copolymer. In the presence of the copolymer,  $k_{obs}$  values were higher than  $10^{-2} \text{ min}^{-1}$  over the temperature range from 37 °C to 63 °C, whereas  $k_{obs}$  was higher than  $10^{-3} \text{ min}^{-1}$  only between 37 °C and 55 °C without the copolymer. At 37 °C in the presence of the copolymer MNzyme activity was higher than that obtained at the optimum temperature of 50 °C without the copolymer. At 50 °C the copolymer facilitated the reaction by approximately 100 fold. To further decrease the working temperature range of the MNzyme we constructed MNzyme B in which the substrate-binding arms were each truncated by 3 nt relative to the arms in MNzyme A (Fig. 1B). As shown in Fig. 4, the optimal temperature for the reaction was lowered about 12 °C by the truncation. The truncation resulted in an extreme decrease in the MNzyme activity in the absence of the copolymer. In the presence of



**Fig. 3** Target concentration dependence of the MNzyme reactions at 50 °C and 200 nM initial substrate concentration under the following conditions: (A) 200 nM MNzyme and 25 mM  $MgSO_4$  in the absence of PLL-g-Dex, (B) 2 nM MNzyme and 25 mM  $MgSO_4$  in the presence of PLL-g-Dex (N/P = 2), and 2 nM MNzyme and 5 mM  $MnCl_2$  in the (C) absence and (D) presence of PLL-g-Dex (N/P = 2). (E) Dependence of the MNzyme reaction rate,  $k_{obs}$ , on the target concentration obtained under the conditions for (A) triangle, (B) square, and (D) circle.



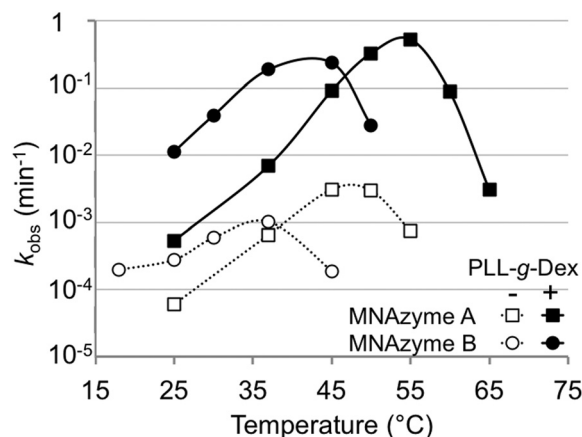


Fig. 4 Temperature dependences of multiple-turnover reactions with MNazyme A (square) and MNazyme B (circle) at 200 nM substrate, 6.7 nM MNazyme, 6.7 nM target, 5 mM  $\text{MnCl}_2$ , in the absence (open) or presence (solid, N/P = 2) of PLL-g-Dex.

PLL-g-Dex, however, the MNazyme was active with  $k_{\text{obs}} > 10^{-1}$  even at 37 °C.

The reactivity of MNazymes was compared with that of their parent DNazymes by analysis of the DNazyme reactions at 200 nM substrate and 6.7 nM DNazyme (Fig. S2†). The copolymer promoted multiple-turnover of the DNazyme with substrate-binding arms comparable to those of MNazyme A by approximately 10 fold, as reported previously.<sup>23</sup> Interestingly, the effect of the copolymer was more pronounced with the DNazyme with shorter substrate-binding arms. The 3 nt truncation considerably decreased stability of the DNazyme/substrate (ES) complex as estimated by prediction of the melting temperature (Table S1†). The fact that the copolymer enhanced the efficiency of the less stable complex is interesting to note. The truncation of the substrate-binding arms should increase the dissociation rate of the DNazyme/product complex, whereas PLL-g-Dex is expected to facilitate association of the substrate with the DNazyme. These effects likely cooperated to facilitate the multiple-turnover reactions of the DNazymes and MNazymes with the short substrate-binding arms.

Finally, the target dependence of the MNazyme B was evaluated at 37 °C with or without PLL-g-Dex under multiple-turnover conditions. As shown in Fig. 5, it took 60 min to cleave

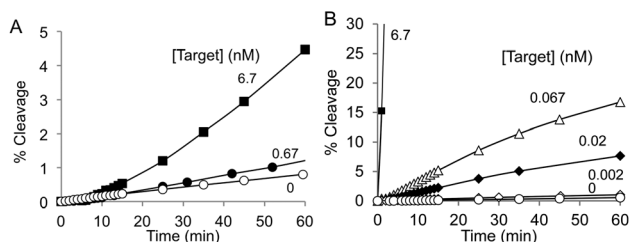


Fig. 5 Target concentration dependence of MNazyme B reactions in (A) the absence and (B) the presence (N/P = 2) of PLL-g-Dex at 37 °C in 5 mM  $\text{MnCl}_2$  at 200 nM substrate and 6.7 nM MNazyme B.

approximately 5% of the substrate in the absence of PLL-g-Dex, but it took less than a minute in the presence of PLL-g-Dex. The target was detected at picomolar concentrations in the presence of the copolymer at 37 °C. The limits of detection (LoD) of MNazyme with and without PLL-g-Dex under these physiological conditions were determined (Table S2, Fig. S3†). The copolymer improved the LoD of MNazyme about 100 times relative to the LoD under the same conditions but without PLL-g-Dex.

## Conclusions

MNazyme activity was significantly reduced under multiple-turnover conditions. In the presence of the cationic copolymer PLL-g-Dex, activity was significantly enhanced. The copolymer likely facilitated target-induced assembly of an active MNazyme complex. Notably, with the copolymer and the short-armed MNazyme DNA was detected at picomolar concentrations at physiological temperature. Although further improvement in the sensitivity will be required to enable utility in point-of-care diagnostic devices, use of the cationic copolymer will facilitate construction of simple and accurate assays using MNazymes.

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