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# DNAzyme-functionalized gold–palladium hybrid nanostructures for triple signal amplification of impedimetric immunosensor

Li Hou [a], Zhuangqiang Gao [a], Mingdi Xu [a], Xia Cao [b,*], Xiaoping Wu [a], Guonan Chen [a], Dianping Tang [a,**]

[a] Key Laboratory of Analysis and Detection for Food Safety, Ministry of Education & Fujian Province, Department of Chemistry, Fuzhou University, Fuzhou 350116, PR China
[b] School of Biochemical and Pharmaceutical Sciences, Capital Medical University, Beijing 100069, PR China

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ABSTRACT

A highly sensitive and selective impedimetric immunosensor with triple signal amplification was designed for ultrasensitive detection of prostate-specific antigen (PSA) by using anti-PSA antibody and DNAzyme-functionalized gold–palladium hybrid nanotags ($Ab_2$–AuPd–DNA). The signal was amplified based on the $Ab_2$–AuPd–DNA toward the catalytic precipitation of 4-choloro-1-naphthol (4-CN). DNAzyme (as a kind of peroxidase mimic) could catalyze the oxidation of 4-CN, whilst AuPd hybrid nanostructures could not only provide a large surface coverage for immobilization of biomolecules but also promote 4-CN oxidation to some extent. The produced insoluble benzo-4-chlorohexadienone *via* 4-CN was coated on the electrode surface, and hindered the electron transfer between the solution and the electrode, thereby increasing the Faradaic impedance of the base electrode. Three labeling strategies including $Ab_2$–AuNP, $Ab_2$–AuPd and $Ab_2$–AuPd–DNA were investigated for determination of PSA, and improved analytical features were obtained with the $Ab_2$–AuPd–DNA strategy. Under optimal conditions, the dynamic concentration range of the impedimetric immunosensor spanned from 1.0 pg $mL^{-1}$ to 50 ng $mL^{-1}$ PSA with a detection limit of 0.73 pg $mL^{-1}$. Intra- and inter-assay coefficients of variation were below 8.5% and 9.5%, respectively. Importantly, no significant differences at the 0.05 significance level were encountered in the analysis of 6 clinical serum specimens and 6 diluted standards between the impedimetric immunosensor and the commercialized electrochemiluminescent method for PSA detection.



## 1. Introduction

A sensitive and accurate detection method of biomarkers is important in early diagnosis and clinical therapy (Wu et al., 2003). Nowadays, various methods and strategies have been developed for detection of cancer biomarkers, such as enzyme-linked immunosorbent assay, fluorescence immunoassay, chemiluminescence immunoassay, electrochemical immunoassay, and radioimmunoassay (Jin et al., 2013; Pei et al., 2013; Tang et al., 2013). Among these methods, electrochemical immunoassay has received a widespread attention owing to its simple instrumentation, fast response time, and inexpensive cost (Tang et al., 2013). Typically, antibody-based immunoassay is a useful tool for the monitoring of biomarkers (Zhang et al., 2012a, 2012b). Normally, the direct reaction between the antigen and the antibody is insufficient to produce a strongly detectable signal (Zhang et al., 2012a, 2012b). Hence, exploiting a novel signal amplification strategy based on the electrochemical principle for sensitive determination of low-abundance protein is very valuable.

Recently, variously labeled methods were employed for the amplification of detectable signal, e.g. by native enzymes, enzyme mimics and nanolables (Gao et al., 2013; Vidal et al., 2013; Yu et al., 2013; Li et al., 2013). Typically, enzyme labels are utilized widely in the electrochemical immunoassays. For example, peroxidase is one of protein-based enzymes that act as catalysts to facilitate a variety of biological processes (Tang et al., 2012). Applying the peroxidase in the biocatalytic precipitation procedure can produce a nonconductive insoluble product on the electrode surface, and the insoluble precipitation can block the electron transfer process of redox probes (Akter et al., 2012), e.g. $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$. The interfacial electron-transfer feature of redox probes such as capacitance and resistance can be monitored by electrochemical impedance spectroscopy (EIS) (Guo et al., 2011). Most recently, our group devised a graphene oxide-labeled impedimetric immunoassay by coupling with horseradish peroxidase-based catalytic precipitation of 4-CN (Hou et al., 2013). However, we later found that partial graphene oxide nanosheets might be reduced during the enzymatic biocatalytic precipitation, and the reduced graphene could promote the electron transfer to

* Corresponding author.
** Correspondence to: Key Laboratory of Analysis and Detection for Food Safety (Ministry of Education), Department of Chemistry, Fuzhou University, Fuzhou 350108, PR China. Tel.: +86 591 2286 6125; fax: +86 591 2286 6135.
*E-mail address:* dianping.tang@fzu.edu.cn (D. Tang).

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some extent, thus decreasing the Faradaic impedance of the electrode. Meanwhile, two-dimensional graphene nanosheets might not facilitate the antigen–antibody reaction owing to the steric-hindrance effect. More importantly, the bioactivity of native enzymes is easily lost under harsh condition (Wild, 2005). To tackle this point, our motivation is to design a new impedimetric immunosensor for determination of low-abundance protein by coupling the nanocatalyst with enzymatic catalytic precipitation technology.

Peroxidase-mimicking split G-quadruplex DNAzyme, formed by guanine-rich nucleic acid sequences and intercalated hemin, has attracted particular interest of researchers due to its distinguishing features, e.g., convenient tagging, low cost, and more stable against hydrolysis and heat treatment (Yuan et al., 2012; Zhu et al., 2013). The hemin-binding DNAzyme can usually exhibit electrocatalytic activity toward $H_2O_2$-mediated oxidation (Pelossof et al., 2010; Tang et al., 2012). Although the catalytic activity of DNAzyme is lower than native HRP, DNAzyme is easier to synthesize, less expensive and more stable (Zhu et al., 2011). Moreover, DNAzyme-labeled gold nanoparticles could display high catalytic activity toward reduction of $H_2O_2$ (Zhou et al., 2013). So, we suspect that DNAzyme can be considered as peroxidase mimics for catalytic oxidation of 4-CN to form an insoluble precipitation on the substrate (Fig. S1 in the Supplementary material).

Palladium nanoparticles, due to their highly efficient catalytic performance for facilitating the reaction of both oxygen reduction and hydrogen oxidation, have been widely applied in fuel cell catalysis, photocatalytic water splitting into hydrogen, and fine chemistry catalysis (Lim et al., 2009; Kobayashi et al., 2008; Das et al., 2009; Tappan and Steiner, 2010). One of the well-known reactions catalyzed by palladium is the Suzuki coupling reaction, which is a powerful and convenient synthetic method in organic chemistry to generate liquid crystals (Li et al., 2010). However, it is usually difficult for pure palladium nanoparticles to directly conjugate with proteins or biomolecules. In contrast, hybrid nanostructures display properties different from their individual constituents to create a new property (Hsieh et al., 2011). Gold nanoparticle is an ideal one in biotechnological systems due to its inherent advantages, such as easy preparation, good biocompatibility, and so on (Zhang et al., 2012b).

Prostate-specific antigen, also known as gamma-seminoprotein or kallikrein-3 (KLK3), is a glycoprotein enzyme encoded in humans by the *KLK*3 gene (Salimi et al., 2013). PSA is present in small quantities in the serum of men with healthy prostates, but is often elevated in the presence of prostate cancer or other prostate disorders (Jeong et al., 2013). Hence, sensitive determination of PSA is very crucial. Herein, we report the proof-of-concept of a new impedimetric immunoassay for the detection of PSA by using gold–palladium hybrid nanostructure (AuPd) and DNAzyme. To construct such an impedimetric immunosensor, AuPd hybrid nanostructures heavily functionalized with DNAzyme and anti-PSA antibody. In the presence of target PSA, the labeled anti-PSA on the AuPd and the immobilized capture antibody on the electrode sandwich the target PSA, generating a complex with a large ratio of DNAzyme and target PSA. Upon addition of 4-CN and $H_2O_2$, DNAzyme and AuPd could catalyze the 4-CN oxidation, and produce an insoluble benzo-4-chlorohexadienone product on the electrode surface, thus increasing the Faradaic impedance. By monitoring the change in the resistance, we might quantitatively determine the level of target PSA in the sample.

## 2. Experimental

### 2.1. Chemicals

Monoclonal mouse anti-human PSA antibody (ab130880, designated as $Ab_1$) was purchased from Abcam Inc. (Cambridge, MA). Polyclonal goat anti-human PSA antibody (designated as $Ab_2$) was purchased from ImmunoReagents, Inc. (Raleigh, NC). PSA standards were obtained from Biocell Biotechnol. Co., Ltd. (Zhengzhou, China). Hemin was purchased from Tokyo Chem. Inc. (Japan). β-Cyclodextrin (CD), bovine serum albumin (BSA), $HAuCl_4 \cdot 4H_2O$ and 4-CN were purchased from Sinopharm Chem. Re. Co. (Shanghai, China). Oligonucleotide designed in this study was synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China), which was purified by high-performance liquid chromatography and confirmed by mass spectrometry. The sequence of the hemin-based aptamer is listed as follows: 5′-SH-GGG TAG GGC GGG TTG GGT-3′. DNA stock solution was obtained by dissolving oligonucleotides in 0.01 M phosphate-buffered saline (PBS, pH 7.4) solution. Each oligonucleotide was heated to 90 °C for 5 min, and slowly cooled down to room temperature before use. Hexadecylpyridinium chloride (HDPC), $K_2PdCl_6$ and ascorbic acid were purchased from Aladdin (Shanghai, China). All other chemicals were of extra pure analytical grade and used without further purification. All solutions were prepared with deionized water obtained from a Milli-Q water purifying system ( ≥ 18 MΩ, Milli-Q, Millipore). The washing buffer solution was 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. The blocking buffer was 0.01 M PBS (pH 7.4) containing 2.5 wt% BSA. Clinical serum samples were made available by Fujian Provincial Hospital, China.

### 2.2. Synthesis of gold–palladium hybrid nanostructures (AuPd)

AuPd hybrid nanostructures were synthesized and prepared according to the literature with a little modification (Huang et al., 2013). Briefly, $K_2PdCl_6$ (10 mM, 0.4 mL), $HAuCl_4$ (10 mM, 0.4 mL) and HDPC (18 mg) were initially added into 5-mL distilled water in turn, and then 0.3-mL ascorbic acid (0.1 M) was quickly injected into the mixture with gentle shaking. Afterwards, the resulting mixture was incubated for 3 h at 35 °C without stirring under the sealed condition until the color of the solution changed from light yellow to deep brown. Following that, the as-produced AuPd hybrid nanostructures were collected by centrifugation at 8,000*g* for 10 min. Finally, the obtained AuPd nanostructures were re-dispersed in 1-mL distilled water.

### 2.3. Conjugation of AuPd with $Ab_2$ and DNAzyme ($Ab_2$–AuPd–DNA)

The $Ab_2$–AuPd–DNA nanocomplexes were synthesized similar to our previous report (Zhang et al., 2012a). Initially, 100 μL of the prepared-above AuPd suspension was adjusted to pH 9.0–9.5 with $NaCO_3$, then 200 μL of $Ab_2$ (100 μg $mL^{-1}$) and 200 μL of hemin-based aptamer (5 OD) were simultaneously added into the suspension, and then incubated overnight at 4 °C with gentle stirring. Following that, the mixture was centrifuged (6000*g*) for 10 min at 4 °C to remove the excess aptamer and antibody. Subsequently, 500 μL of 0.2 mM hemin aqueous solution was added into the precipitate, and incubated 35 min at room temperature (RT) to form the DNAzyme. Finally, the resulting suspension was centrifuged (6000*g*) for 10 min at 4 °C, and the obtained $Ab_2$–AuPd–DNA were re-dispersed into 1.0-mL PBS (0.01 M, pH 7.4) containing 1.0 wt% BSA and stored at 4 °C for further use.

### 2.4. Fabrication of impedimetric immunosensor

A glassy carbon electrode (GCE, 3 mm in diameter) was polished repeatedly with 1.0 and 0.3 μm alumina slurry, followed by successive sonication in distilled water and ethanol for 5 min, and dried in air. After washing, the cleaned GCE was cycled for five times in a 0.1 M $H_2SO_4$ within the potential range from 0 to 2 V. During this process, the anodization of the GCE surface resulted in a multilayer oxide film with –OH or –COOH (Won et al., 2005).