Electronic Supplementary Information (ESI) for Chemical Communications

Facile synthesis of novel reduced graphene oxide@polystyrene nanospheres for sensitive label-free electrochemical immunoassay

Qingchun Lan,[a] Huifang Shen,[a] Juan Li,*[a] Chuanli Ren,[b] Xiaoya Hu*[a] and Zhanjun Yang*[a]

[a] *College of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou 225002, P.R. China*

[b]*Department of Laboratory Medicine and Clinical Medical College of Yangzhou University; Subei Peoples' Hospital of Jiangsu Province, Yangzhou, 225001, P.R. China.*

Experimental

Materials and Reagents. AFP ELISA reagent kit was purchased from CanAg Diagnostics, which consists of a series of AFP standard solutions (0-500 ng/mL) and biotinylated mouse monoclonal AFP antibodies (1.0 μg/mL). Graphite powder, potassium permanganate ($KMnO_4$), sulfuric acid (98%, H_2SO_4), sodium nitrate ($NaNO_3$), styrene, azodiisobutyronitrile (AIBN) hydrazine hydrate, hydrochloric acid and hydrogen peroxide (30%, H_2O_2) were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Streptavidin, chitosan, 2-methylacryloylxyethyl trimethyl ammonium chloride (DMC) and bovine serum albumin (BSA) were bought from Sigma (St. Louis, MO). Phosphate buffer solution (PBS) was a mixture of 0.1 M Na_2HPO_4 and NaH_2PO_4 and its pH was adjusted with H_3PO_4 or NaOH solution. The clinical serum samples were supplied by Jiangsu Institute of Cancer Research. All other reagents were analytical grade.

Apparatus. Electrochemical measurements were performed on a CHI852C electrochemistry workstation (Shanghai CH Instruments Co., China) with a conventional three-electrode system. All experiments were carried out with a three-electrode system with a glassy carbon electrode (GCE, Φ=3 mm) as the working electrode, a platinum wire as the auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. Transmission electron micrographs (TEM) were obtained on

Philips Tecnai-12 transmission electron microscope using an accelerating voltage of 120 kV. Scanning electron micrographs (SEM) were obtained by a Hitachi S-4800 (Japan) scanning electron microscope with an acceleration voltage of 15 kV. Zeta potentials of the graphene oxide solution and PS nanospheres emulsion were measured using a zetasizer (Nano ZS 90, Malvern Instruments). Raman spectra were recorded using a Renishaw InVia microRaman system. Fourier transform infrared (FTIR) spectrum measurements were performed by a Tensor 27 spectrophotometer (Bruker Co., Germany). The electrochemical impedance spectroscopy (EIS) analysis was performed on an Autolab PGSTAT30 (The Netherlands) using a solution of 0.10 M KCl containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$. The amplitude of the applied sine wave potential was 5 mV and the frequency range was from 0.1 to 10 kHz at a bias potential of 190 mV. The static water contact angle measurements were done at 25 ^oC using a contact angle meter (Rame-Hart-100). X-ray photoelectron spectroscopic (XPS) spectrum was obtained with an ESCALAB 250Xi spectrometer (USA).

Preparation reduced graphene oxide@polystyrene Nanospheres. Graphene oxide (GO) was synthesized according to a typical Hummers synthesis method.[S1] Firstly, 2.0 g of graphite and 1.0 g of $NaNO_3$ were mixed into 46 mL of 98% H_2SO_4, and stirred for 1 h at 0 ^oC. Afterwards, 6.0 g of $KMnO_4$ was slowly added into the mixture and continued to keep reaction temperature at 0 ^oC for 2 h. Then, the mixture was taken away from the ice bath, and stirred for 30 min at 38 ^oC. Next, 160 mL deionized water was added into above mixture, and its temperature increased to 95 ^oC. After keeping a reaction temperature at 95 ^oC for 30 min, 120 mL deionized water and 30 mL 3% H_2O_2 were added to reduce the residual permanganate and manganese dioxide. The mixture was filtered when the color changed to brilliant yellow and washed with 80 mL 5% HCl aqueous solution and deionized water. After several centrifugations the final dry GO products were obtained in vacuum at 50 ^oC.

Polystyrene (PS) nanospheres were synthesized by the modified dispersion polymerization method.[S2] Typically, 20 mL styrene monomer, 0.5g AIBN and 136 g 30% ethanol aqueous solution were added to the three-neck flask with a stirring speed of 400 rpm and kept bubbling with N_2 for 30 min. Then, its temperature increased to 75 ^oC and continued to keep reaction for 2 h. After pre-polymerization, 0.75 g

DMC (dissolved in 12.5 g H_2O) was added to the solution. After reaction at N_2 atmosphere for 22 h, the monodisperse PS nanospheres emulsion could be obtained. Subsequently, the PS nanospheres were collected by repeated centrifugation and redispersion process, and final dilution with deionized water to 10 wt% solid content before use.

5.0 g (10 wt%) PS aqueous dispersion and 10.0 mL (0.1 wt%) GO suspensions were added to three-neck flask and sonicated for 30 min. Then 120 mL deionized water was added into the above mixture and stirred at room temperature for 6 h. Subsequently, 200 μL 80 wt% hydrazine hydrate was added to the mixture and continued to keep reaction temperature at 95 °C for 4 h. The resultant black rGO@PS NSs were purified by several centrifugations and redispersions. Finally, the nanocomposite NSs were re-dispersed in deionized water.

Preparation of Electrochemical AFP Immunosensor. The schematic illustration of the fabrication of the label-free electrochemical immunosensor and detection of tumor marker was shown in Scheme 1B. The GCE was respectively polished with 0.3 and 0.05 mm alumina slurry, followed by sonication in ethanol solution and deionized water successively, and dried under a stream of nitrogen. 2.0 mg of rGO@PS NSs was firstly dispersed in 1.0 wt% chitosan (CS) solution with sonication. Then chitosan-dispersed rGO@PS NSs solution was mixed with streptavidin solution (300 μg mL^{-1}) at 1:1 ratio under gentle stirring for 2 h. Subsequently, 5.0 μL of the resulting mixture was dropped on the pretreated GCE, and allowed to dry at 4 °C overnight. Next, 10 μL of 1 μg mL^{-1} biotin-anti-AFP was dropped onto streptavidin/rGO@PS NSs modified GCE for the reaction for 1 h at room temperature, followed by washing with buffer solution to remove physical adsorption. Finally, the resulting electrode was incubated with 1% BSA solution for 1 h at room temperature to block nonspecific binding sites. After washing three times with buffer solution, the label-free immunosensor was stored at 4 °C prior to use.

Label-Free Immunoassay Procedure. The label-free electrochemical immunosensor was incubated with 50 μL of the incubation solution which consisted of different concentrations of AFP for 40 min at room temperature. After washing the immunosensor with PBS several times, electrochemical measurements were carried out in 10.0 mL of pH 6.5 PBS containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$, and the

differential pulse voltammetry (DPV) measurements were performed from -0.2 to 0.5 V with pulse amplitude of 50 mV and pulse width of 0.2 s.

Photographs of the resultant samples

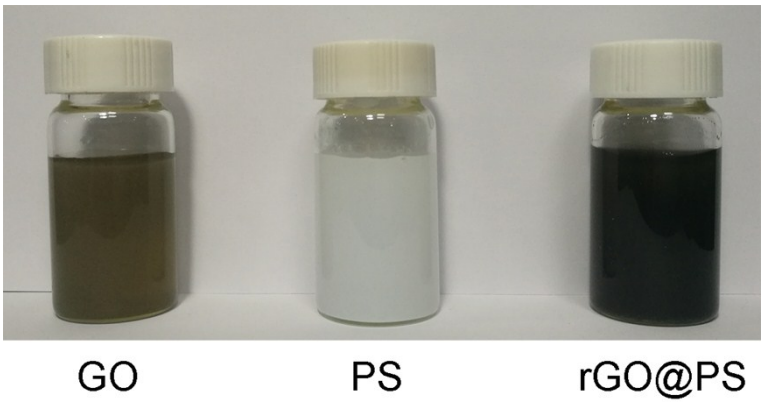

Fig. S1 Photographs of GO dispersion, PS and rGO@PS suspensions.

The characterization of the fabrication of the immunosensor

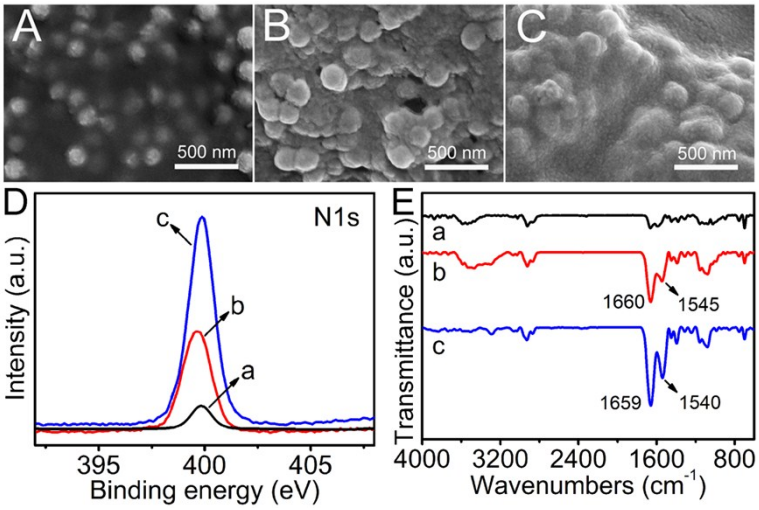

Fig. S2 SEM images of rGO@PS NSs/chitosan (A), streptavidin/rGO@PS NSs/chitosan (B), and biotin-anti-AFP/streptavidin/ rGO@PS NSs/chitosan (C) modified electrode. XPS of N 1s peak (D) and FT-IR spectra (E) of rGO@PS NSs/chitosan (a), streptavidin/rGO@PS NSs/chitosan (b) and biotin-anti-AFP/streptavidin/rGO@PS NSs/chitosan (c) modified electrode.

The characterization of contact angles of different modified electrode

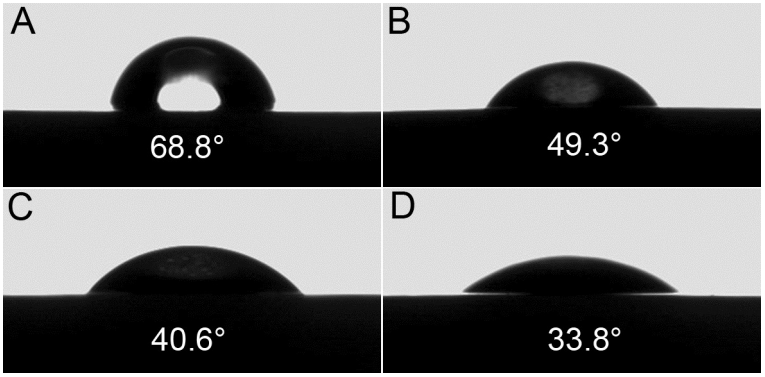

Fig. S3 Contact angles of bare GCE (A), rGO@PS NSs/chitosan /GCE (B), streptavidin/ rGO@PS NSs/chitosan/GCE (C), biotin-anti-AFP/streptavidin/rGO@PS NSs/chitosan/GCE (D).

The characterization of electrochemical impedance spectrum of different modified electrodes

Electrochemical impedance spectroscopy (EIS) was used to investigate the fabrication process of the label-free immunosensor.[16] The electron transfer resistance (*R*et) is related to the semicircle diameter of the impedance curve. As shown in Fig. S4, the pretreated electrode GCE shows a small semicircle with a *R*et value of about 185 Ω (curve a). Following modification with the rGO@PS NSs composite, the electrode exhibits a much lower *R*et value of about 76 Ω (curve b), indicating that rGO@PS NSs composite is an excellent electrically conducting material which accelerates the electron transfer. When the electrode was modified with rGO@PS NSs/chitosan composite, the electrode exhibits a bigger semicircle with a R_{et} value of about 385Ω (curve c). After the electrode was further modified with streptavidin and biotinylated anti-AFP, the R_{et} values increased to 724 Ω (curve d) and 847 Ω (curve e), respectively. This was attributed to the non-conductive bioactive proteins which blocked the electron transfer. These results also demonstrate the successful fabrication of the label-free immunosensor.

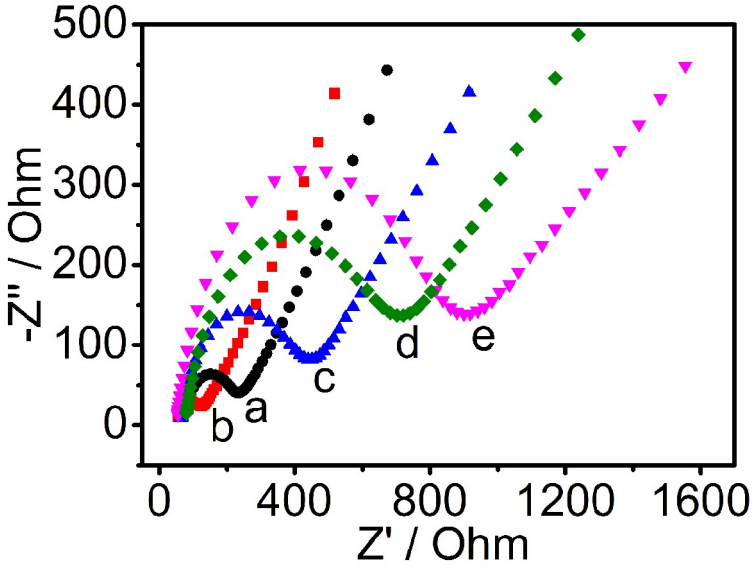

Fig. S4 Nyquist plots of EIS for bare GCE (a), rGO@PS NSs (b), rGO@PS NSs/chitosan (c), streptavidin/ rGO@PS NSs/chitosan (d), and biotin-anti-AFP/streptavidin/ rGO@PS NSs/chitosan (e) modified electrode measured in PBS solution containing 0.1 M KCl and 5.0 mM $[Fe(CN)_6]^{3-/4-}$.

The electrochemical behavior of the label-free immunosensor

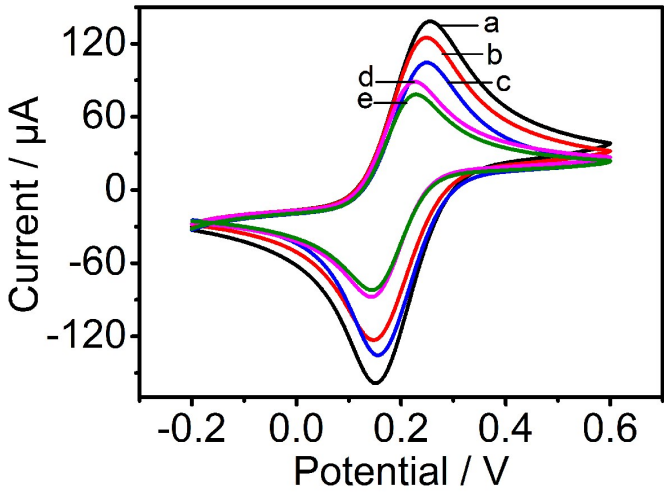

Fig. S5 Cyclic voltammgrams of rGO@PS NSs/chitosan/GCE (a), streptavidin/rGO@rGO@PS NSs/chitosan/GCE (b), biotin-anti-AFP/streptavidin/rGO@PS NSs/chitosan/GCE (c), BSA/biotin-anti-AFP/streptavidin/rGO@PS NSs/chitosan/GCE (d), and AFP/BSA/biotin-anti-AFP/streptavidin/rGO@PS NSs/chitosan/GCE (e) in pH 6.5 PBS solution containing 0.1 M KCl and 5.0 mM$[Fe(CN)_6]^{3-/4-}$.

Optimization of the pH and incubation time

To achieve an optimal assay performance, the pH value of the detection solution and the incubation time were investigated at AFP concentration of 10 ng/mL. In order to optimize the pH, the current signal of immunosensor was tested in PBS solution containing 0.1 M KCl and 5.0 mM $[Fe(CN)_6]^{3-/4-}$ with different pH values. As shown in Fig. S6A, the peak current reached the maximum value at pH 6.5, and then decreased. Therefore, PBS with pH value of 6.5 was selected for further evaluation of this label-free immunoassay. In the case of incubation time, the current response quickly declined with the prolonged incubation time, and tended to a relatively constant value within 40 min (shown in Fig. S6B), showing a saturated reaction between capture antibodies and target analytes on the sensing interface. Therefore, 40 min was chosen as the optimized incubation time for the label-free immunoassay.

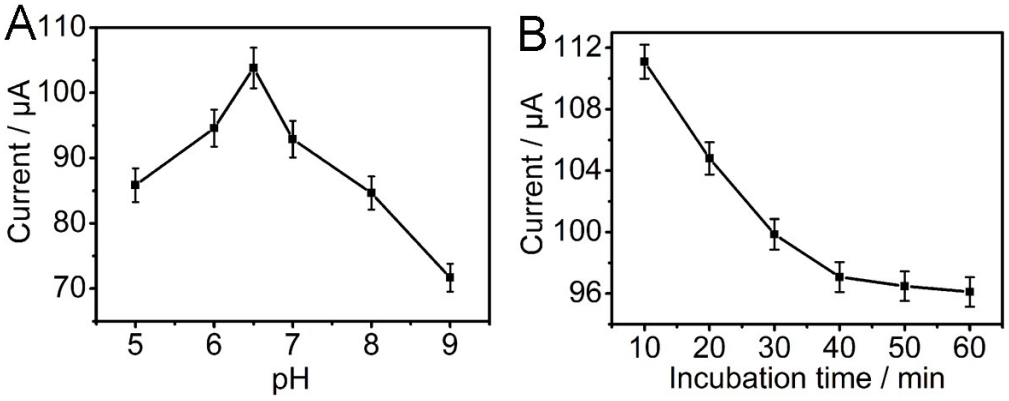

Fig. S6 Dependence of current response of the immunosensor on pH of detection solution (A), and incubation time (B). (n = 5 for each point).

Table S1 Comparison of AFP detection with some different methods

Analytical methods	Linear range (ng/mL)	Detection limit (ng/mL)	References
Enzyme-linked immunosorbent assay	2-200	0.23	S3
Fluoroimmunoassay	0.1-750	0.08	S4
LA-ICPMS	1-500	0.2	S5
Electrochemistry	0.2-200	0.5	S6
Chemiluminescence	10-100	2.4	S7
Electrochemistry	0.1-100	0.03	This work

LA-ICPMS: Laser ablation inductively coupled plasma mass spectrometry

Table S2 Detection results of clinical serum samples using proposed and reference methods (n = 5).

Sample	Proposed method (ng/mL)	Reference method (ng/mL)	Relative error (%)
1	2.08	2.16	-3.7
2	3.17	3.47	-8.7
3	3.80	3.51	8.3
4	15.83	15.22	4.0
5	640.3	618.3	3.6

References

S1 W. S. Hummers and R. E. Offeman, *J. Am. Chem. Soc*., 1985, **80**, 1339.

S2 P. F. Zhao, Y. Y. Luo, Y. L. Yang, D. N. He, L. X. Kong, P. Zheng and Q. Yang, *Mater. Lett*., 2014, **121**, 74.

S3 Z. H. Xuan, M. M. Li, P. F. Rong, W. Wang, Y. J. Li and D. B. Liu, *Nanoscale*, 2016, **8**, 17271.

S4 J. Hou, T. Liu, Z. Ren, M. Chen, G. Lin and Y. Wu, *Analyst*, 2013, **13**, 3697.

S5 S. Hu, S. Zhang, Z. Hu, Z. Xing and X. Zhang, *Anal. Chem*., 2007, **79**, 923.

S6 C. L. Hong, R. Yuan, Y. Q. Chai and Y. Zhuo, *Electroanal*., 2008, **20**, 989.

S7 Y. F. Wu, Y. F. Zhuang, S. Q. Liu and L. He, *Anal. Chim. Acta,* 2008, **630**, 186.