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Encapsulation in cooling-induced coacervates augments DNA enzyme activity†

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When two types of polymers are mixed in aqueous media, liquid droplets resulting from liquid–liquid phase separation are formed. These complex coacervates can entrap biomolecules including protein enzymes. Nucleic acid enzymes entrapped in coacervates have altered activities relative to those in dilute solution. We previously reported that the ureido polymer alone can form a simple coacervate that dissolves upon heating and reforms upon cooling. In this study, we examined the effect of entrapment of a DNA enzyme, the 10–23 DNAzyme, in the simple coacervate induced by cooling of a ureido polymer functionalized with amino groups, poly(allylamine-*co*-allylurea) (PAU). The copolymer formed coacervate droplets containing DNAzyme and its substrate upon cooling. The activity of the DNAzyme in the droplets was markedly enhanced compared to the reaction in the absence of polymer due to a significant decrease in K_m, implying that entrapment promotes formation of the enzyme–substrate complex. Thus, cooling-induced liquid droplets formed by the PAU are efficient reaction media for DNAzymes.

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Introduction

Under certain conditions, polymer solutions undergo liquid–liquid phase separation (LLPS) into two liquid phases, a polymer-poor phase and a polymer-rich phase. The latter is termed a coacervate. Coacervates formed by one type of polymer are called simple coacervates, whereas those formed by two or more types of polymers are called complex coacervates. Polymers that form simple coacervates are less common than those that form complex coacervates. Biomolecules can be entrapped in the coacervates, and previous work has shown that enzyme activities are altered by entrapment due to the crowding environment inside the coacervate.[1–3] For example, the activity of horseradish peroxidase is decreased 3 fold by entrapment in the complex coacervate formed by a positively charged amylose and negatively charged amylose.[4] The enzymatic activities of ribozymes and DNAzymes, which are nucleic acids that have enzymatic activity,[5] are also altered when these enzymes are trapped in complex coacervates.[6,7] For example, activities are enhanced in the complex coacervate composed of poly(diallyldimethylammonium chloride) and oligoaspartic acid.[8] Thus, the microenvironment of complex coacervates influences activities of both protein and nucleic acid enzymes. The stabilities of complex coacervates depend on ionic strength and pH[9] as well as charge stoichiometry of the two polymers.[10] Practical applications using the complex coacervate require strategies such as chemical crosslinking to stabilize the coacervate.[11]

We previously reported that ureido polymers such as polyallylurea,[12–14] polyvinylurea,[15,16] and poly(2-ureidoethylmethacrylate)[17,18] exhibit LLPS over wide ranges of pH and salt concentrations including in physiologically relevant conditions. As the ureido polymers form simple coacervates, we reasoned that these coacervates would be useful in biological media. Moreover, LLPS properties of the ureido polymers are thermosensitive.[12–18] The coacervate droplets formed by ureido polymers dissolve upon heating, and the coacervates form again when cooled below the phase separation temperature (T_p); T_p is dependent on molecular weight and ureido content. Due to this unique upper critical solution temperature (UCST)-type thermoresponsive, ureido polymers can capture proteins in coacervates upon cooling[13] and can induce a switch between monolayer and spheroid cell culture upon a change in temperature.[19] Recently, we examined the effect of the simple coacervation on activities of protein enzymes.[20] β-galactosidase (β-gal), which is an anionic protein enzyme, was spontaneously entrapped in simple coacervates formed by the cationic ureido polymer, poly(allylamine-*co*-allyurea) (PAU; Fig. 1A) below T_p. Entrapment significantly decreased β-gal activity, and the activity was recovered by heating above T_p. Thus, cooling-inducible LLPS of ureido polymers enables regulation of protein enzyme activity.

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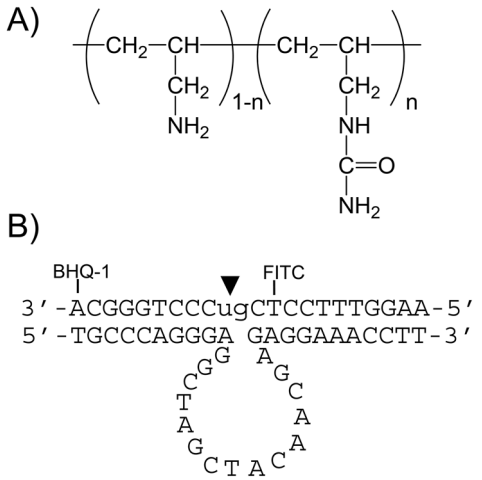

Fig. 1 (A) Chemical formula of PAU. (B) Sequences of 10–23 DNAzyme and substrate. Locations of fluorescent dye, FITC, and quencher, BHQ-1, on the substrate are indicated. Cleavage site on substrate is indicated by arrowhead.

In this study, we investigated the effect of the cooling-inducible simple coacervates of PAU with T_p values near 37 °C on the activity of the previously described 10–23 DNAzyme[21,22] (Fig. 1B), which cleaves RNA and which has been integrated into various DNA nanodevices such as metal sensors[23,24] and logic gates.[25] This investigation could lead development of temperature-responsive DNA nanodevices that operate under physiologically relevant conditions.

Experimental

Materials

HPLC-purified oligonucleotides were obtained from Fasmac. Sodium hydroxide, sodium chloride, Tween20, and manganese chloride were purchased from Wako Pure Chemical Industries. 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) was obtained from Nacalai Tesque. Poly(allylamine hydrochloride) (PAA·HCl, $M_w = 1.5 \times 10^4$, $M_w/M_n = 1.4$) was purchased from Nittobo. PAUs were prepared by post-modification of PAA·HCl as we previously reported.[12] In brief, various amounts of potassium cyanate were added to PAA·HCl dissolved in water and then incubated at 60 °C for 24 h. The resulting PAUs were purified by dialysis against water and then lyophilization. Ureido contents of the PAU were determined by ^{1}H NMR (Fig. S1†) as described previously.[12]

Thermoresponsive behaviour of PAUs

PAU was suspended in 50 mM HEPES-NaOH (pH 7.3) containing 150 mM NaCl and 0.1% Tween20 (Tween prevented adsorption of the DNA and the copolymer on the quartz cell surface) at a concentration of 1 mg mL^{-1} with or without 2 nM DNAzyme and 100 nM substrate. Transmittance at 500 nm of the solution in a quartz cell was measured on a V-630 UV-visible spectrophotometer (JASCO) equipped with a Peltier temperature controller. Transmittances were recorded from 70 to 10 °C at a cooling rate 1 °C min^{-1}. T_p was defined as the temperature at intersection between a line at 100% transmittance and a tangent line at an inflection point of the transmittance curve. The T_p values were reproducible with temperature differences less than 2 °C.

Entrapment of oligonucleotides into coacervates of PAU

PAU (1 mg mL^{-1}) was mixed with 5′ FITC-labelled DNAzyme (100 nM) or FITC-labelled substrate without quencher (100 nM) in 50 mM HEPES-NaOH(pH 7.3) containing 150 mM NaCl and 0.1% Tween20 at 35 °C for 10 min. Images were acquired on a spinning disk confocal microscope (IX71, Olympus) equipped with a scanner unit (CSU-X1, Yokogawa), an EMCCD camera (iXon 893, Andor), a dish heater (Petri Dish Heater, JPK Instruments), and an objective lens (100× UPlanS Apo NA 1.4, Olympus). For polyacrylamide gel electrophoresis (PAGE), the mixture was centrifuged at 4 °C, and supernatant was collected. A 5 μL aliquot of supernatant was mixed with 5 μL of loading buffer (80 vol% formamide, 10 vol% glycerol, 50 mM EDTA, 0.025 wt% bromophenol blue, and 0.6 wt% poly (sodium vinylsulfonate)) and then analysed on a 15% polyacrylamide gel containing 7 M urea. The gel was visualized with a Pharos FX™ Molecular Imager (BIO-RAD).

Estimation of enzymatic activity

DNAzyme (2 nM) and various concentration of substrate modified with FITC and BHQ-1 were mixed without or with 1 mg mL^{-1} PAU in 50 mM HEPES-NaOH (pH 7.3) containing 150 mM NaCl and 0.1% Tween20 at 35 °C or 55 °C, and $MnCl_2$ was added to a final concentration of 5 mM to initiate the reaction. Cleavage of the substrate by the DNAzyme separated the fluorescent donor from quencher resulting in fluorescence. The fluorescence (λ_{ex}: 450 nm, λ_{em}: 520 nm) was measured using an FP-6500 spectrophotometer (JASCO) equipped with a Peltier temperature controller. The percent substrate cleavage over time was obtained from the following equation:

$$\text{Reaction } (\%) = [(I_t - I_0)/(I_\infty - I_0)] \times 100. \quad (1)$$

where I_t, I_∞, and I_0 are fluorescence intensities at a given reaction time t, fluorescence intensity when the intensity plateaued, and initial fluorescence intensity, respectively. The observed rate constant (k_{obs}) was calculated by fitting the experimental data to the following equation:

$$I_t = I_0 + (I_\infty - I_0)\ (1 - e^{-k_{obs}t}). \quad (2)$$

Thermal melting of DNA duplex

The melting curves of the DNA duplex 5′-FITC-TCATAATC-3′/5′-GATTATGA-Dabcyl-3′ (100 nM) in the presence or the absence of PAU (1 mg mL^{-1}) in 50 mM HEPES-NaOH (pH 7.3) containing 150 mM NaCl were measured by monitoring of fluorescence (λ_{ex}: 450 nm, λ_{em}: 520 nm) as a function of temperature.

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Results and discussion

Entrapment of DNAzyme into coacervate of PAU

We prepared PAUs with molecular weight of 1.5×10^4 and 93–100 mol% ureido content because T_ps of polymers with these compositions were close to body temperatures (Table 1). Transmittance of a solution of the polymer with 93 mol% ureido and 7 mol% amino groups (PAU93) solution dropped precipitously at 41 °C, which was defined as T_p, and the addition of DNAzyme and substrate did not significantly affect the transmittance curve (Fig. 2). At 35 °C, a temperature below T_p, coacervate droplets were observed in the presence of DNA for PAU93 as well as PAU95, PAU97, and PAU100, which had 95 mol%, 97 mol%, and 100 mol% ureido content, respectively (Table 1 and Fig. S2†). In solutions of FITC-labelled DNAzyme with PAUs at 35 °C, coacervate droplets with green fluorescence were observed for PAU93 and PAU95 but not PAU97 and PAU100 (Fig. 3A). When compared to fluorescence intensity per unit area of the coacervate and solution without PAU, the intensity of the droplets was 16-fold higher than that in solution without PAU indicating that DNAzyme was considerably concentrated in the coacervate relative to the concentration in solution. Supernatants were collected by centrifugation and then analysed by PAGE for the presence of DNAzyme. A band corresponding to the DNAzyme was clearly observed for samples prepared with PAU97 and PAU100, but intensities of the bands corresponding to the DNA from supernatants of PAU93 and PAU95 samples were less than 5% of the intensity of control DNAzyme sample (Fig. 3B). Substrate DNA was also entrapped in droplets of PAU93 and PAU95 with high efficiency (Fig. S3†). These data indicate that entrapment of DNA in the droplets required electrostatic interactions as entrapment was only observed when the PAU with amino content was 5% or more (Fig. 3C).

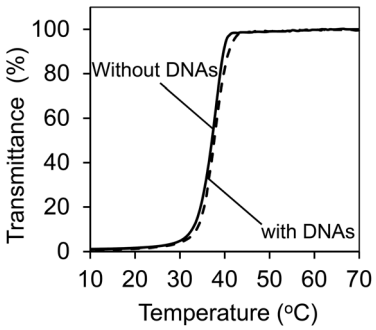

Fig. 2 Transmittance curves for 1 mg mL^{-1} PAU93 in the absence (solid line) or the presence of 2 nM DNAzyme and 100 nM substrate (dashed line) in 50 mM HEPES-NaOH (pH 7.3) containing 150 mM NaCl.

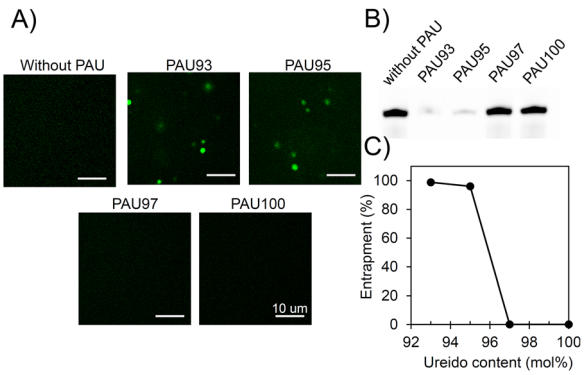

Fig. 3 (A) Microscopic images of 100 nM FITC-labelled DNAzyme in the presence of 1 mg mL^{-1} PAUs at 35 °C. Scale bars indicate 10 μm. (B) PAGE analysis of supernatants prepared from FITC-labelled DNAzyme alone or DNAzyme in the presence of PAUs. (C) Percent entrapped DNAzyme as a function of ureido content of PAU.

Activity of the DNAzyme in coacervates of PAU

The activity of the DNAzyme in the presence of PAU was evaluated under multiple-turnover conditions at 55 °C and at 35 °C (Fig. 4A and B). At 55 °C, where the polymer solution dissolved, the addition of PAUs did not affect the DNAzyme reaction profile significantly with the exception of PAU93, which enhanced k_{obs} by 2.5 fold (Fig. 4C). At 35 °C, the DNAzyme reaction in absence of PAU was slower than that at 55 °C; 55 °C is close to the optimal temperature of the DNAzyme reaction.[26] The presence of PAU93 significantly enhanced the reaction with k_{obs} 7.2 fold higher at 35 °C in the presence of PAU93 compared to its absence (Fig. 4C). PAU95 enhanced k_{obs} by 2.1 fold, although the difference was not significant, and PAU97 and PAU100 had no effect (Fig. 4C). Although both PAU93 and PAU95 coacervates encapsulated the DNAzyme and substrate, PAU93 enhanced DNAzyme activity more than PAU95. A previous report suggested that the enhancement of the DNAzyme reaction by highly cationic polymers with amino groups is due to polymer-mediated enhancement of DNAzyme/substrate complex formation and to acceleration of turn over.[26] PAU93, which has a higher amino content than PAU95 (Table 1), likely increases DNAzyme activity to a greater extent than PAU95 due to higher percentage of cationic amino groups. The k_{obs} for DNAzyme with PAU93 at 35 °C (3.6×10^{-3} s^{-1}) was similar to that for DNAzyme alone at 55 °C (4.3×10^{-3} s^{-1}). This indicated that cooling-inducible coacervates of PAU93 increased the activity of DNAzyme at near physiological temperature to close to that observed at its optimal temperature.

Table 1 T_ps of ureido polymers used in this study

Abbreviation	Ureido content (mol%)	Amino content (mol%)	T_p (°C) Without DNA	T_p (°C) With DNA
PAU93	93	7	41	42
PAU95	95	5	42	43
PAU97	97	3	43	42
PAU100	100	0	44	44

Kinetic parameters of DNAzyme in coacervates of PAU

To understand the observed enhancement of the DNAzyme activity by the cooling-induced coacervation of PAU93, enzy-

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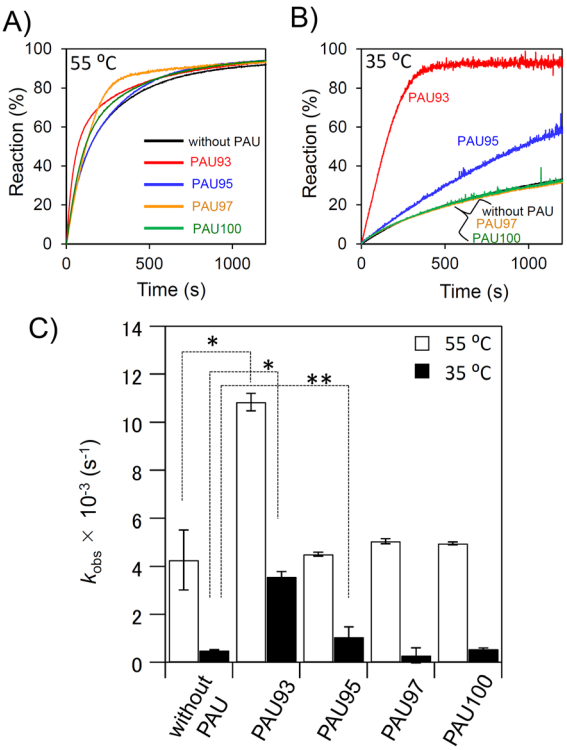

Fig. 4 (A and B) Reaction profiles of 2 nM DNAzyme with 100 nM substrate in the absence or the presence of 1 mg mL^{-1} PAUs at (A) 55 °C and (B) 35 °C. (C) Observed rate constants of the DNAzyme in the absence or the presence of PAUs at 55 °C and 35 °C. *P < 0.05, **P < 0.15.

matic kinetic parameters were determined (Fig. S4† and Table 2). At 55 °C and 35 °C, the addition of PAU93 decreased maximum velocity (V_{max}), which reflects how fast enzymes can catalyse the reaction, by 2.8 fold and 1.2 fold, respectively, compared to the V_{max} in the absence of PAU93. It is possible that PAU93 changes the conformation of the DNAzyme or hampers binding of the cofactor Mn^{2+}, resulting in the decrease in V_{max}. Values of Michaelis constant (K_m), an inverse measure of affinity of the enzyme for the substrate, were decreased by the addition of PAU93. We hypothesized that the decrease in K_m could result from stabilization of the duplex formed by the DNAzyme with its substrate by the cationic polymer; cationic polymers are known to stabilize DNA structures.[27] In support of this hypothesis, the melting temperature of an 8-base pair DNA duplex was increased by 20 °C by the addition of PAU93 and by 16 °C by addition of PAU95, although the other PAUs tested did not alter melting significantly (Fig. 5). PAU93 decreased K_m by 15.3 fold at 35 °C but

Table 2 Kinetic parameters of DNAzyme

Temperature (°C)		K_m (μM)	V_{max} (μmol s^{-1})
55	Without PAU	1.2	3.4
	With PAU93	0.5	1.2
35	Without PAU	2.3×10^{-1}	3.5×10^{-1}
	With PAU93	1.5×10^{-2}	3.0×10^{-1}

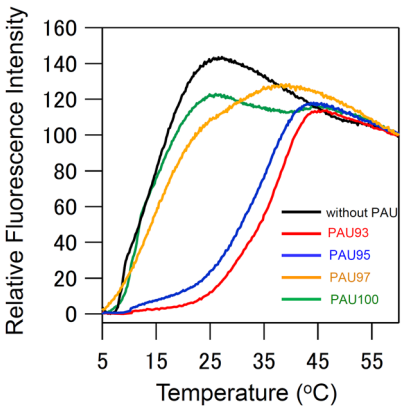

Fig. 5 Melting curves of an 8-base pair DNA duplex (100 nM total strand concentration) in the absence or the presence of 1 mg mL^{-1} indicated PAU in 50 mM HEPES-NaOH (pH 7.3) containing 150 mM NaCl.

by only 2.4 fold at 55 °C. At 35 °C, more than 95% of DNAzyme and substrate were recruited into the coacervates (Fig. 3), meaning that the DNAs were locally concentrated, promoting formation of the enzyme–substrate complex. Thus, both an increase in local DNA concentration and the stabilization of the DNAzyme/substrate complex by PAU93 could lead to the marked decrease in K_m. We previously reported that activity of β-gal was decreased by entrapment in PAU coacervates due to an increase in K_m.[20] The increase in K_m was due to limited molecular diffusion under the molecular crowding conditions of the coacervate. Entrapment would also limit diffusion of the DNAzyme and its substrate; however, the effect of promotion of DNAzyme/substrate complex presumably predominates, resulting in a decrease in K_m and enhancement of the DNAzyme activity in the coacervate. Although determined enzymatic parameters provided clues to understanding the reaction mechanism, the influence of the polymer on reaction processes that were not reflected as changes in the enzymatic parameter should be also examined in the future.

Conclusions

PAU showed UCST-type solution behaviour in the presence of the 10–23 DNAzyme. Below the T_p value of PAUs having amino content of 5 mol% or more, DNAzyme and substrate were entrapped in coacervate droplets. Above T_p, the addition of PAU did not affect DNAzyme activity significantly, although PAU93 slightly enhanced the activity. Below the T_p, PAU93 considerably enhanced DNAzyme activity. The observed 100-fold decrease in K_m implied that the enhancement of DNAzyme activity upon the cooling was due to stabilization of complex between DNAzyme and substrate resulting from the increase in local concentration in the coacervate. The simple PAU93 coacervate enhanced DNAzyme activity to the same extent as previously reported for a complex coacervate.[8] Simple coacervates of PAU are more robust than complex coacervates in biological media and are induced by cooling, making simple coa-

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cervates optimal for biological applications. We believe that cooling-inducible coacervate of ureido polymers will prove useful in optimization of DNA nanodevices under biological conditions.

Conflicts of interest

There are no conflicts to declare.

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