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A Reusable Pb^{2+} Detecting Aptasensor Employing a Gold Nanorod-DNAzyme Conjugate

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Here, we demonstrated a Pb^{2+} detecting aptasensor using Pb^{2+}-sensitive DNAzyme-conjugated gold nanorods (GNRs). Fluorescent DNA substrates that were initially quenched by GNRs, are released in response to Pb^{2+} ions to give a substantial fluorescence signal. The GNR-tethered DNAzyme is reusable at least three times with a LOD of 50 nM.

Keywords : DNAzyme, Heavy metal ions, Gold nanorod, Aptamer

I. Introduction

Humankind has used lead (Pb) for thousands of years either in its pure metallic state or in the cationic form, to constitute various compounds. In most countries, lead is pervasive in the environment as it has been widely used in various kinds of industries such as paints for building construction, cosmetics, and batteries. As lead became a common element in many industrial products, *lead-poisoning* emerged as a new threat to public health. Accordingly, its content in the daily supplies is thus strictly regulated. It has been reported that lead causes intellectual disability and retardation of neurodevelopment in infants and children [1]. Lead also has adverse effects on adults, causing high blood pressure, cardiovascular disease, cancer, and even death [2,3]. Hence, there have been many attempts to reduce its use and to decrease the contamination level in the environment. For example, the U.S. Environmental Protection Agency (US-EPA) has set the maximum contaminant limit of lead in drinking water to 15 ppb or 72 nM (http://water.epa.gov/drink/contaminants/index.cfm#List).

For lead detection, atomic absorption/emission spectrometry (AAS/AES) and inductively coupled plasma mass spectrometry (ICP-MS) are well-known conventional methods with high sensitivity [4,5]. Simultaneously, colorimetric and electrochemical methods have also been extensively pursued to meet the demands of low-cost and on-site detection, not only for lead ions (Pb^{2+}) but also for other hazardous heavy metal ions (Hg^{2+} and Cd^{2+}). [6,7] Meanwhile, since the finding of the lead-specific DNAzyme (termed 17E), the enzymatic oligonucleotide has been applied to *on-site* Pb^{2+} detection as an alternative lead monitoring system [8]. The DNAzyme can cleave the complementary substrate strand (termed 17DS) when Pb^{2+} ion interacts with it. This catalytic reaction was usually monitored by spectrophotometric methods and largely satisfies the demands of detecting Pb^{2+} ions in aqueous environments. For most applications using free DNAzyme-substrate

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pairs, the issue of re-collecting (or recycling) the DNAzyme has not garnered the attention of many researchers [9-12].

II. Materials and Methods

The 5'-fluroescein-substrate strand (cy5-$(CH_2)_6$-5'-ACTCACTATrAGGAAGAGATG) and 5'-thiolated DNAzyme (SH-$(CH_2)_6$-5'-AAAAAAAAACATCTCTTCTCCGAGCCG GTC-GAAATAGTGAGT) were purchased from Bioneer Inc. (Daejeon, Korea) and used as received. Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), hexadecyltri-methylammonium bromide (CTAB), sodium borohydride, ascorbic acid, silver nitrate, and lead acetate trihydrate were purchased from Sigma- Aldrich. All other chemicals were also purchased from Sigma-Aldrich.

1. Synthesis of gold nanorods

Gold nanorods (GNRs) were synthesized by seed-mediated growth method in aqueous CTAB solution [14]. GNRs are washed twice with distilled water, using centrifugation at 15,000 rpm for 30 min, and preserved in distilled water for further modification.

2. DNAzyme immobilization on GNRs

The thiolated DNAzyme was mixed with GNRs for 24 hr at room temperature. Then, unbound DNAzymes were removed by centrifugation at 15000 rpm for 30min. GNR (-DNAzyme) conjugates were stored in HEPES buffer (50 mM HEPES-NaOH, 50 mM NaCl, pH 7.3).

3. Pb^{2+} detection and reusability test

GNR conjugates and Cy5-substrate DNA strands were incubated in 1:1,000 molar ratio at room temperature for 24 hr. Unbound Cy5-substrates were removed by centrifugation at 10,000 rpm for 30 min. Pb^{2+} samples were prepared in 10 nM to 1 μM range of concentration. Each Pb^{2+} ion samples were then added to 2nM of substrate-hybridized GNR conjugates and then incubated for 20 min. The fluorescence intensity (FL intensity) of the mixture was measured by using LS45 luminescence spectrometer (Perkin Elmer, USA). Once used GNR conjugates were recollected by centrifugation and stored in $4^{o}C$ for 3 days. Those GNR conjugates were again hybridized with Cy5-substrate as described above, and then used in Pb^{2+} detection. Each trial was carried out at 3 day interval.

III. Results and Discussion

Among fluorescence-mediated detection schemes with DNAzymes, Kim et al. employed a 5'-fluorescent substrate strand that was conjugated with gold nanoparticles (GNPs) [9]. Since GNP functions as an excellent fluorescent quencher, only weak background fluorescence was observed in its resting mode, with non-reactive substrates strands or in the absence of Pb^{2+} ions. However, strong '*turn-on*' fluorescence could be observed in the presence of Pb^{2+} ions by breaking substrate strands on GNPs (DNAzyme activity). The sensitivity of this sensor was high enough to satisfy the safety limit in drinking water [9]. Guided by their work, we herein demonstrate a reusable fluorescence Pb^{2+} sensor by tethering the DNAzyme part instead of its substrate. As depicted in Fig. 1, this simple modification helps conserve the core DNAzyme moiety after one measurement, by simply centrifuging it along with anchoring nanoparticles.

In a previous study, GNPs (~2 nM) were conjugated with fluorescent substrate strands is sufficient quantity to cover the whole surface area of GNPs, and free DNAzymes (~1 μM) in a buffer solution were allowed to hybridize with the GNP-conjugated substrates and to cleave them for 20 min. The most noticeable advantage

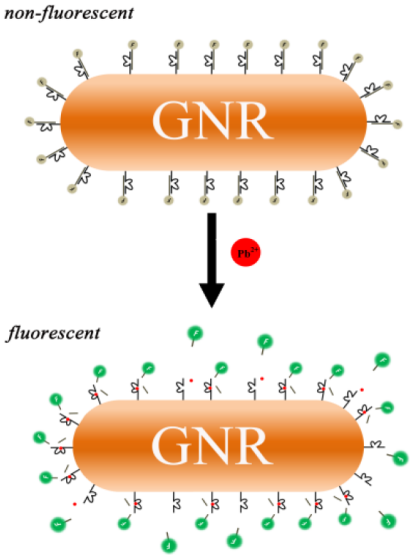

Figure 1. A schematic illustration of Pb^{2+} detection by cleaving the fluorescent substrate strand with the DNAzyme conjugated gold nanorod (GNR).

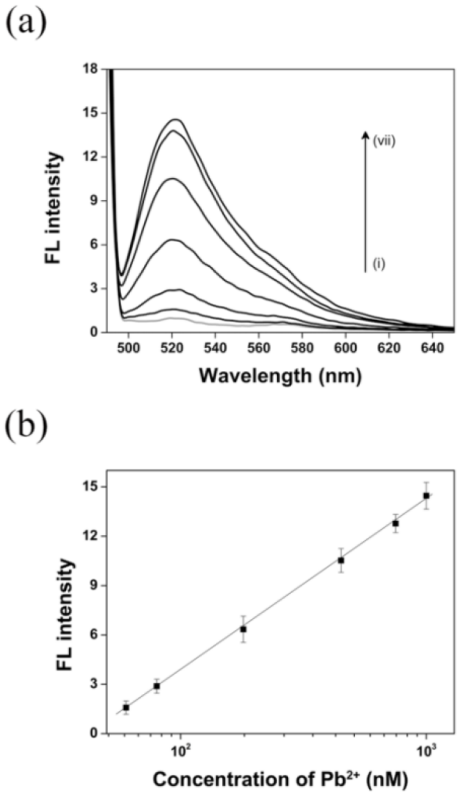

Figure 2. (a) Pb^{2+}–dependent fluorescence intensity changes over emission wavelength. The concentrations of Pb^{2+} ions: (i) 0 nM, (ii) 60 nM, (iii) 80 nM, (iv) 180 nM, (v) 450 nM, (vi) 750 nM, (vii) 1 μM, and (b) the corresponding plot against concentrations of Pb^{2+} ions in logarithmic scale ([GNR]=2 nM; reaction time, 20 min).

in this scheme is that sophisticated equipment is not required, and it is thus assumed to be suitable for *on–site* detection with high sensitivity (limit of detection, LOD=5 nM). As mentioned earlier, we simply switched the tethered substrate strand to the DNAzyme; GNPs were conjugated with thiolated DNAzyms (17E) and the fluorescent substrate strands (17DS) were exogenously provided to hybridize with the tethered DNAzymes. The fluorescent 17DS was believed to be initially quenched by GNPs and diffused out in the presence of Pb^{2+} ions. The other change was the use of heavier gold nanorods (GNRs) instead of GNPs so that recollection of GNR conjugates could be achieved by simple centrifugation.

As shown in Fig. 2, we demonstrated that this minor change contributes to a reusable Pb^{2+} detection sensor. Various concentrations of Pb^{2+} samples (ranging from 10 nM to 1000 nM) were incubated with GNR–dsDNA (17E and 17DS pairs) conjugates and fluorescence changes were recorded after 20 min of incubation time. The fluorescence intensity was gradually increased as was the concentration of Pb^{2+} ions (Fig. 2(a)), showing good linearity over the Pb^{2+} concentrations used (see Fig. 2(b)). The measured LOD (~50 nM) with this scheme satisfied the EPA regulation, but the value was ten times higher than that in a previous study by Kim et al. (~5 nM). We assumed that this higher LOD might be caused by crowded DNAzymes on the GNR, which reduce the chance of 17DS to bind to the DNAzyme. Since the maximum fluorescence intensity in the present study is ultimately determined by the total amount of 17DS (fluorescent tagged) bound to the DNAzymes on the GNR surface, the high DNAzyme population appeared to induce steric hindrance and to lower the hybridization efficacy. In fact, according to other studies, tethered oligonucleotides require some space between each other to form a

complementary base pairing with substrate strands, and an adequate spacer sequence on the thiolated end, therefore, helped to enhance the hybridization efficiency [13]. The total amount of fluorescent strands (17DS) thus always would be less than that of tethered DNAzymes and the maximum hybridization efficiency consequently would be less than 100 percent. As such, the amount of fluorescent probes liberated by the addition of Pb^{2+} ions should be substantially lower than that of the previous study.

On the contrary, when 17DS was directly tethered on GNPs by thiol chemistry, as in a previous study [9], a substantially large amount of 17DS could be immobilized on GNPs. At the initial stage of the reaction, free DNAzymes would also be confronted with the same steric hindrance as the tethered DNAzymes would be, owing to the crowded substrates on the surface of GNPs. However, once a few DNAzymes succeeded in the cleavage reaction, the next incoming DNAzymes would overcome the obstacle easily by finding less steric hindrance. Moreover, DNAzymes could act several times repeatedly so that only a small amount of DNAzymes would be needed to cleave the substrates. However, that scheme is not always superior to the reverse strategy for sensor applications. Since the amount of DNAzymes is one of the rate-determining factors in the cleaving reaction, the concentration of DNAzymes has to be strictly controlled at the working concentration of Pb^{2+} ions. Moreover, GNP conjugates synthesized in the same batch should be used to avoid errors from batch-to-batch variation of the tethered DNA density on the GNP surface, as the production yield and/or the size distribution of GNP were hardly reproducible.

Although our new scheme has some drawbacks, they will be quickly solved through several optimization strategies that will provide more freedom in the conformational space between the GNR and DNAzyme-substrate pairs. The optimization of the sequence could be made either by extension of the thiolated tail of DNAzyme to prevent interaction with the GNR surface, or by co-immobilizing poly(ethyleneglycol) chains (or thiolated poly(A) sequences) onto GNR surfaces with the thiolated DNAzyme to give more space between adjacent DNAzymes.

In an attempt to estimate the reusability of the GNR conjugates, once used GNR samples were re-collected by repeated centrifugation three days after the previous usage and incubated with a fresh substrate strand with fluorescent tags. As shown in Fig. 3, GNR-DNAzyme conjugates were reusable at least three more times without any significant function losses. However, an obvious functional loss was observed after the fourth usage, and only half the signaling for the same concentration of Pb^{2+} ions was measured at the sixth recycling. The gradual loss of Pb^{2+}-detecting capability might be caused by partial aggregation of GNRs upon repetitive centrifugation or detachment of the thiolated DNAzymes, due to long-term storage (>14 days) of GNR conjugates. When the re-cycling interval was shortened to 12 hr, no noticeable degradation was observed with repetitive usage of the GNR conjugates, suggesting that the long storage in an aqueous solution might be the main reason for the functional degradation. Although

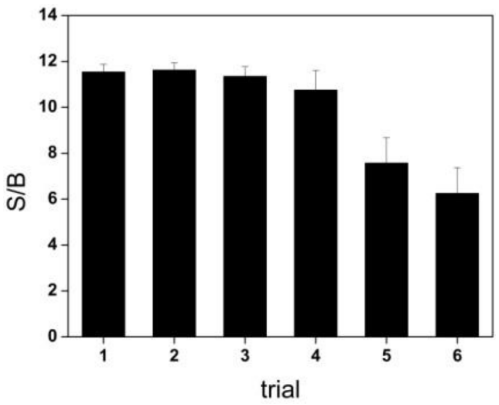

Figure 3. Reusability test of DNAzyme-GNR conjugates, accomplished by monitoring the fluorescence signal at a fixed concentration of Pb^{2+} ions (500 nM). Each trial was performed with 3 day-intervals at a concentration of GNR (2 nM).

it was shown that a reusable aptasensor of Pb^{2+} ions is possible, reloading the substrate strands (17DS) after single assay appears to be another bottleneck to overcome in realizing an '*on-site*' aptasensor since it took at least 12 hrs and special care was required to maintain sufficient reproducibility. Obviously, the reloading of substrate strands is also related with the crowded DNAzymes on the GNR surface and a simple scheme that can mitigate the crowded condition is being pursued. For controlling the immobilization density of DNAzyme, loading inert spacer groups and applying longer DNAzymes with poly(A) are now respectively under investigation.

IV. Conclusions

In summary, we have demonstrated a reusable and fluorescent Pb^{2+} ion detector that has potential to be employed for *on-site* monitoring of hazardous ions by tethering a DNAzyme oligonucleotide onto GNRs. The DNAzyme-GNR conjugate showed a LOD of 50 nM, and could be easily recollected by simple centrifugation and recycled at least three times while retaining almost the same sensitivity. Further recycling resulted in some deterioration of Pb^{2+} detecting capability, which was partially ascribed to the loss of the tethered DNAzymes with long storage in the aqueous solution.

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