Label-free Detection of Zn\textsuperscript{2+} Based on G-quadruplex

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Herein, we developed a sensing strategy for the label-free detection of Zn\textsuperscript{2+} based on G-quadruplex. In the absence of Zn\textsuperscript{2+}, there was a fluorescence enhancement of thioflavin T by interaction with human telomere sequence. On the addition of Zn\textsuperscript{2+}, Zn\textsuperscript{2+} induced a more compact G-quadruplex structure to release thioflavin T, resulting in a fluorescence decrease. This simple “mix-then-detect” method gave the detection limit of 0.91 \(\mu\text{M}\) with linear dynamic ranges from 0 to 10 \(\mu\text{M}\). Because it does not require the use of expensive and unstable DNAzyme systems, or need synthesis and modification of nanomaterials, this label-free biosensor is simple, fast, cost-effective and applicable for real samples taken from lake water.

Keywords G-quadruplex, thioflavin T, Zn\textsuperscript{2+} detection, label-free

Introduction

Zinc ion plays a vital role in biological systems\textsuperscript{1}, with the multiple Zn\textsuperscript{2+-}bound proteins acting as regulators of enzymes and transcription-related factors. Zinc homeostasis is required for normal functioning of the immune system, insulin secretion/action, and anti-oxidant systems.\textsuperscript{2} Free Zn\textsuperscript{2+} is also a neurotransmitter and neuromodulator in the central nervous system. Diseases such as Alzheimer’s, Parkinson’s and stroke are associated with abnormal levels of zinc.\textsuperscript{3} At the same time, large amounts of Zn\textsuperscript{2+} in the environment have been found to be a threat to fish, cattle and plant species. Therefore, it is environmentally important to minimize Zn\textsuperscript{2+} contamination arising from its widespread usage in industry.\textsuperscript{4,5}

Various efficient and reproducible methods, such as atomic absorption spectrometry,\textsuperscript{6,7} inductively coupled plasma mass spectroscopy,\textsuperscript{8,9} and inductively coupled plasma-atomic emission spectrometry (ICP-AES),\textsuperscript{10,11} have been developed for the detection of Zn\textsuperscript{2+}. However, these methods are usually complicated, time-consuming, and costly. In recent decades, Zn\textsuperscript{2+} chemical sensors based on luminescence have been developed and applied to Zn\textsuperscript{2+} monitoring in various biological systems.\textsuperscript{12,13} However, they suffer from drawbacks including complicated organic synthesis of the sensing platform on these sensors, harmful systems, insufficient selectivity and short excitation wavelength.\textsuperscript{14}

The application of functional nucleic acids has recently facilitated development of detection methods that do not require expensive instruments. Various DNA constructs stimulated by different external signals, G-quadruplex/i-motif formation, binding of aptamer-target or activation of deoxyribozymes, etc., have been used for sensing in analytical chemistry and biomedicine.\textsuperscript{15-20}

Guanine-quadruplexes (denoted hereafter as G4s) are unique structures formed by G-rich nucleic acid sequences and based on stacked arrays of G-quartets connected by Hoogsteen-type base pairing.\textsuperscript{21-24} G4s not only play vital roles in cancer research, but are also regarded as robust tools in analytical chemistry and biomedicine.\textsuperscript{25-28} A number of sensors have been developed based on G4s for the detection of metal ions, such as K\textsuperscript{+},\textsuperscript{29,30} Pb\textsuperscript{2+},\textsuperscript{31,32} Ca\textsuperscript{2+},\textsuperscript{33} Cu\textsuperscript{2+},\textsuperscript{34} Hg\textsuperscript{2+},\textsuperscript{35-37} Ag\textsuperscript{+},\textsuperscript{38-41} Sr\textsuperscript{2+}.\textsuperscript{42} Herein, we first developed a label-free detecting strategy for the assay of Zn\textsuperscript{2+} based on G4s; Zn\textsuperscript{2+} can induce the structural transition of the human telomere sequence AG\textsubscript{3}(T\textsubscript{2}AG\textsubscript{3})\textsubscript{3} (denoted hereafter as H\textsubscript{22}) from a random coil to an antiparallel G4 structure.\textsuperscript{43} It was reported that thioflavin T (denoted hereafter as ThT), a benzothiazole fluorogenic dye, could exhibit striking fluorescence enhancement when binding to the human telomeric G-quadruplex structure.\textsuperscript{44} So ThT was employed as the label-free fluorescent indicator in the sensoring system. The presented sensoring system consists of G-rich oligomer H\textsubscript{22} and G4-binding dye ThT, which eliminates the need for complex and expensive synthesis procedure and also for nanomaterial fabrication.

Experimental

Reagents and chemicals

Oligonucleotides were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). Sequences of the oligonucleotides are listed in Table S1 (Supporting Information). The stock solution of DNA samples was prepared in ultrapure water, and...
the DNA concentration was accurately quantified based on UV absorbance measurement at 260 nm. ThT was purchased from Sigma-Aldrich. All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). All solutions were prepared with water purified by a Milli-Q system (Millipore, Bedford, MA) and stored at 4°C. Measurements were performed in 50 mM Tris-HCl buffer (pH 8.0), unless stated otherwise.

Apparatus

The absorption of DNA solution at 260 nm was recorded on an Eppendorf biophotometer (Eppendorf AG 22331 Humburg, Germany). A fluorescence spectrometer (F-4600, Hitachi Co., Ltd., Japan) with a Xenon lamp excitation source was employed to record fluorescence spectra. In these experiments, a 10 nm slit width for excitation and a 10 nm slit width for emission were used. The accurate concentrations of Zn\(^{2+}\) in real samples were determined by ICP-AES (PerkinElmer, Optima 8000), using the most recommended wavelength (213.856 nm).

Fluorescence measurements

In all Zn\(^{2+}\) detection experiments, an appropriate aliquot of 0.1 M Zn(NO\(_3\))\(_2\) standard solution was added to the reaction solution containing 1 μM H22 before this was incubated for 10 min; 10 μL ThT (25 μM) was added to the reaction solution for a final concentration of 2.5 μM. Fluorescence spectra were recorded after the reaction solution was incubated for another 10 min. By applying an excitation wavelength of 425 nm, the solution fluorescence was monitored from 450 to 560 nm. Inorganic metal salts including Li\(_2\)CO\(_3\), NaCl, KCl, Mg(NO\(_3\))\(_2\)-6H\(_2\)O, CaCl\(_2\), Mn(NO\(_3\))\(_2\)-4H\(_2\)O, FeSO\(_4\), Co(NO\(_3\))\(_2\)-6H\(_2\)O, CuSO\(_4\)-5H\(_2\)O, Pb(NO\(_3\))\(_2\), AlCl\(_3\) and Fe(NO\(_3\))\(_3\)-9H\(_2\)O were used for evaluating the selectivity of the sensing system towards Zn\(^{2+}\).

Lake water samples were collected from Dushu Lake in Suzhou, and were filtered by 0.45 μm membrane before assay, then the concentration of Zn\(^{2+}\) in the lake water samples was determined by performing a standard addition calibration method. Different concentrations of Zn\(^{2+}\) standard solution (0, 1.0, 4.0, 7.0, and 10 μM) were spiked in the filtered samples, then the samples were added to the detecting system respectively and the data were collected according to the same procedure mentioned above.

Results and Discussion

Principle of operation

ThT is a commercially available fluorescent dye and has shown pronounced structural preference to G4s other than single-stranded DNAs, duplexes or triplexes. The binding to most G4s could bring out greatly enhanced emission in the fluorescence intensity of ThT.\(^{45}\) Recently, by the merit of its excellent property, ThT has been employed as G4s fluorescent indicator for the label-free detection of Tb\(^{3+}\),\(^{46}\) K\(^{+}\),\(^{47}\) Hg\(^{2+}\),\(^{48}\) biothiols,\(^{49}\) cancer gene\(^{50}\) and protein.\(^{51}\) As shown in Scheme 1a, the biosensor consists of a G-rich DNA H22 and fluorescent dye ThT. The fluorescence spectra were recorded in the absence and presence of Zn\(^{2+}\), respectively. As shown in Scheme 1(B), in the absence of Zn\(^{2+}\), there was a fluorescent enhancement of ThT owing to binding to the H22 G-quadruplex structure; after adding 50 μM Zn\(^{2+}\), a sharp decrease in the fluorescence intensity was observed.

Circular dichroism (denoted hereafter as CD) spectroscopy is employed for the study of G4s polymorphism since it is sensitive to the conformational changes of G4s.\(^{52,53}\) CD measurement was conducted to investigate the principle of the decrease. As shown in Fig. 1, in the 50 mM Tris-HCl buﬀer without ThT and Zn\(^{2+}\), H22 itself folded into a parallel structure having a characteristic CD peak at 260 nm. Upon the addition of ThT, H22 transformed into a mixture of parallel (CD peak at 260 nm) and antiparallel (CD peak at 295 nm) quadruplexes.\(^{54,55}\) Then a clear structural change was observed after the addition of Zn\(^{2+}\), the resulting complex presented a positive peak near 290 nm and negative peak near 265 nm, which declared an antiparallel G4 structure and was accordant to the Zn\(^{2+}\)-mediated H22 G-quadruplex (dotted line in Fig. 1).\(^{43}\) The results of CD measurement showed a clear explanation that the fluorescence decrease in Scheme 1(B) was caused by the structural change of

![Scheme 1](image1)

![Fig. 1](image2)
the G-quadruplex triggered by Zn\(^{2+}\). We speculated that the decrease might be the result of ThT released from the Zn\(^{2+}\)-stabilized G4. Since the ionic radius of Zn\(^{2+}\) (\(r = 0.74\) Å) is much smaller than Pb\(^{2+}\) (\(r = 1.29\) Å) and K\(^{+}\) (\(r = 1.51\) Å) (inset in Scheme 1(B)),\(^{36}\) G4s mediated by a smaller ion have been demonstrated to show more compact structures and are unfavorable for the binding to the G4 specific dyes.\(^{57,58}\)

Besides H22, five most-reported G4 sequences (Table S1, Supporting Information) were also employed to further validate the principle of the Zn\(^{2+}\)-induced fluorescence decrease. As shown in Fig. 2, the addition of Zn\(^{2+}\) into G4-ThT systems all resulted in a fluorescence decrease, providing evidence for our aforementioned speculation. On the other hand, ionic radius is not the definitive element for the binding to dye. The binding also quite depends on the sequences of oligonucleotides, for example, a kind of iridium(III) complex preferentially bound to a G4 (sequence: 5'-G4T4G4-3') structure mediated by Ca\(^{2+}\) rather than Pb\(^{2+}\), though Ca\(^{2+}\) has a smaller ionic radius than Pb\(^{2+}\);\(^{33}\) and crystal violet preferred a G4 (sequence: T30695) structure mediated by Pb\(^{2+}\) rather than K\(^{+}\).\(^{31}\) The data collected from five other G4 sequences could not provide sufficient evidence for our speculations, but the fastidiousness of the Zn\(^{2+}\)-mediated compact G4 structure to ThT binding was most likely responsible for the decrease in fluorescence intensity based on the present data.

Another G4s-binding dye berberine (Fig. S1, Supporting Information) was known hereafter as BBR) was employed to prove our speculation. BBR is known to interact with DNA and to have an effect on telomerase activity,\(^{59,60}\) and it could cause a fluorescence increase of BBR when binding to G4s.\(^{61,62}\) Then the fluorescence intensities of the H22-BBR complex were collected in the presence of increasing concentrations of Zn\(^{2+}\). As shown in Fig. S2a (Supporting Information), there were corresponding decreases when the H22-BBR complex was challenged with increasing concentrations of Zn\(^{2+}\). Besides H22, the other five G-rich sequences were also employed to verify the principle of the turn-off detection for Zn\(^{2+}\) based on G4s. As shown in Fig. S2b (Supporting Information), the addition of Zn\(^{2+}\) to the G4s-BBR systems all caused decreases of fluorescence intensities. All the collected data seemed directed towards the same explanation, but more investigations are needed since research about the Zn\(^{2+}\)-G4 structure is rare.

Selection of optimal ThT concentration

The fluorescence intensity was first studied as a function of ThT concentration on the assay. Different concentrations of ThT were mixed with H22 in the presence of 40 \(\mu\)M Zn\(^{2+}\), then these mixtures were allowed to react for 30 min at room temperature, and finally the fluorescence intensity at 490 nm was measured. The blank sample was treated in the same way without Zn\(^{2+}\). As shown in Fig. 3, the changes of ThT fluorescence intensity increased with increasing concentration of ThT in the range from 0.5 to 2.5 \(\mu\)M, and then leveled off from 2.5 to 3.0 \(\mu\)M. Therefore, 2.5 \(\mu\)M of ThT was used in this assay.

The linear correlation and the detection limit

As shown in Fig. 4, based on aforementioned results, we developed a label-free biosensor for Zn\(^{2+}\) detection by employing a G4 sequence H22 and a G4-binding light-up dye ThT. The binding to H22 initially brought out increased fluorescence of ThT, after introducing Zn\(^{2+}\), H22 transformed to a compact G4, which resulted in a fluorescence decrease of ThT. The relationship between fluorescence intensities of ThT and concentrations of Zn\(^{2+}\) was firstly studied, a series of spectra of the solutions with increasing concentrations of Zn\(^{2+}\) were recorded (Fig. 4(A)). Upon treatment with increasing concentrations of Zn\(^{2+}\), the fluorescence intensities of ThT gradually decreased. The fluorescence intensities were dependent linearly on the concentrations of Zn\(^{2+}\) over a range of 0 to 10 \(\mu\)M (Fig. 4(B)), and the detection limit for Zn\(^{2+}\) was estimated to be 0.91 \(\mu\)M based on the following formula: \(C_m = 3\times S_0/m\), where \(C_m\) is the detection limit, \(S_0\) is the standard deviation from the blank samples in the absence of Zn\(^{2+}\) and \(m\) is the slope of linear relationship (Zn\(^{2+}\) concentration vs. fluorescence intensity).

Selectivity

Figure 5 displays the fluorescence intensities observed upon addition of different metal ions. As shown, compared with other ions a significant fluorescence decrease was observed upon the addition of Zn\(^{2+}\). These results indicated that this label-free biosensor could also provide the capability to differentiate Zn\(^{2+}\) from many metal ions, demonstrating the good selectivity of this biosensor. This high specificity might be due to the more compact G4 fold mediated by Zn\(^{2+}\) and its weak binding with ThT.

Real sample analysis

The meaningful determination of Zn\(^{2+}\) by the biosensor was
conducted with a standard addition method in serum and lake water samples, which aimed at investigating the detecting ability of the biosensor in complex matrices. The testing results showed that the biosensor was applicable to the analysis of lake water samples, but could not determine the concentration of $\text{Zn}^{2+}$ in a serum sample without pre-treatment (data not shown). Serum introduced a complex matrix to our system, which might have prevented the interaction between $\text{H}_2\text{~22}$, $\text{ThT}$ and $\text{Zn}^{2+}$. The results shown in Fig. 6 were obtained by firstly filtering the lake water sample and then diluting by 10-fold. The quantity of $\text{Zn}^{2+}$ in the lake water sample was estimated by an external standard method. There was good linear correlation between fluorescence intensities and the spiked $\text{Zn}^{2+}$ concentrations. The detection result ($2.4 \mu\text{M}$) of the lake water sample was in accordance with the data determined by ICP-AES. The result suggested that the proposed method has potential for real sample analysis.

Conclusions

In this work, we first demonstrated a label-free strategy for the detection of $\text{Zn}^{2+}$ based on $\text{Zn}^{2+}$-stabilized compact G4s, and developed a fluorescent biosensor by employing a G4s-binding light-up dye $\text{ThT}$. The biosensor had high sensitivity and good linearity for the quantitative analysis of $\text{Zn}^{2+}$, and could be applied for real samples from lake water without complicated pre-treatment. Most importantly, no sophisticated experimental techniques or any chemical modification of DNA were required, which offered the advantages of cost efficiency. It was simple in design, fast in operation and possessed high sensitivity and selectivity.

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Supporting Information

Response of the G4s-berberine system towards $\text{Zn}^{2+}$. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

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