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SHORT COMMUNICATION

Fluorescence Based Investigation of Temperature-Dependent Pb^{2+}-Specific 8–17E DNAzyme Catalytic Sensor

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Abstract

The 8–17E DNAzyme is a temperature-dependent DNA metalloenzyme catalyzing RNA trans esterification in the presence of Pb^{2+} metal ions. Labeling the stems of the substrate and DNAzyme with the Cy3 and Cy5 respectively, the considered DNAzyme was studied by the fluorescence spectroscopy. The temperature-dependent variability of the Pb^{2+}-specific 8–17E DNAzyme catalytic sensor was investigated trough a number of successive temperature fluctuations from 4 to 25 °C to obtain information. Investigating underlined biochemical system reveals that in this sensor, free single strands Enzyme (Cy5-E) and Substrate (Cy3-S) have higher fluorescence intensities than hybridized forms, suggesting that the fluorophores are in a contact quenched. Increasing the temperature has three effects: 1) Fluorescence intensities for the free fluorophores were reduced, 2) stability of the hybridized form was reduced and cleavage of substrate in presence of $Pb^{2+}$was occurred, and 3) conformation of ES hybridized form was changed (before cleavage). As a result of conformation changes in ES, S was more affected than E in the ES. Pb^{2+} ion shows quenching effect on both fluorophores and in the absence of N_2(g) purge the effect was more considerable. A main goal that we had in mind was to find if significantly lower concentrations of Pb^{2+} and ES, compared to previous reports, can generate any observable cleavage in substrate. Analysis of the cleavage reaction for 50 nM ES indicates that S is cleaved at 25 °C in presence of N_2(g) and 0.5 μM Pb^{2+}, while in same condition no apparent change occurs in the 4 or 10 °C. The rapid, sensitive and low cost strategy presented here can be applicable to study temperature-dependent behavior of other nucleic acid-based biosensors.

Keywords DNAzyme · Metal biosensor · Fluorescence sensor · Pb^{2+}

Introduction

Recent discovery of catalytically active DNAs (deoxyribozymes) [1] leads to a wide-spread interest in their use as a simple, stable, and cost-effective alternatives to proteins and ribozymes in biochemical and pharmaceutical applications [2, 3]. DNAzymes are particularly attractive as a platform for designing biosensors. Long considered as a strictly genetic information storage material, DNA was shown in 1994 to carry out catalytic functions [1], and thus became the newest member of the enzyme family after proteins and RNA. Since then, DNAzymes have been shown to catalyze a series of reactions as RNA or protein enzymes [3, 4]. DNAzymes are catalytically active DNA molecules that are obtained via in vitro selection and, the presence of the DNAzymes in nature have not reported yet. RNA-cleaving DNAzymes have attracted significant attention for sensing applications due to their excellent programmability, stability, and activity [5]. In addition to being biocompatible, biodegradable, and amenable to in vitro selection, DNAzymes are relatively less expensive to produce and more stable to hydrolysis than RNA and protein enzymes. Unlike proteins, most DNAzymes can be denatured and renatured many times without losing binding ability or activity. DNA/RNAzymes that are highly specific for Pb^{2+} [1, 6, 7], Cu^{2+} [8–10], Zn^{2+} [11], and Co^{2+} [12, 13], have been obtained through in vitro selection [14]. Upon combining the selected DNAzymes with either fluorophore/quencher pairs [14, 15], nanoparticles [16–19] or electro-active tags

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[20–22], new and powerful classes of fluorescent, colorimetric and electrochemical sensors have emerged that can be applied for detecting a wide range of metal ions, including both diamagnetic and paramagnetic metal ions. DNA-based catalytic beacons have demonstrated fluorescent sensing of many targets with high sensitivity and selectivity [14, 15, 23–27]. DNAzymes can also function as promising therapeutics, when designed to complement target mRNAs or viral RNAs, and consequently lead to down-regulation of protein expression. So far, DNAzymes have shown potential as therapeutic agents for various diseases, such as antiviral, antibacterial, anticancer and anti-inflammatory [28–30], as well as atherosclerosis [31]. DNAzyme was successfully tested for the HIV RNA cleavage in vitro [2, 32]. Besides, it can be applied for anti-cancer treatment by targeting the *bcr-abl* oncogene, which is associated with chronic myelogenous leukaemia [33].

Lead (Pb), a typical heavy metal pollutant, is non-degradable and can exist in the environment for a long time. Human exposure to environmental Pb is harmful to the hemopoietic system, the nervous system and the kidneys, causing many types of disease [34]. Thus, the simple, rapid and reliable detection of Pb^{2+} is of great significance for environmental protection, human health and food safety. To date, several methods have been reported for the determination of Pb^{2+}, including atomic absorption spectrometry [35], plasma mass spectrometry [36], electrochemical [37], fluorescence sensor [38], and stripping voltammetry [39]. Current methods often require sophisticated equipment or sample treatment. As a supplementary technique to overcome certain limitations of the previous ones, DNAzymes have been isolated and developed into sensors using general applicable methods to detect a wide range of metal ions [40, 41].

Fluorescence based methods have high sensitivity and good selectivity, and one of the first DNAzyme biosensors was based on 8–17, and exploited its Pb^{2+}-dependent RNA cleavage activity [7]. A Pb^{2+} biosensor was constructed with a fluorescent reporting system, by simply labeling the 5′ end of the substrate oligonucleotide with a fluorophore, and the 3′ end of the DNAzyme strand with a fluorescence quencher. In the uncleaved state, the substrate binds to the DNAzyme, positioning the fluorophore and quencher in maximal fluorescence static quenching state. When Pb^{2+} is introduced into the solution, the DNAzyme becomes active and cleaves the substrate, which subsequently dissociates from the DNAzyme to generate a fluorescence signal. The signaling properties of this original design have been improved by modifying the number, arrangement, and type of fluorophores and quenchers [14, 42]. Most of these modifications cause to complexity of the designed system. Other point is that, the applied concentrations for lead ion and the DNAzyme are much lower than previous studies.

In the present report, we are particularly interested in study of simple fluorescence based Pb^{2+} sensor and cleavage of substrate S labeled with Cy3, largely because of the adverse effects of Pb^{2+} exposure to human health, especially to children [43, 44]. On the other hand, DNAzymes can be used to cleave RNA for treating various diseases, such as viral infection, cancer, inflammation and atherosclerosis, and the sensor or drug performance is vulnerable to temperature variation [14]. The effect of some variables such as N_2(g) purge, temperature, Pb^{2+} on cleavage of RNA were investigated. To do these, fluorescence intensity of Cy5-E and Cy3-S were monitored when fluorophores are free or in the hybridized form. Significantly lower concentrations of Pb^{2+} and ES were used to generate observable cleavage of RNA. We made successive temperature fluctuations of temperature-dependent Pb^{2+} 8–17E DNAzyme biosensor around 4 °C, 10 °C and 25 °C to obtain statistically more reliable information. It is because the sensor performance is vulnerable to temperature variations and is expected to show temperature-dependent changes from 4 to 25 °C. Besides, Pb^{2+}effect as quencher or cofactor were surmised, depending on the experimental condition. Furthermore, this simple and rapid approach could provide a new platform for temperature dependent DNAzyme investigations.

Experimental

Reagents and Instruments

Materials and Measurements The oligonucleotides were purchased from BIONEER, Korea. Na-HEPES was purchased from ACRON and other chemicals were purchased from Sigma. Lead(II) acetate trihydrate was purchased from Merk (Darmsted, Germany). The oligonucleotide sequences used in our experiments are as follows: 24S-Cy3, 5′-Cy3-CAACTCACTAT rAGGAAGAGATGTC; 38E-Cy5, 5′-Cy5-GAC ATC TCT TCT CCG AGC TCG GTC GAA ATA GTG AGT TG. The oligonucleotide S-Cy3 could form a hybridized structure with E-Cy5. The fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer. Temperature of the contents of the quartz cuvettes was adjusted with a thermostat controlled cell holder. All solutions were prepared with ultrapure water purified using a Millipore filtration system.

Hybridization and Assay Procedures

Both Cy5-E and Cy3-S were dissolved in a buffer of 50 mM Na-HEPES (pH = 7.0) with a concentration of 50 nM. The sensor solution including E-Cy5 and S-Cy3 was kept in 90 °C for 5 min and subsequently cooled to 4 °C slowly over 120 min, to ensure complete hybridization. Then, through three different experiments solutions were treated with aliquots of Pb^{2+} (0–20 $\mu molL^{-1}$) at about 8 steps and

experiments were replicated at 4, 10 and 25 °C. When the cell was outside the compartment Pb^{2+} was added to cell and cell was purged with N_2(g) for two minutes in each step of Pb^{2+} addition. Two other experiments were performed at 10 °C. First, after purging of cell by N_2(g), solutions of buffer were added instead of Pb^{2+}. Second, solutions of Pb^{2+} were added without purging of N_2(g). The fluorescence excitation-emission matrix (EEM) spectra of these samples were recorded by addition of different concentration of the Pb^{2+} during 120 min on a fluorescence spectrophotometer by changing excitation wavelengths from 500 to 700 with a 10 nm increment emission of each sample has been recorded from 500 to 700 nm with a 10 nm increment.

Result and Discussion

The fluorescence experiments were done in the water bath to control temperature at 4, 10 and 25 °C. Additionally, N_2(g) was purged into the solutions and the cuvette holder area to push the dissolved oxygen out from the solution. The N_2(g) purge was out of controlled temperature cell compartment, that resulted in a temperature variation of cuvette during the purge. Successive temperature changes during the purge of N_2(g) allowed us enough temperature variation replicates and statistically more reliable information from each experiment. Although reported concentration of Pb^{2+} and DNAzyme in previous reports were around 50 μM and 4 μM, respectively [45], the considered concentrations are 0.83 μM and 50 nM in this report which are far lower than that in previous works.

The considered nucleic acid strands based system in this study is the 8–17E DNAzyme which was used for the Pb^{2+} sensing, through labeling the substrate strand with a fluorophore such as 5' Cy3 and the enzyme strand with quencher such as 5' Cy5 (Fig. 1a). In the presence of the Pb^{2+}, the substrate strand containing a single ribonucleotide base is cleaved, resulting in the release of the substrate arms containing the fluorophore (Fig. 1b).

There are at least two mechanisms for fluorophore-quencher interactions. The first mechanism is through fluorescence resonance energy transfer (FRET), when the fluorophore and quencher distance is about 10-100 Å. The emission of the fluorophore should have large overlap with the absorption spectrum of the quencher for higher quenching efficiency. Another, static quenching mechanism acts through ground-state complex formation when the fluorophore-to-quencher distance is too short. In this case, the spectral overlap is not an important issue [46]. Quenching of a fluorophore by the FRET can occur if the fluorophore and quencher pair has spectral overlap and they remain within sufficient distance of each other for efficient energy transfer. This mechanism is highly dependent on the dipole orientations of the molecules and it is limited by the distance between the donor and the acceptor molecules. Typical effective distances between the donor and acceptor molecules are in the 10–100 Å range. On the other hand, quenching of the fluorophore by the static quenching can occur if the fluorophore comes in close proximity to the quencher molecule or to a nucleotide, owing to internal hybrid formation within the probe. In contact (static) quenching, the donor and acceptor molecules interact by a proton-coupled electron transfer through the formation of hydrogen bonds. In aqueous solutions, electrostatic, steric, and hydrophobic forces control the formation of hydrogen bonds. When this complex absorbs energy of light, the excited state immediately returns to the ground state without emission of a photon and the molecules do not emit fluorescent light. A characteristic of contact quenching is a change in the absorption spectra of the two molecules when they form a complex. In contrast, in the FRET mechanism, the absorption spectra of the molecules do not change [46].

Temperature Effect on Fluorescence

For investigating the designed fluorophore-quencher system, the fluorescence intensity of 50 nM S and 50 nM E as free and hybridized form (ES) were monitored at 565 and 665 nm

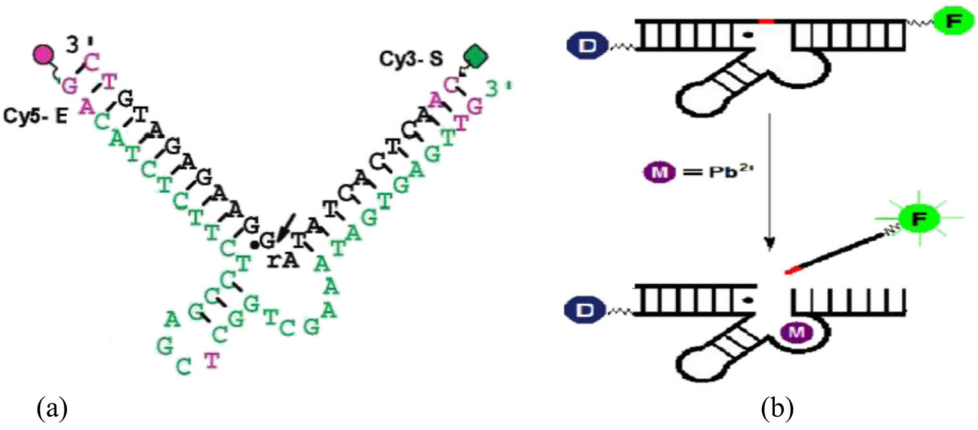

Fig. 1 **a** Secondary structure of the 8–17E (prepared for enzyme assay). Cleavable Cy3-labeled substrate (5'Cy3-S) hybridized with Cy5-labeled enzyme (5'Cy5-E). **b** Schematic representation of the catalytic DNAzyme sensor

(selective wavelengths), which are the emission wavelengths of Cy3 and Cy5, respectively. This experiment is replicated in 4, 10 and 25 °C. Figure 2 shows the intensities from E and S in the same concentrations 50 nM S and 50 nM E at 4, 10 and 25 °C experiments.

Single strands with the fluorophores have higher fluorescence intensities in the free forms. Upon hybridization of strands, the fluorescence intensity decreases. Closeness of the fluorophores on one strand to the other strand and some types of contact or interaction quenching can be the reason. Because the fluorophore (Cy3)-to- quencher (Cy5) distance is too short (~8 Å) and fluorescence is quenched due to the close proximity to the quencher. Furthermore, Fig. 2 shows fluorescence of single strands increase with decreasing temperature regardless to hybridized or free forms. It should be highlighted that temperature changes is more effective parameter on the fluorescence of the S compared to E. In this way, raising the temperature to 25 °C reduces the fluorescence of the two fluorophores. Fluorescence intensities in the Fig. 2 are recorded 5 min after placing the sample cell in the temperature controlled compartment to obtain the temperature equilibrium with the compartment.

Quenching Effect of Lead Ion

Two experiments were performed to confirm the quenching effect of the lead ion. First, the effect of Pb^{2+} on fluorescence intensity of E and S in free forms with purging of N_2(g) at 4 °C were studied. Figure 3a shows at 4 °C free fluorophore Cy3-S and Cy5-E by addition of Pb^{2+} (0.33 and 0.50 μM) are quenched and it is proportional to Pb^{2+} concentration. Second, the effect of Pb^{2+} on fluorescence intensity of ES-hybridized form without purging N_2(g) at 10 °C were investigated. Figure 3b shows that Pb^{2+} is quencher for fluorophores in ES-hybridized form and against of Fig. 2, at 10 °C and in presence of O_2(g) fluorescence intensity of Cy3-S is more than Cy5-E. It seems O_2(g) had considerable effect on fluorescence intensity of Cy3-S in hybridized form. The experiment was operated at 10 °C in presence of 0–13.04 μM of $Pb^{2+}$and without N_2(g) purge.

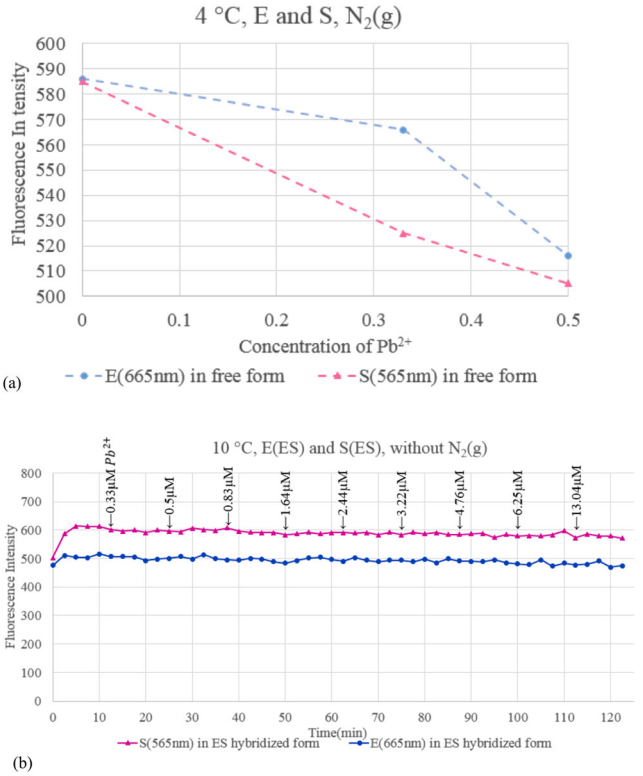

Fig. 3 Quenching effect of lead ion. **a** Fluorescence intensities of Cy5-E and Cy3-S in the free form in different concentrations of Pb^{2+}, with purge of N_2(g) at 4 °C. Concentrations of lead cation in the three solutions were 0.00, 0.33, and 0.50 μM. **b** Fluorescence intensity versus the incubation time for 50 nM Cy5-E and 50 nM Cy3-S in 50 mM HEPES (in ES form), without purge of N_2(g), at 10 °C and pH = 7, at different concentrations of Pb^{2+}.The concentration (μM) of Pb^{2+} in each step is marked in the figure. For each step of Pb^{2+} addition, cell was bring out from cell compartment and when the cell was outside the compartment some Pb^{2+} was added to cell and solution was purged with N_2(g) for two minutes in each step. The concentration (μM) of Pb^{2+}in each step is marked in the figure, which is 6.6 times as ES in 12.5 min, 10 times as ES in 25 min, 16.6 times as ES in 37.5 min, 32.8 times as ES in 50 min, 48.8 times as ES in 62.5 min, 64.4 times as ES in 75 min, 95.5 times as ES in 87.5 min, 125 times as ES in 100 min, and 260.8 times as ES in 112.5 min

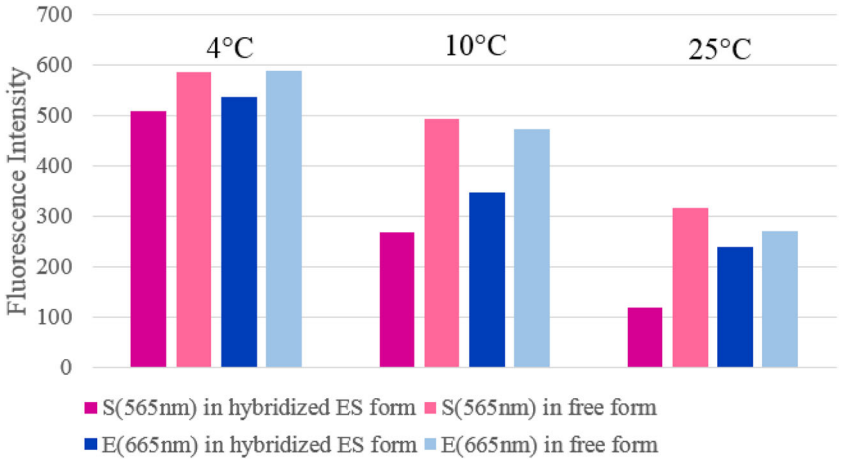

Fig. 2 Fluorescence intensity of 50 nM S and 50 nM E in free and hybridized forms and different temperatures of 4, 10and 25 °C. Solutions were prepared in 50 mM Na-HEPES, pH = 7 and purged with N_2(g) for two minutes

Figure 3 shows that Pb^{2+} can be a quencher for free fluorophore forms with purge of N_2(g) and the ES hybridized fluorophore forms without purge of N_2(g). Experiments were performed with purge of N_2(g) all except for Fig. 3b for investigation of effect of O_2(g) on fluorescence intensity of fluorophores. Quenching effect of Pb^{2+}on ES in the absence of N_2(g) is considerable. In these experiments by addition of Pb^{2+}, ES-Pb^{2+} complex is formed and weak quenching for fluorophores are observed. But, without purge with N_2 and at 10 °C ES-Pb^{2+} complex was not in a suitable condition for cleavage. The melting temperature of the uncleaved substrate is designed to be far above room temperature (42 °C) so that the substrate will not melt from the enzyme strand in this hybridized state [47]. Upon cleavage, the melting temperatures of the shorter parts of the substrate are designed to be below room temperature so that it will melt and dissociate from the enzyme strand. Also, previous studies show that there is a perfect match between the dissociation kinetics and melting temperatures of oligonucleotides.

Cleavage and Temperature

Since DNA hybridization and dehybridization play a critical role in almost all DNAzyme sensor design, the sensor performance is vulnerable to temperature variations [48]. Therefore, Pb^{2+}-specific 8–17E DNAzyme catalytic sensor shows a temperature-dependent fluorescence variations from 4 to 25 °C. Here, we report a simple strategy for studying effect of temperature change from 4 °C to 25 °C on fluorescence signal of free and hybridized form of the E and S.

Figure 4 shows the effect of the Pb^{2+}as a cofactor for the enzymatic cleavage of hybridized fluorophores which is monitored as a function of time in presence of N_2(g) and Pb^{2+} in 4, 10 and 25 °C.

Figure 4 confirms the effect of temperature changes on fluorescence intensity of E and S in hybridized form which was observed previously in Fig. 2. By raising the temperature fluorescence intensity of E and S in hybridized form is begun to decrease and fluorescence changes of S is much more than fluorescence changes of E. In the Fig. 4 the effects of temperature changes and Pb^{2+} addition are merged in each step. If Pb^{2+} is added to ES hybridized form, complex of ES-Pb^{2+} is formed and in a proper condition cleavage reaction of S is begun. Figure 4a, b show the results at 4 and 10 °C that, by addition of Pb^{2+}, ES-Pb^{2+} complex is formed. However, because of strong hybridization of ES, ES-Pb^{2+} complex is not in a suitable condition for cleavage and no trend is observed by addition of Pb^{2+}. At 4 and 10 °C the temperature difference between cell and surrounding was higher compared to 25 °C, therefore, fluorescence fluctuations due to temperature changes were larger. Most of the observed fluorescence changes are expected to be from temperature variations. We also surmised that introduction of Pb^{2+} in each step is another reason for fluorescence decrease. In this way, an experiment including 50 nM ES-hybridized form in 50 mM Na-HEPES (pH = 7) with purging of N_2(g) at 10 °C in absence of Pb^{2+} was performed and compared to the results in Fig. 4b. Mann–Whitney *U*-test and Tukey's quick test [49] as non-parametric distribution-free statistical tests proved that Pb^{2+}have significant

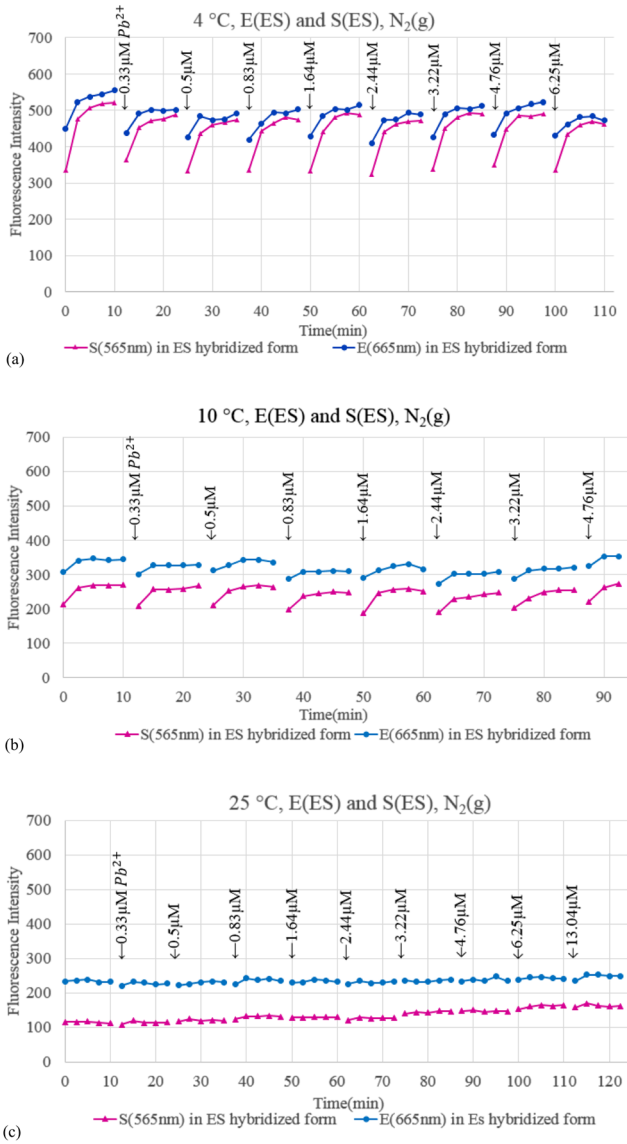

Fig. 4 Scaled fluorescence intensity versus the incubation time for the ES hybridized form at different concentrations of Pb^{2+}. The ES hybridized form was prepared with 50 nM Cy5-E and 50 nM Cy3-S in 50 mM Na-HEPES, pH = 7. For each step of Pb^{2+} addition, cell was brought out from cell compartment and when the cell was outside the compartment some Pb^{2+} was added to cell and solution was purged with N_2(g) for about two minutes in each step. The concentration (μM) of Pb^{2+}in each step is marked in the figures, which is 6.6 times as ES in 12.5 min (step 1), 10 times as ES in 25 min (step 2), 16.6 times as ES in 37.5 min (step 3), 32.8 times as ES in 50 min (step 4), 48.8 times as ES in 62.5 min (step 5), 64.4 times as ES in 75 min (step 6), 95.5 times as ES in 87.5 min (step 7), 125 times as ES in 100 min (step 8) to 260.8 times as ES in 112.5 min (step 9), and 400 times to 125 min(step 10). Experiments were performed (**a**) at 4 °C in 8 steps, (**b**) at 10 °C in 7 steps, and (**c**) at 25 °C in 9 steps

decreasing effect on fluorescence of both Cy3-S and Cy5-E. Emphasizing that the effect of fluorescence reduction on S is greater than E. Figure 4c shows that at 25 °C difference between cell temperature and surrounding was lower, therefore, fluorescence fluctuation due to temperature changes is smaller. Figure 4c shows an incremental trend of fluorescence at 25 °C, which indicate substrate breakdown. At 25 °C by addition of Pb^{2+}, the substrate is cleaved at the RNA base site. So that, at 25 °C breaking of substrate strand happens and separation of the complementary DNA molecules into separate single strands that lead to increase in fluorescence. According to Fig. 1b, in the presence of the target metal ion, such as Pb^{2+}, the substrate is cleaved and the fluorophore bearing fragment is separated from its quencher, resulting in fluorescence recovery. And the rate of fluorescence increase be proportional to metal concentration [50]. By addition of Pb^{2+} at 25 °C, fluorescence of substrate was increasing, even when concentration of added lead ions is up to 400 times greater than that of ES hybridized form. Therefore, S was cleaved at 25 °C and in presence of Pb^{2+} and N_2(g) by slow kinetic, that can be related to very low concentration of ES. When the same experiment was carried out at 4 °C and 10 °C, no significant trend was observed. The cleavage of substrate and overall fluorescence increment was not observed in 4 °C and 10 °C experiments (Fig. 4a, b). Since this sensor exhibited high activity at 25 °C, it was referred to as the RT (room temperature) sensor. For the efficient functioning of the catalytic sensor, a fine balance is struck between the high stability of the substrate–enzyme strands for obtaining low fluorescence of E and S in the absence of Pb^{2+}, and the low stability of the product–enzyme strands for fast product release and high fluorescence increase in the presence of Pb^{2+}. For the RT sensor, the stability of the product–enzyme strand was so high at 4 °C that the product was not released even after Pb^{2+}-dependent cleavage. Increasing the temperature provided sufficient energy to facilitate the release of the product strand, and fluorescence increase were observed at 25 °C. Above 25 °C, however, the stability of the substrate–enzyme complex decreases more, causing more de-hybridization and higher fluorescence [48].

Results of fluorescence experiments at 4, 10 and 25 °C (Figs. 2 and 4) show that increasing temperature has three effects. First, fluorescence of the free forms of fluorophores are decreased. Second, in ES hybridized form the decrease in S fluorescence is more than decrease in E signal. It means there are changes in conformation of ES during temperature variation that affect S more than S. Third, cleavage for S and de-hybridization of ES complex occurred when temperature is proper. In order to observe the repetitions of E and S fluorescence changes in ES as a function of temperature, the cell was placed in and out of thermo-stated compartment a number of times. Using alternative temperature variations, statistically more reliable information was obtained in a simple and rapid way.

Cy3 and Cy5 were fluorophore labels on 5'-S and 5′-E, respectively, and in contrast of many studies, simply the one quencher system was used [14]. A central feature of this investigation is correlation between enzyme activity and increased temperature from 4 to 25 °C in the same metal concentration range and under identical conditions. Concentrations of Pb^{2+} and ES complex used in this report ((0-20 μM) and 50 nM, respectively) are much lower than similar reports [44, 48]. Under such conditions, only significant enzyme activity was observed at 25 °C with purge of N_2(g) and in the same metal concentration range as used at 4 and 10 °C. It was found that by raising the temperature, the changes in conformation of ES is more effective on S compared to E. We observed thatPb^{2+} can play quencher role for free single E and S strands, and especially for ES hybridized form at 10 °C without N_2(g). While, $Pb^{2+}$is enzyme cofactor for enzymatic cleavage of ES-Pb^{2+} complex at 25 °C and in presence of N_2(g). The effect of temperature changes on fluorescence signal of a considered samples were observed a number of times in each experiment and statistically more trustable information was obtained. A suggestion for future work is considering this temperature-dependent sensor for temperature measurement, as a sensitive nano-thermometer from 4 to 25 °C, in very small samples such as a living cell [51].

Conclusion

A simple, sensitive, informative and rapid approach for the study of temperature-dependent spectral behavior of fluorescence labeled Pb^{2+}-8–17 DNAzyme is presented. The cleavage of the substrate (S) by the enzyme (E) in the presence of $Pb^{2+}$and at 25 °C causes ES-Pb^{2+}conversion into ssDNA and fluorescence increase, especially in case of S. Concentration of E and S in free/hybridized forms and Pb^{2+} were lower than that in most of the previous reports. Sufficient data for performing statistical tests was obtained easily by repeated placement of cell, in each step of Pb^{2+} addition and N_2 purge. Effect of different factors such as Pb^{2+}concentration, N_2(g) purge and temperature changes on fluorescence intensity of Cy5-E and Cy3-S were studied. We found that the presence of Pb^{2+} had no significant cofactor effect on the DNAzyme activity at 4 and 10 °C and showed quencher effect at 10 °C and in absence of N_2(g) purge. Decreasing effect of temperature on fluorescence intensities of free fluorophores was more than hybridized fluorophores. Conformation changes for ES hybridized form was observed by raising the temperature, from which S fluorophore was more affected than E. Finally, at 25 °C and in presence of Pb^{2+} and N_2(g) cleavage reaction for substrate was begun and fluorescence intensity was increased moderately. The methodology developed in this study can be applied for other nucleic acid based sensors that are temperature-dependent. Moreover, this strategy provides

promise for the study of other DNAzymes and metal ions in aqueous solution.

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