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A new label-free strategy for highly efficient chemiluminescent immunoassay

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Experimental

Materials and Reagents. HIgG and rabbit anti-HIgG were purchased from Beijing Boisynthesis Biotechnology Co. Ltd. (Beijing, China). HRP, bovine serum albumin (BSA), chitosan, and 3-glycidoxypropyltrimethoxysilane (GPTMS, 98%) were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Chioroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), tween-20, trisodium citrate dehydrate and hydrogen peroxide (30%, H_2O_2) were obtained from Sinopharm Chemical Reagent Co. Ltd. (China). Luminol and p-iodophenol (PIP) were bought from Acros (Belgium) and Alfa Aesar Ltd. (China), respectively. Stock solutions (0.01 M) were prepared in 100 mL of 0.1 M NaOH. Prior to their use, the mixture of luminol and PIP stock solutions was diluted in 0.1 M pH 8.5 Tris-HCl buffer. The CL substrate solution was obtained by mixing with 5 mM luminol, 0.6 mM PIP and 4 mM H_2O_2. All other reagents were of analytical grade and used as received. Distilled water was used in all assays.

Buffers. Distilled water was used in the preparation of all buffers. Coupling buffer was phosphate buffer solution (PBS; 0.01 M, pH 7.4), which was used for antibody immobilization. Blocking buffer, which was used to block the residual reactive sites on the prepared arrays, was 0.01 M PBS containing

1% BSA (BSA; pH 7.4). To minimize unspecific adsorption, 0.05% Tween-20 was spiked into 0.01 M PBS as a washing buffer (PBST, pH 7.4).

Apparatus. The flow-through CL label-free assay system was constructed as illustrated in Fig. S3. The flow device was composed of a Teflon cover (4.0 cm × 2.5 cm × 0.8 cm) with an inlet and outlet, a silicon slice rubber spacer (2.0 mm thickness), and a transparent plexiglass slice (0.5 cm thickness). Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all of components in this system. All fluids were delivered with a multichannel bidirectional peristaltic pump. The introductions of different solutions into the flow system were performed using a multiposition valve with five inlets and one outlet. The flow device was positioned in front of photomultiplier (PMT), and the CL signals produced in the flow device were measured with the PTM operated at -500 V. Instrument control and data recording were performed using the IFFM software package run under Windows 2003.

An IFFM-E Luminescent Analyzer (Remex Analytical Instrument Co. Ltd., Xi'an, China) was used to perform the flow-through CL label-free measurements. The reference electrochemiluminescence immunoassay was performed using a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH). Scanning electron micrographs (SEM) were obtained with a Hitachi S-4800 (Japan) scanning electron microscope at an acceleration voltage of 5 kV. The static water contact angles were obtained with a contact angle meter (Rame-Hart-100) using droplets of the distilled water at 25 °C. Electrochemical impedance spectroscopy (EIS) measurements were recorded in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ with an Autolab/PGSTAT30 (The Netherlands), and the amplitude of the applied sine wave potential was 5 mV. The impedance measurements were performed at a bias potential of 190 mV within the frequency range from 0.05 to 10 kHz.

Synthesis of AuNPs. AuNPs were synthesized according to the classical citrate reduction method.[S1] Prior to the synthesis of AuNPs, all glassware was thoroughly cleaned with Aqua regia (3:1 HNO_3-HCl), rinsed extensively with distilled water and dried in air. Typically, 100 mL of 0.01% (w/w) aqueous $HAuCl_4$ solution was heated to boiling, and 2.5 mL of 1% (w/w) trisodium citrate solution was quickly added to boiling solution with vigorous stirring for 10 min. When a wine-red color was observed, the

reaction kettle was removed from the heating equipment and stirred continually for 10 minutes to cool to room temperature. The prepared AuNPs showed a mean diameter of 16 nm (shown in Fig. S4) and were stored at 4 °C prior to use.

Preparation of the label-free immunosensor. The glass slide (2.1 cm × 0.4 cm × 0.1 cm) was firstly soaked in the piranha solution (H_2SO_4/30% H_2O_2, 7:3 v/v) for 12 h. Then, it was washed thoroughly with distilled water and dried under a stream of nitrogen. The pretreated glass slide was silylanized by immersing it in toluene solution containing 1% GPTMS overnight at room temperature.[S2] Subsequently, the glass substrate was successfully epoxy-activated after washing three times with pure toluene and ethanol to remove physically absorbed GPTMS, and dried under a stream of nitrogen. Next, 1.0 mL AuNPs were first dispersed in 2.0 wt% chitosan solution with the assistance of sonication. 50 μL of the suspension was then mixed with 8.0 μL of 1.0 mg/mL anti-HIgG, 5.0 μL of 100 μg/mL HRP and 37 μL PBS. The resulting mixed solution (20 μL) was dropped onto the silylanized glass slide for the reaction at room temperature for 1 h, and then stored at 4 °C overnight. After washing several times with PBST and blocking with blocking buffer for 12 h at 4 °C, the label-free immunosensor was finally obtained.

The anti-HIgG-HRP modified glass slide was put in the flow device (Fig. S3) by fixing it on the center area of the inner side of the Teflon cover (4.0 cm × 2.5 cm × 0.8 cm). The total thickness of the immobilized slide and film was approximately 1.1 mm. The volume of the fabricated flow cell was approximately 80 μL (2.1 cm × 0.4 cm × 0.09 cm). The prepared immunosensor was stored in 0.01 M pH 7.4 PBS at 4 °C prior to use.

Label-Free CL immunoassay protocol. A detailed description of the flow-through label-free CL assay is illustrated in Scheme 1 and shown in Table S1. First, 80 μL HIgG standard solutions or serum samples was delivered into the flow device and incubated under stop flow at room temperature for 25 min. PBST was then introduced into the system at an optimal flow rate of 1.0 mL/min to wash the immunosensor. Subsequently, the CL substrate was injected into the flow device. The CL signals were

measured after the HRP catalyzed CL reaction was triggered for 50 s under stop flow. The procedure required 30 min, from sample injection to signal collection.

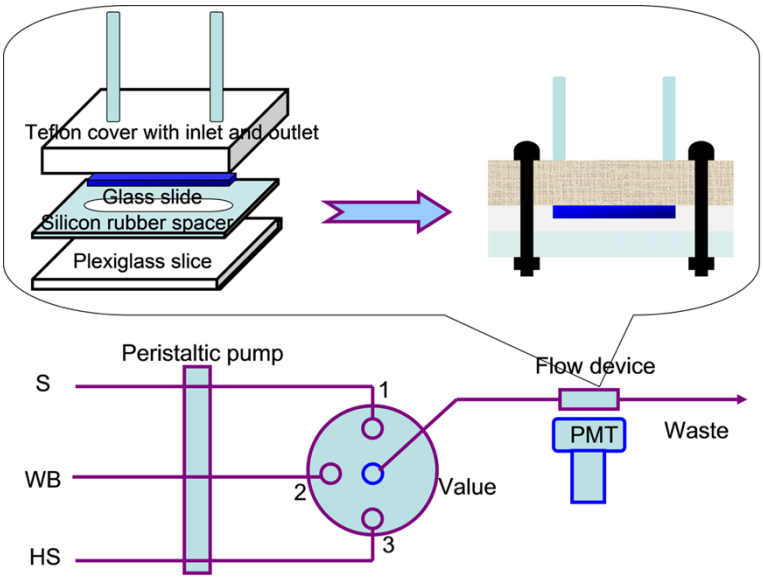

Fig. S3 Scheme of the label-free chemiluminescent immunoassay for HIgG. (S) sample, (WB) wash buffer, (HS) HRP substrate, (PMT) photomultiplier.

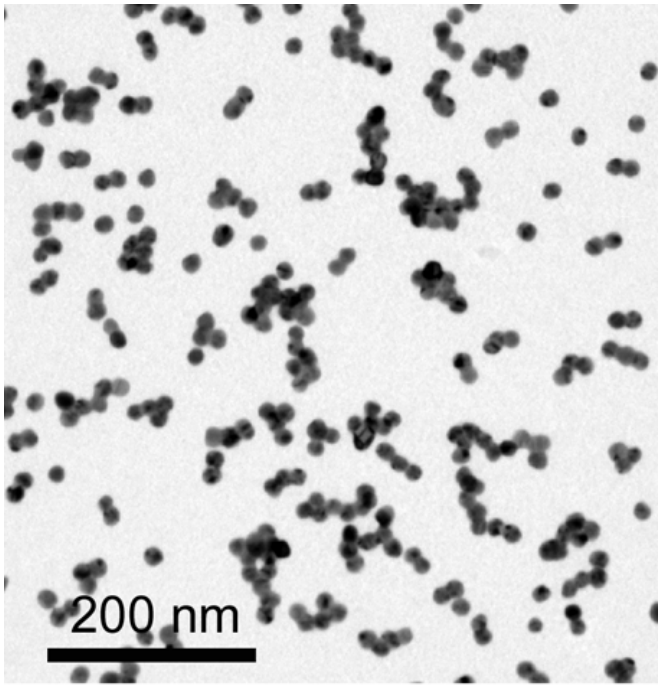

Fig. S4 TEM image of the synthesized AuNPs

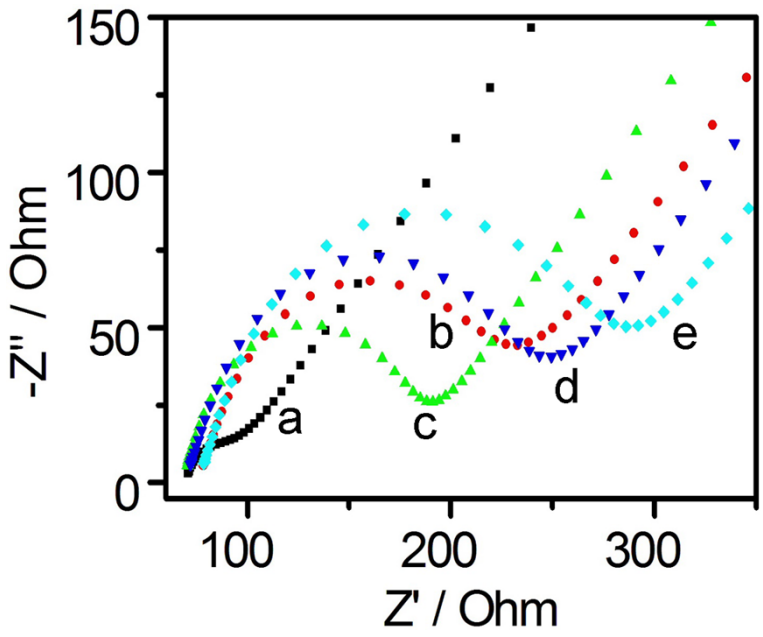

Fig. S1 Nyquist plots of EIS for bare GCE (a), chitosan/GCE (b); AuNPs-chitosan /GCE (c), anti-HIgG-HRP/AuNPs-chitosan/GCE (d) and BSA/ anti-HIgG-HRP/AuNPs-chitosan/GCE (e) measured in PBS solution (0.1 mM, pH 7.0) including 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KCl at a scan rate of 100 mV/s.

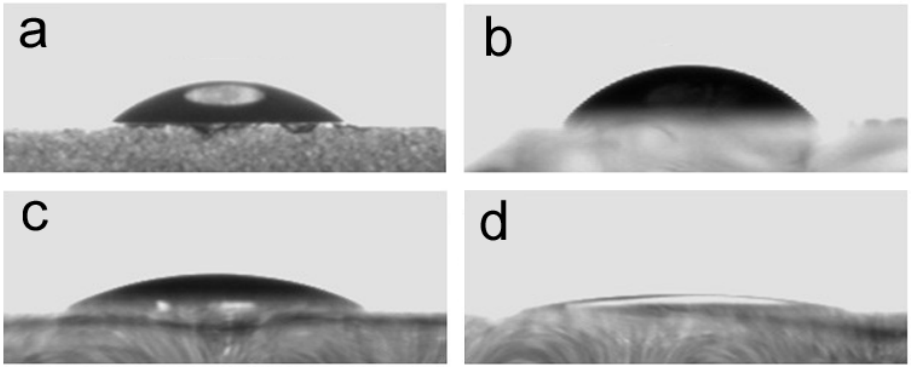

Fig. S2 Contact angles of piranha-treated (a), GPTMS-silylanized (b), AuNPs-chitosan modified (c) and anti-HIgG-HRP/AuNPs-chitosan immobilized (d) glass substrates.

Table S1 Details of the proposed label-free CL immunoassay for HIgG

Step No.	Valve position	Step	Starting time (min:second)
1	1	Introduce 80 μL sample into the flow device	00: 00
2	1	Stop flow and incubation at room temperature	00: 30
3	2	Wash the flow device with PBST at a flow rate of 1.0 mL/min	25: 30
4	3	Introduce 80 μL HRP substrate into the flow device and stop flow to collect data	27: 30
5	1	Introduce PBST to wash flow device at 1.0 mL/min and renew the immunosensor	28: 30
6	1	Ready for the next assay cycle	30: 00

Table S2 Recoveries for HIgG by the proposed label-free CL immunoassay system (n= 5)

Sample	Spiked (ng mL^{-1})	Measured (ng mL^{-1})	Recovery (%)	RSD (%)
1	4	4.01	100.2	2.1%
2	10	10.31	103.1	4.2%
3	30	30.56	101.9	3.2%
4	50	48.54	97.1	3.5%
5	60	60.31	100.5	3.6%

References

S1 A. Doron, E. Katz and I.Willner, *Langmuir*, **1995**, 11, 1313.

S2 Z. J. Yang, J. Shen, J. Li, J. Zhu and X. Y. Hu, *Anal. Chim. Acta*, **2013**, 774, 85.