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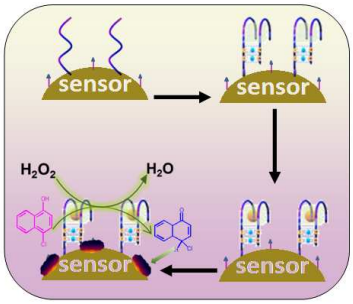

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ARTICLE TYPE

Target-initiated impedimetric proximity ligation assay with DNAzyme design for *in situ* amplified biocatalytic precipitation†

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Target-initiated proximity ligation assay (PLA) protocol accompanying DNAzyme formation was for the first time designed for ultrasensitive impedimetric monitoring of heavy metal ion (silver ion used in the case) coupling with enzymatic biocatalytic precipitation.

Environmental pollution caused by heavy metal ions has been an important worldwide concern for decades. Probing traces requires ever more sensitive and selective methods, suitable for acquiring information about interactions and modifications of participating molecules.[1] For development of new methods or improvement of existing ones, there are two basic aspects for obtaining low limits of detection/quantification.[2] The first key point is to exploit a highly efficient signal-amplification strategy. Proximity ligation assay (PLA) is a powerful method capable of detecting proteins, protein-protein interactions and post-translational modifications.[3] Typically, protein-recognition event is converted into detectable DNA molecules. Binding of two or more conjugates to the target results in the assembly of an assay-specific DNA molecule. Unfortunately, the present PLA mainly focuses on detecting proteins, and there is no report on the electrochemical monitoring of metal ions.

Inspiringly, recent researches have demonstrated that cytosine-Ag^+-cytosine (C-Ag^+-C) coordination chemistry and the Ag^+-stabilized hybridization of oligonucleotides with C-C mismatches can be considered as a selective and versatile scheme for the detection of silver ion.[4] Such an assay protocol is usually based on the conformational change of the labelling oligonucleotides with molecular tags (*e.g.* ferrocene and methylene blue).[5] Unfavourably, most methods reported were the 'signal-off' assay systems, demanding a high background signal for development of the prepared sensors. Certainly, a few electrochemical sensors were also constructed through the 'signal-on' assay mode. However, they usually involved in the nanomaterials and enzyme labels. In this regard, our motivation is to combine the C-Ag^+-C coordination chemistry with the PLA technique for the formation of electroactive materials, *e.g.* DNAzyme, upon target analyte introduction.

Another important issue is to adopt a simple and sensitive signal-transduction method. Impedimetric measurement is an effective tool to probe the interfacial properties of a modified electrode.[6] Frequency dependence in the impedance yields useful information about the adsorption kinetics and the dynamics of charge transfer at the interface are strongly influenced by the nature of the electrode surface and the structure of the electrical double layer.[7] To acquire a high sensitivity, the amplification of electronic signal is extremely important. Enzymatic biocatalytic precipitation, involving in the formation of insoluble product on the electrode, is a feasible way for the extension of the detection limits *via* mass amplification in the biosensing field.[8] The precipitation of the product onto the electrode provides a means to amplify the biorecognition (biosensing) event occurring on the electrode, which can block the electron transfer process of a redox probe (such as ferricyanide ion).

Herein, we report the proof-of-concept of a novel and powerful impedimetric sensing strategy for sensitive detection of silver ion (Ag^+) coupling target-induced PLA with DNAzyme formation for *in situ* amplified biocatalytic precipitation (Fig. 1, see ESI† for experimental details). Initially, the sensing platform is simply prepared by immobilizing probe S_1 (5'-SH-AAAAATCTCTGTG GAGGG-3', one split part of DNAzyme) on the gold electrode through the classical Au-S bond. Then, the as-prepared electrode is used for challenging with target silver ion in the presence of probe S_2 (S_2: 5'-GGGCAGGGGACACA-3', another split part of DNAzyme). During this process, two split parts can be ligated together to form the DNAzyme upon addition of hemin. The formed DNAzyme can catalyze 4-chloro-1-naphthol (4-CN) to produce an insoluble benzo-4-chlorohexadienone precipitate on the electrode in the presence of H_2O_2, thus resulting in the increasing impedimetric signal. By monitoring the change in the resistance, we can quantitatively determine the concentration of target silver ion in the sample. In the absence of target Ag^+, the hemin/G-quadruplex-based DNAzyme can not be formed, thereby displaying a weak resistance (background signal).

To realize our design, one precondition for development of the PLA-based sensing strategy was whether the insoluble precipitate could be achieved in the presence and absence of target Ag^+. To monitor this point, the topology of the S_1-modified gold electrode was studied by using atomic force microscope (AFM) before and after precipitation toward zero analyte and 10 nM Ag^+ (used as an example), respectively (Fig. 2). Fig. 2a and Fig. 2c show AFM images of S_1-modified gold substrate after incubation with (10 nM Ag^+ + S_2 + hemin) and (S_2 + hemin), respectively. It is obvious that the surface in the presence of Ag^+ (Fig. 2a) was rougher than that in the absence of target analyte (Fig. 2c). The reason might be the fact that the added Ag^+ ion could promote the ligation of probes S_1 and S_2 to form the hemin/G-quadruplex-based DNAzyme. To further investigate that the formed complex

could readily catalyze 4-CN to produce an insoluble product, the above-obtained gold substrates were incubated with excess 4-CN in the presence of H_2O_2, respectively. Significantly, numerous particles were observed on the surface of gold substrate in the presence of 10 nM Ag^+ (Fig. 2b). In contrast, the topology was not almost changed in the absence of Ag^+ (Fig. 2d). These results preliminarily revealed that (i) target Ag^+ analyte could induce the formation of DNAzyme in the simultaneous presence of probe S_2 and hemin, and (ii) our design could be utilized for the detection of silver ion.

Logically, another question arises as to whether the PLA-based assay protocol could be conducive to the amplification of the impedimetric signal with the aid of enzymatic biocatalytic precipitation (BCP). To clarify this issue, the as-prepared sensor was utilized for detection of 10 nM Ag^+ (as an example), which was studied using electrochemical impedance spectroscopy (EIS) before and after precipitation (Fig. 3A). The judgment was based on the change in the resistance relative to background signal. As seen from Fig. 3A, use of BCP could caused a 402.7 ± 39% signal increase of the sensor, while the signal only increased 119.1 ± 15% without the BCP. The strong signal shift in the resistance mainly derived from the formation of insoluble benzo-4-chlorohexadienone precipitate on the electrode. However, one puzzling question to be produced was whether the stronger signal derived from target-initiated DNAzyme formation for the development of BCP. To verify this concern, the newly prepared sensor was used for the determination of zero analyte, and the Nyquist diagrams were investigated before and after using the BCP. Relative to curve '*a*', the resistance of S_1-modified electrode after reaction with S_2 and hemin was slightly increased (curve '*b*'). The reason might be most like a consequence of the fact that partial S_2 probes were assembled to probe S_1. Significantly, the resistance after precipitation with 4-CN (data not shown) was almost the same as that curve '*b*'. The results revealed that (i) hemin/G-quadruplex-based DNAzyme could not be formed in the absence of target Ag^+ ion, and (ii) the amplification of impedimetric signal mainly originated from the formed DNAzyme toward the catalytic precipitation of 4-CN.

As control test, the impedimetric signals of variously modified electrodes were investigated in the absence of hemin toward 4-CN (10 nM Ag^+ used in this case). Curve '*a*' in Fig. 3B shows the EIS of the bare gold electrode. There was a very small semicircle domain, implying a low resistance to the redox probe dissolved in the electrolyte solution. When probe S_1 was modified onto the gold electrode, the resistance increased because of the repulsive interaction between negatively charged DNA and $[Fe(CN)_6]^{4-/3-}$ (curve '*b*'). Moreover, the resistance increased again when the as-prepared electrode reacted with 10 nM Ag^+ and probe S_2 (curve '*c*'). The reason might be attributed to the fact that the formation of the C-Ag^+-C coordination chemistry hindered the transfer of $[Fe(CN)_6]^{4-/3-}$ from the solution to the electrode. More inspiringly, the absence of hemin in the G-quadruplex could not cause the significant increase in the impedimetric signal (curve '*d*') [For comparison, the S_1-modified electrode was used for incubation with 4-CN/H_2O_2 system, and the resistance was almost the same as that of curve '*b*' (curve '*e*'), indicating that probe S_1 could not catalyze the precipitation of 4-CN]. The results further revealed that the amplification of the impedimetric signal mainly derived from target-induced formation DNAzyme.

To acquire an optimal analytical performance, some factors influencing the analytical properties of the PLA-based assay method were also studied. Experimental results indicated that the optimal hybridization time for the $S_1/S_2/Ag^+$ and the catalytic precipitation time for 4-CN were 60 min and 10 min, respectively (Fig. S1†). Under optimal conditions, the sensitivity and dynamic range of PLA-based impedimetric sensor were monitored with Ag^+ standards by using the developed strategy. It is obvious that the resistances displayed a dependence upon the level of the target Ag^+ (Fig. 4a). As seen from Fig. 4a, the electron-transfer resistances, R_{et}, increased with the increment of target Ag^+ concentration in the sample. A linear dependence between the electron-transfer resistances and the target Ag^+ concentrations were obtained in the range from 0.1 to 25 nM. The detection limit (LOD) was estimated to be 0.08 nM at the signal-to-noise ratio of 3. Although the system has not yet been optimized for maximum efficiency, the linear range and detection limit were comparable with other Ag^+ assay methods reported previously (Table S1†). Importantly, the LOD toward Ag^+ ion was largely lower than that defined by the United states Environmental Protection Agency (~460 nM).

The selectivity of the impedimetric sensor was monitored by challenging the system with other metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{3+}). In this case, the comparison was performed by assaying 1 μM of interfering ions with 10 nM target Ag^+, respectively. As shown in Fig. 4b, low signals were obtained toward these components only at much higher concentrations compared to the target analyte. Hence, the specificity of the PLA-based assay scheme was acceptable.

The precision and reproducibility of the PLA-based impedimetric sensor were also evaluated by repeatedly assaying three different Ag^+ levels, using identical batches of impedimetric sensors. Experimental results indicated that the coefficients of variation (CVs) of using the same-batch sensors were 8.9%, 8.6%, and 9.2% at 0.5 nM, 1 nM and 10 nM Ag^+, respectively, whilst the CVs of using various-batch sensors were 10.4%, 8.8% and 9.7% toward the mentioned-above analytes, respectively. The low CVs revealed the possibility of batch preparation.

The high specificity and reproducibility of the PLA-based impedimetric sensor suggest that the developed method might be directly applied for analysis of silver ion in the complex sample matrix. To exploit this point, 4 Ag^+ standards including 0.5 nM, 1 nM, 5 nM and 10 nM were initially spiked into blank tap-water solution, and then the spiked water samples were measured by using the developed impedimetric sensor. The concentration of Ag^+ ions in these samples were calculated according to the above-mentioned regression equation. Experimental results indicated that the recoveries were 99.3%, 103.5%, 88.9% and 94.2%, respectively. Hence, the PLA-based impedimetric senor could be considered as an optional strategy for Ag^+ detection in real samples.

As a proof-of-principle demonstration, we have successfully built a simple and convenience impedimetric sensor for sensitive Ag^+ detection coupling with target-activated proximity ligation assay with DNAzyme formation for *in situ* amplified biocatalytic precipitation. The assay was based on a DNA assembly with C-C

mismatched base-pairs as Ag^+-recognition sites in the appended duplex and the interactions between two G-quadruplex halves for signal transductions. The C-Ag^+-C base pairs strengthened the G-quadruplex for binding hemin, presenting high efficiency in the accelerating oxidation of 4-CN by H_2O_2 to yield the insoluble product benzo-4-chlorohexadienone on the transducer surface. The signal was highly enhanced by the biocatalytic precipitation amplification route, allowing the determination of silver ions down to 0.08 nM. Moreover, the impedimetric sensor afforded exquisite selectivity for target silver ions against other metal ions owing to the specific C-Ag^+-C binding reaction. Therefore, the PLA-based impedimetric sensor holds great practicality for the detection of heavy metal ions in environmental samples.

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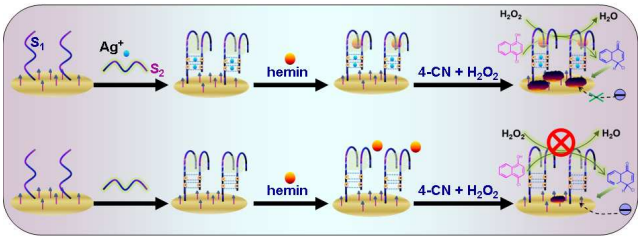

Fig. 1 Schematic illustration of PLA-based impedimetric sensing platform for *in situ* amplified biocatalytic precipitation coupling with target-induced DNAzyme formation.

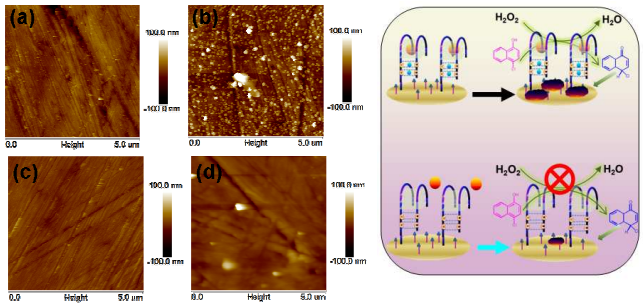

Fig. 2 AFM images of (a) S_1-modifed gold substrate after incubation with 10 nM Ag^+ + S_2 + hemin, (b) substrate '*a*' after reaction with 4-CN + H_2O_2, (c) S_1-modifed gold substrate after incubation with S_2 + hemin, and (d) substrate '*c*' after reaction with 4-CN + H_2O_2.

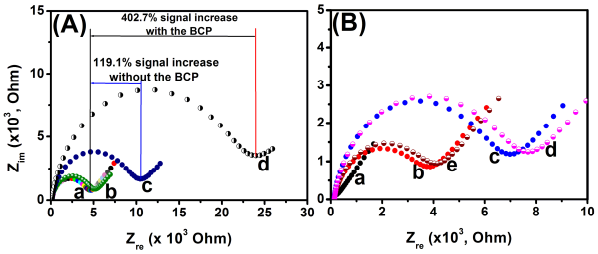

Fig. 3 (A) Nyquist diagrams for (a) the prepared impedimetric sensor, (b) sensor '*a*' + S_2 + hemin, (c) sensor '*a*' + 10 nM Ag^+ + S_2 + hemin, and (d) sensor '*c*' + 4-CN + H_2O_2, and (B) Nyquist diagrams for (a) bare gold (Au) electrode, (b) S_1/Au, (c) S_2/Ag^+/S_1/Au, (d) sensor '*c*' + 4-CN + H_2O_2, and (e) sensor '*b*' + 4-CN + H_2O_2 in 2 mM $Fe(CN)_6^{4-/3-}$ + 0.1 M KCl with the range from 10^{-2} to 10^6 Hz at an alternate voltage of 10 mV.

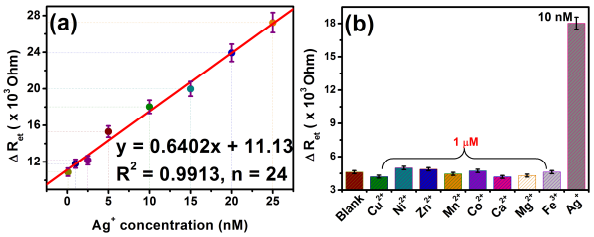

Fig. 4 (a) Calibration plots of PLA-based impedimetric sensor toward Ag^+ standards in 2 mM $Fe(CN)_6^{4-/3-}$ + 0.1 M KCl with the range from 10^{-2} to 10^6 Hz at an alternate voltage of 10 mV (error bars: SD, n = 3), and (b) resistance changes of PLA-based impedimetric sensor against Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+} and target Ag^+.

Notes and references

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† Electronic supplementary information (ESI) available: Experimental procedures and condition optimization. See DOI: 10.1039/xxxxxxx

1 (*a*) H. Chang and H. Wu, *Energ. Environ. Sci.*, 2013, **6**, 3483; (*b*) S. Papirio, D. Villar-Gomez, G. Esposito, F. Pirozzi and P. Lens, *Crit. Rev. Env. Sci. Technol.*, 2013, **43**, 2545; (*c*) T. Huynh, B. Sun, L. Li, K. Nichols, J. Koyner and R. Ismagilov, *J. Am. Chem. Soc.*, 2013, **135**, 14775.

2 (*a*) B. Zhang, D. Tang, R. Goryacheva, R. Niessner and D. Knopp, *Chem. Eur. J.*, 2013, **19**, 2496; (*b*) D. Tang, B. Liu, R. Niessner, P. Li and D. Knopp, *Anal. Chem.*, 2013, **85**, 10589.

3 (*a*) R. Nong, D. Wu, J. Yan, M. Hammond, G. Gu, M. Kamali-Moghaddam, U. Landegren and S. Darmanis, *Nat. Protoc.*, 2013, **8**, 1234; (*b*) I. Weibrecht, E. Lundin, S. Kiflemariam, M. Mignardi, I. Grundberg, C. Larsson, B. Koos, M. Nisson and O. Soderberg, *Nat. Protoc.*, 2013, **8**, 355; (*c*) J. Hu, T. Wang, J. Kim, C. Shannon and C. Easley, *J. Am. Chem. Soc.*, 2012, **134**, 7066; (*d*) V. Castro-Lopez, J. Elizalde, M. Pacek, E. Hijona and L. Bujanda, *Biosens. Bioelectron.*, 2014, **54**, 499; (*e*) G. Rishi, E. Crampton, D. Wallace and V. Subramaniam, *PloS One*, 2013, **8**, e77267.

4 (*a*) W. Xie, W. Huang, N. Li and H. Luo, *Chem. Commun.*, 2012, **48**, 82; (*b*) C. Tang, N. Bu, X. He and X. Yin *Chem. Commun.*, 2011, **47**, 12304; (*c*) D. Feng, G. Liu, W. Zheng, J. Liu, T. Chen and D. Li, *Chem. Commun.*, 2011, **47**, 8557; (*d*) S. Shimron, J. Elbaz, A. Henning and I. Willner, *Chem. Commun.*, 2010, **46**, 3250; (*e*) A. Porchetta, A. Vallee-Belisle, K. Plaxco and F. Ricci, *J. Am. Chem. Soc.*, 2013, **135**, 13238; (*f*) T. Funai, Y. Miyazaki, M. Aotani, E. Yamaguchi, O. Nakagawa, S. Wade, H. Torigoe, A. Ono and H. Urade, *Angew. Chem., Int. Ed.*, 2012, **51**, 6464.

5 (*a*) Z. Lin, X. Li and H. Kraatz, *Anal. Chem.*, 2011, **83**, 6896; (*b*) Y. Wang, J. Li, H. Wang, J. Jin, J. Liu, K. Wang, W. Tan and R. Yang, *Anal. Chem.*, 2010, **82**, 6607; (*c*) A. Ono, H. Torigoe, Y. Tanaka and I. Okamoto, *Chem. Soc. Rev.*, 2011, **40**, 5855; (*d*) C. Tang, N. Bu, X. He and X. Yin, *Chem. Commun.*, 2011, **47**, 12304.

6 (*a*) J. Zhuang, L. Fu, W. Lai, D. Tang and G. Chen, *Chem. Commun.*, 2013, **49**, 11200; (*b*) J. Tang, D. Tang, J. Zhou, H. Yang and G. Chen, *Chem. Commun.*, 2012, **48**, 2627.

7 (*a*) C. Macgriff, S. Wang, P. Wiktor, W. Wang, X. Shan and N. Tao, *Anal. Chem.*, 2013, **85**, 6682; (*b*) T. Alonso-Garcia, M. Rodriguez-Presa, C. Gervasi, S. Moya and O. Azzaroni, *Anal. Chem.*, 2013, **85**, 6561; (*c*) C. Ocana, N. Malashikhina, M. del Valle and V. Pavlov, *Analyst*, 2013, **138**, 1995.

8 (*a*) J. Zhou, J. Tang, G. Chen and D. Tang, *Biosens. Bioelectron.*, 2014, **54**, 323; (*b*) L. Hou, Y. Cui, M. Xu, Z. Gao, J. Huang and D. Tang, *Biosens. Bioelectron.*, 2013, 47, 149; (*c*) W. Zhao, Z. Ma, P. Yu, X. Dong, J. Xu and H. Chen, *Anal. Chem.*, 2012, **84**, 917.