ELECTRONIC SUPPORTING INFORMATION (ESI)

New voltammetric immunosensing platform for prostate-specific antigen based on Cu(II)-pyrophosphate ion chelation reaction

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EXPERIMENTAL SECTION

Chemical and reagent. Monoclonal mouse anti-human prostate-specific antigen antibody (mAb_1; clone 8301, unconjugated), rabbit polyclonal to prostate-specific antigen (pAb_2; unconjugated) and human total prostate-specific antigen ELISA kit containing PSA standards with different levels (sensitivity: 9.4 pg mL^{-1}; range: 31.25–2000 pg mL^{-1}; cat# ab188388) were purchased from Abcam (Shanghai, China). All high-binding polystyrene microplates were acquired from Greiner Bio-One GmbH (Frickenhausen, Germany). Gold chloride, bovine serum albumin (BSA; Vetec™, reagent grade, ≥98%), inorganic pyrophosphatase (PPase, EC 3.6.1.1, MW 71 kDa, p*I*: 4.75) from baker's yeast (*S. cerevisiae*) (≥ 90%, HPLC, lyophilized powder, ≥ 1000 units mg^{-1} protein) (note: One-unit PPase can liberate 1.0-μmol inorganic orthophosphate per min at pH 7.2 at 25 ºC referring to Sigma's unit definition), Nafion® 117 solution (~5% in a mixture of lower aliphatic alcohols and water), and citric acid were acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All synthetic procedures were conducted by using ultrapure water obtained from a Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA). HEPES buffer [10 mM 4-(2-hydroxyerhyl) piperazine-1-erhanesulfonic acid containing 50 mM KNO_3, Shanghai Chem. Re. Inc.] was used as the supporting electrolyte. All other chemicals were of analytical grade and used as received. Ultrapure water obtained from a Millipore water purification system at 18.2 MΩ cm (Milli-Q, Millipore) was used throughout this work. A pH 9.6 coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3) and a pH 7.4 phosphate-buffered saline (PBS, 10 mM) (2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.24 g KH_2PO_4, 0.2 g KCl and 8.0 g NaCl) were prepared by dissolving these chemicals into 1000 mL ultrapure water, respectively. The blocking buffer and washing buffer were obtained by adding 1.0% BSA (w/v) and 0.05% Tween 20 (v/v) in PBS (pH 7.4, 10 mM) (PBST), respectively.

Preparation of gold nanoparticles (AuNPs). All glassware used in the following procedures was cleaned in a bath of freshly prepared solution (3:1 $K_2Cr_2O_7$-H_2SO_4), thoroughly rinsed with double distilled water, and dried prior to use. The 16-nm-diam Au colloid was prepared by adding 2 ml of 1% (w/w) sodium citrate solution into 50 mL of 0.01% (w/w) $HAuCl_4$ boiling solution.[1] The maximum adsorption of the synthesized colloidal gold in UV-vis spectrum was at 520 nm and the solution was stored in a refrigerator with a dark-colored glass bottle before use. The particle sizes were confirmed by transmission electron microscopy (TEM).

Preparation of PPase/pAb$_2$-conjugated gold Nanoparticles. The PPase/pAb$_2$-conjugated gold nanoparticles (PPase-AuNP-pAb$_2$) were prepared according to previous report.[2] The process could be simply summarized as follows: (i) gold colloids with 18 nm in diameter (AuNP, $C_{[AuNP]}$ = 24 μM) were tuned to pH 8-9 by using 0.1 M Na_2CO_3; (ii) pAb$_2$ (100 μL, 0.1 M) and HRP (100 μL, 0.5 M) were injected into 1.0 mL of the resulting gold colloids (note: The molar ratio of pAb$_2$ and PPase was optimized), and incubated 12 h at 4 °C under gentle stirring; and (iii) the suspension was centrifuged (12,000*g*) for 20 min at 4 °C to obtain the PPase/pAb$_2$-conjugated nanogold particles. Finally, the obtained PPase-AuNP-pAb$_2$ bioconjugates was dispersed into 1.0 mL of pH 7.2 HEPES buffer (10 mM) containing 1.0 wt % BSA ($C_{[AuNP]}$ = 24 μM), and stored at 4 °C until use.

Preparation of Nafion-modified gold-disk electrode. A QCM gold-disk electrode (Φ = 3.0 mm) was cleaned and sonicated in ultrapure water water and ethanol for 5 min and dried in air. Prior to the experiment, the gold electrodes were cleaned with hot piranha solution (a 3:1 mixture of H_2SO_4 and H_2O_2. *Cautions*!) for 10 min, and then continuously scanned within the potential range of –0.3 to 1.5 V in freshly prepared deoxygenated 0.5 M H_2SO_4 until a voltammogram characteristic of the clean gold electrode was established. After the cleaned electrode was thoroughly rinsed with water and absolute ethanol, 10 μL of Nafion ethanol solution (1.0%, v/v) was initially dropped onto the cleaned gold-disk electrode. Then, the resulting electrode was removed to parch with an infrared light for 20 min. After being washed with distilled water, the negatively charged electrode was formed owing to the presence of $–SO_3^-$ groups on Nafion molecules.

Immunoreaction protocol and electrochemical measurement. Prior to electrochemical assay, Cu^{2+}-coordinated pyrophosphate ion ($P_2O_7^{4-}$, PPi) complex (Cu^{2+}-PPi) was prepared by means of mixing 2-mM Cu^{2+} ion and 2-mM PPi at a volume ratio of 4 : 9 (optimized) in 500-μL HEPES buffer (10 mM, pH 7.2). All electrochemical measurements were carried out on an AutoLab electrochemical workstation (μAUTIII, Eco Chemie B.V., The Netherlands) with a conventional three-electrode system comprising a modified gold-disk working electrode, a platinum wire auxiliary electrode and an Ag/AgCl reference electrode. In this detection cell, the QCM gold-disk electrode was installed on the bottom. A high-binding polystyrene 96-well microtiter plates (Ref. 655061, Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with 50 μL per well of mAb$_1$ antibody at a concentration of 10 μg mL^{-1} in 0.05 M sodium carbonate buffer (pH 9.6). The microplates were covered with adhesive plastics plate sealing film to prevent evaporation. On the

following day, the plates were washed three time with PBST, and then incubated with 300 μL per well of blocking buffer for 1 h at 37 °C with shaking. The plates were then washed as before. Following that, a mixture containing 50-μL PSA standard/sample and 50-μL PPase-AuNP-pAb_2 ($C_{[AuNP]}$ = 24 μM) was injected in the well, and incubated for 35 min at room temperature with slight shaking. After being washed as before, a 100-μL aliquot of the above-prepared Cu^{2+}-PPi complexes was added into each well, and reacted for 25 min at 37 °C. During this process, pyrophosphate ions were hydrolyzed into phosphate ions and released the electroactive copper ions from Cu^{2+}-PPi complexes). Subsequently, the resulting solution was transferred in a homemade detection cell for square-wave voltammetric (SWV) measurement in an applied potential range from -200 to 200 mV with a potential step of 4 mV, a frequency of 25 Hz and an amplitude of 25 mV. The peak current at +68 mV was collected as the sensor signal. All determinations were made at least in duplicate. The sigmoidal curves were calculated by mathematically fitting experimental points using the Rodbard's four parameter function with Origin 6.0 software. Graphs were plotted in the form of peak current against the logarithm of PSA concentration.

Measurement with PSA ELISA kit for human serum specimens. A commercially available ELISA assay was utilized for method comparison studies (*i.e.*, evaluation of this method accuracy). In sandwich ELISA with standard polystyrene 96-well plates, 50 μL serum sample suspension was incubated at 37 °C for 30 min, and the wells were rinsed 3 times (3 min each) with 0.1 M PBS (pH 7.4) containing 0.5 M NaCl and 1.0 mL L^{-1} Tween 20. Then we added 50 μL conjugate solution and incubation continued for 60 min at room temperature. The wells were again rinsed and 50 μL 3,3',5,5'-tetramethylbenzidine (TMB) reagent was added and incubated at 37 °C for 10 min. The enzymatic reaction was stopped by adding 50 μL of 2.0 M H_2SO_4 to each well. The results of ELISA were measured by a spectrophotometric ELISA reader at a wavelength of 450 nm.

Statistical Analysis. A statistical data analysis was performed using Statistics Analysis System (SAS) ver. 9.0 and Statistical Program for Social Sciences (SPSS) ver. 9.0 software packages. Comparisons between dependent variables were determined using analysis of variance (ANOVA), Duncan multiple range test, correlation analysis and multiple regression analysis. Results are expressed as mean value ± standard deviation (SD) of three determinations and statistical significance was defined at $P \leq 0.05$.

Calculation Method for *t*-Test Statistics. To investigate the method accuracy between two

methods, statistical comparison based on the experimental results was first carried out with an unpaired Student's *t*-test preceded by the application of an *F*-test. The statistics for each sample was calculated by using independent two-sample t-test with equal sample sizes and equal variance as follows:

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{s_{x1x2}} \sqrt{\frac{n}{2}} \quad (1)$$

Where

$$s_{x1x2} = \sqrt{\frac{{s_{x1}}^2 + {s_{x2}}^2}{2}} \quad (2)$$

The $\bar{x}$, S_x and n represent the mean, standard deviation and times of parallel detection of the sample (1 means the data obtained from the proposed method and 2 means the data obtained from referenced method), respectively.

PARTIAL RESULTS AND DISCUSSION

Optimization of experimental conditions. To ensure an optimal analytical performance of the developed electrochemical immunoassay, some experimental parameters including immunoreaction time, molar ratio of PPase and pAb_2 for preparation of PPase-AuNP-pAb_2 and the hydrolytic time of PPase toward the Cu^{2+}-PPi complexes should be investigated (note: 0.1 ng mL^{-1} PSA was used as an example in these cases). Usually, the antigen-antibody reaction is adequately carried out at human normal body temperature (37 °C). Considering the possible application of the proposed immunoassay in the future, we selected room temperature (25 ± 1.0 °C) for the antigen-antibody interaction throughout the experiment. At this condition, we monitored the effect of incubation time on the current of the electrochemical immunoassay from 10 min to 50 min (not: To avoid confusion, the incubation times of mAb_1 with PSA were paralleled with those of the mAb_1-PSA with PPase-AuNP-pAb_2). As shown in Fig. S1-A, SWV peak currents increased with the increment of incubation time, and tended to level off after 35 min. Hence, an incubation time of 35 min was selected for sensitive determination of PSA.

Owing to the co-immobilization of PPase and pAb_2 on the gold nanoparticles, the conjugated ratio of pAb_2 and PPase Ab_2 is one of the most important factors influencing the sensitivity of the electrochemical immunoassay. Usually, a high amount of pAb_2 antibody on the gold nanoparticles

can increase the possibility of antigen-antibody reaction, but it is not conducive to the enzymatic catalytic reaction. As shown in Fig. S1-B, the optimum current was obtained at the molar ratio of 1 : 5. So, a molar ratio of 1 : 5 for pAb_2 and PPase was used for preparation of pAb_2-AuNP-PPase.

Usually, it takes some time for PPase to hydrolyze the PPi into Pi. Fig. S1-C gives the effects of different hydrolytic times on the voltammetric peak current of electrochemical immunoassay. The currents initially increased with the increasing hydrolytic time, and then tended to level off after 25 min. To save the assay time, 25 min was utilized for PPase-induced Cu^{2+} release from the Cu^{2+}-PPi complexes.

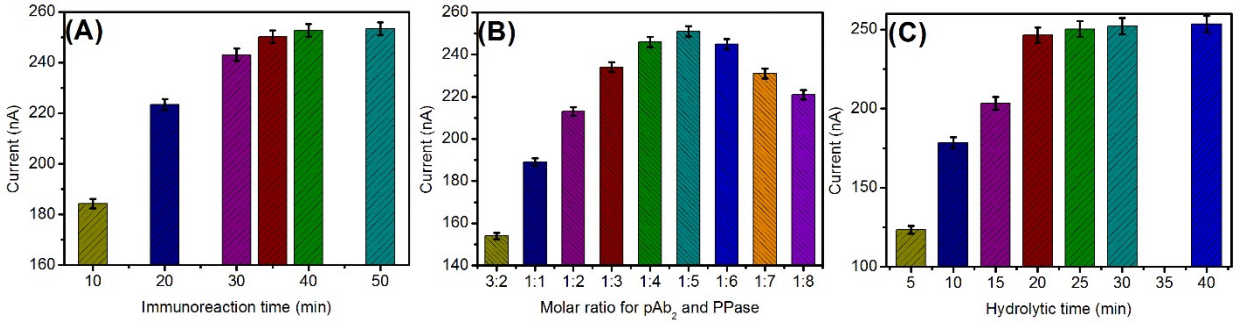

Fig. S1. Effect of (A) immunoreaction time, (B) molar ratio between pAb_2 and PPase for the preparation of pAb_2-AuNP-PPase and (C) hydrolytic time of PPase toward Cu^{2+}-PPi complex on the current of electrochemical immunoassay (0.1 ng mL^{-1} PSA used in these cases).

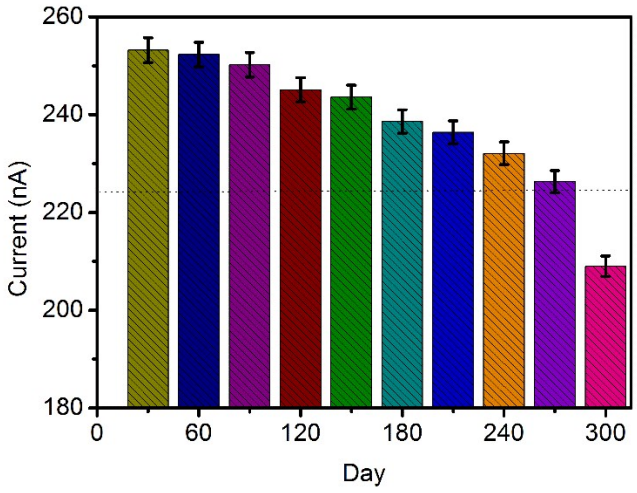

Fig. S2. The storage stability of mAb_1-coated microplate and PPase-AuNP-pAb_2 for the detection of 0.1 ng mL^{-1} PSA during a 300-day period.

Table S1. Reproducibility of electrochemical immunoassay for the detection of target PSA

Item	$C_{[PSA]}$ (ng mL^{-1})	Current (nA)					RSD (%)
		Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	
Intra-assay for the same batch	0.01	92.3	88.6	93.5	91.7	86.4	3.2
	1.0	417.5	402.3	423.7	398.7	416.5	2.6
	100	750.2	756.3	768.3	731.4	749.2	1.8
Batch-to-batch inter-assay	0.01	80.4	96.7	93.1	89.6	82.5	7.8
	1.0	432.3	406.7	418.9	393.4	421.2	3.6
	100	732.3	774.4	743.2	754.3	711.2	3.2

Table S2. Comparison of electrochemical immunoassay on the analytical properties with other PSA detection schemes

Method	Linear range (ng mL^{-1})	LOD (pg mL^{-1})	Ref.
Chemiluminescence immunoassay	1.0 – 50	300	3
Fluorescence aptasensor	0.25 – 70	35	4
Colorimetric immunoassay	0.01 – 20	9.0	5
Capacitance immunosensor	0.1 – 50	31	6
Impedance immunosensor	0.01 – 100	5.4	7
Multicolor and photothermal biosensor	1.0 – 128	310	8
Fluorescence aptasensor	0.1 – 2.5	10	9
Fluorescence aptasensor	1.0 – 100	360	10
Fluorescence bioassay	0.1 – 200	10	11
Electrochemiluminescence immunosensor	0.001 – 150	0.6	12
SWV immunoassay	0.01 - 100	5.2	This work

Table S3. Detection of human serum samples by using electrochemical immunoassay and human PSA ELISA kit

	Method; Conc. (mean ± SD, ng mL^{-1}, n = 3)[a]		
Sample no.	Electrochemical immunoassay	PSA ELISA kit	t_{exp}
1	4.5 ± 0.4	5.1 ± 0.2	2.32
2	12.4 ± 0.9	10.2 ± 1.1	2.68
3	1.3 ± 0.2	1.5 ± 0.1	1.55
4	36.7 ± 3.5	30.2 ± 2.3	2.69
5	213.2 ± 9.8	232.5 ± 10.4	2.34
6	112.3 ± 10.2	123.1 ± 3.6	1.73
7	56.7 ± 4.9	50.8 ± 5.3	1.42
8	24.9 ± 1.2	23.1 ± 0.6	2.32

[a] The value of high-concentration samples were calculated on the basis of the dilution ratio.

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