Int. J. Electrochem. Sci., 13 **(2018)** 8905 – 8914, doi: 10.20964/2018.09.29

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

High Sensitive Sandwich-type Electrochemical IgG Immunosensor Based on a Nanocomposite of Carbon Nanotube and Multifunctional Ionic Liquid Containing Aldehyde and Ferrocene Groups as Labels

Youming Shen[1], *Guangyu Shen*[1], *Youyu Zhang*[2,*]

[1] Hunan Province Cooperative Innovation Center for The Construction & Development of Dongting Lake Ecological Economic Zone, College of Chemistry and Material Engineering, Hunan University of Arts and Science, Changde, 415000, PR China
[2] Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, PR China
[*]E-mail: sgyrab@163.com, zhangyy@hunnu.edu.cn

Received: 21 May 2018 / *Accepted:* 14 June 2018 / *Published:* 5 August 2018

Using a nanocomposite of carbon nanotube and multifunctional ionic liquid as labels and chitosan as platform, we fabricated a sandwich-type electrochemical immunosensor. The multifunctional ionic liquid was anchored with both aldehyde groups and ferrocene groups. Aldehyde groups were used to bond the second antibody and ferrocene groups were used as signal probes. Due to the large surface area of carbon nanotube, increased-loading of ferrocene was obtained, which improved the sensitivity of the immunosensor. In addition, the high conductivity of carbon nanotube and ionic liquid also enhanced the sensitivity of the immunosensor. The fabricated immunosensor showed a good performance. The linear range was from 0.05 to 30 ng mL^{-1} with a detection limit of 0.01 ng ml^{-1} (*S*/*N*=3), which is 40 times higher than that of reported immunsensor for human IgG based on ferrocene as labels. We anticipate this strategy may provide a new platform for the clinic application.

Keywords: Multifunctional ionic liquid; Ferrocene; Carbon nanotube; Sandwich-type electrochemical immunosensor; Human IgG.

1. INTRODUCTION

Electrochemical immunosensors based on antibody–antigen interactions have been widely developed because they are simple, fast, sensitive and inexpensive [1,2]. Ferrocene (Fc), an organometallic molecule, has a special structure, where ferrous ion is sandwiched by two cyclopentadiene rings [3]. It is well known that Fc and its derivatives are good reversible redox

mediators. For example, a reversible redox signal of the Fc/carbon nanotubes modified glassy carbon electrode was observed [4]. The peak was attributed to the electrochemical process of Fc^+/Fc because carbon nanotubes did not have redox electrochemical activity in this potential range. Sung constructed an effective electron transfer mediating layer using ferrocene-containing multifunctional redox copolymer [5]. Thus, many biosensors were fabricated by using Fc and its derivatives to produce detection signals [6-8]. Using ferrocene-modified carbon nanotubes as building block, Qiu and co-workers developed an amperometric sensor for glucose detection [9]. A facile strategy for the preparation of hydrogel based on Fc functionalized amino acid was developed. Due to the incorporation of a large number of Fc moieties into the hydrogel, the hydrogel displays strong redox activity [10]. Non-covalent or covalent functionalization was a good strategy of introducing ferrocene on the surface of electrode. For example, Kanyong and co-workers constructed a biosensor using ferrocene carboxylic acid-based film formed by non-covalent interaction for the detection of hydrogen peroxide residues [11]. Using ferrocenecarboxaldehyde covalently bound to chitosan, Liang fabricated a label-free electrochemical immunosensor for determination of hepatitis B surface antigen in human serum [12]. Fc and its derivatives can be entrapped into film-forming materials and used as building block for the construction of label-free electrochemical biosensors [7,8]. They also can be loaded on carbon nanotubes, graphene, nanoparticles and used as labels for the fabrication of sandwich-type immunosensors [15-17].

Carbon nanotubes (CNT) have been widely applied in electrochemical biosensor due to their good electrical conductivity, high chemical stability and modifiable sidewalls. They were usually used not only as additive to improve direct electron transport of sensing interface [16,19], but also as substrate to carry a lot of probe molecules for amplifying electrochemical signals [20]. However, CNT were easy to entangle with each other and formed agglomerates due to π–π stacking [21], which decreased the amount of probe molecules loaded on their surface. Ionic liquids (IL) are composed of large organic cation and a relatively small anion. Due to the existence of cation, IL can prevent the entangling of CNT via cation–π interactions [22]. IL was widely applied in electrochemical biosensors because of their good features including biocompatibility, high ionic conductivity, electrochemical stability and biocompatibility. Usually, they were incorporated into conventional matrixes including chitosan, cellulose, nanoparticles, CNT, and graphene to form composite materials for modifying electrode [23-25]. The introducing of IL provided a good microenvironment which was good for immobilizing biomolecules and at the time improved the conductivity of sensing interface. Although the application of IL-based composite materials in electrochemical biosensors was highlighted, little attention was paid to the functionalization of IL with carboxyl, amino, aldehyde for capturing label biomolecules and redox mediators for the development of electrochemical immunosensor.

Recently, our group prepared an "all-in-one" ionic liquid functionalized with both aldehyde and ferrocene groups (Fc-IL-CHO). Casting this multifunctional ionic liquid entrapped in Nafion matrix on gold electrode, we fabricated a label-free immunosensor with good performances [26]. In this work, using nanocomposite of CNT and Fc-IL-CHO, we developed a sandwich-type immunosensor for human IgG. Herein, Fc as a redox mediator produces detection signals; The –CHO groups directly bond secondary antibodies for convenient preparing labels, in which no other link reagents were required. Due to the large surface area of carbon nanotube, the amount of Fc immobilized on the

electrode surface increased, which enhanced the signals of detection. In addition, carbon nanotube and ionic liquid improved the conductivity of sensing interface. Thus, high sensitive immunosensor was obtained. This study expanded the application of ferrocene derivatives in biosensors and this immunosensor provided a new platform for the detection of human IgG and other biomolecules.

2. EXPERIMENTAL

2.1. Reagents and apparatus

We purchased rabbit anti-human IgG antibody (Ab), human immunoglobulin G (IgG), and bovine serum albumin (BSA) from Beijing Dingguo Biotechnology Company (Beijing, China). Carboxylic CNT was purchased from Nanjing Xianfeng nanomaterial Technology Ltd. Other reagents were come from Sigma-Aldrich (St. Louis, MO). 0.1 M phosphate buffer solution (PBS, pH 7.0) was prepared using Na_2HPO_4 and KH_2PO_4. A 5 mg/mL chitosan solution was prepared by dissolving 10 mg chitosan in 2.0 mL of 0.1 mol/L acetic acid.

Electrochemical measurements including differential pulse voltammetry (DPV), cyclic voltammetric (CV), and electrochemical impedance spectra (EIS) were carried out using a CHI 660E electrochemistry workstation (Shanghai CH Instruments, China) with a conventional three-electrode cell, in which Platinum sheet was used as the auxiliary, saturated calomel was used as reference, and gold electrode (Au) was used as working electrode.

2.2. Preparation the nanocomposite of CNT/Fc-IL-CHO

The composite of CNT/Fc-IL-CHO was prepared by adding 2.0 mg of CNT-COOH into 10 mL of Fc-IL-CHO (2 mg/mL) ethanol solution. After the mixture was ultra-sonicated for 30 min followed by strongly stirring for 3 h, the resulting homogeneous mixture was subsequently centrifuged at 12000 rpm for 15 min, washed three times with water. The CNT/Fc-IL-CHO nanocomposite was obtained and then redispersed in PBS (0.1 M, pH7.0). The concentration of CNT/Fc-IL-CHO was 2 mg/ L.

2.3. Preparing the bioconjugates of CNT/Fc-IL-CHO/Ab2

Because protein contains amino groups, it can be cross-linked with –CHO groups of Fc-IL-CHO to form CNT/Fc-IL-CHO/Ab2 complexes. Typically, 200 μL antibody solution was added into 1 mL CNT/Fc-IL-CHO solution (2 mg L^{-1}) incubated for 2 h at room temperature under slow stirring. The CNT/Fc-IL-CHO/Ab2 complexes were subsequently centrifuged at 12000 rpm for 10 min. Washed three times with PBS (0.1 M, pH 7.0), the resulted complexes were dispersed in 1 mL PBS (0.1 M, pH 7.0)

2.4. Fabrication of the immunosensor

Before modifying, the gold electrode (3 mm in diameter) was polished with 0.3 μm alumina slurries and then washed with water. The cleaned electrode was modified as following: (1) 10 μL of chitosan solution was cast on the working area, dried in the air; (2) 2.5% glutaraldehyde (GA, 10 μL) solution was added on Chit film-modified electrode; (3)10 μL of anti-IgG solution (Ab1, 50 μg mL^{-1}) was cast on the electrode to be incubated at 37 ^{o}C for 40 min; (4) In order to blocked the nonspecific reaction sites, 10 μL BSA (2.0 wt%) was dropped on Ab1-modified electrode to be incubated for 40 min at 37 ^{o}C ; (5) 10 μL of antigen solution was added on the electrode and incubation for 40 min at 37 ^{o}C; (6) 10 μL of CNT/Fc-IL-CHO/Ab2 solution was added on the electrode and incubation for 60 min at 37 ^{o}C. The schematic of a stepwise modification of the immunosensor is shown in Fig. 1.

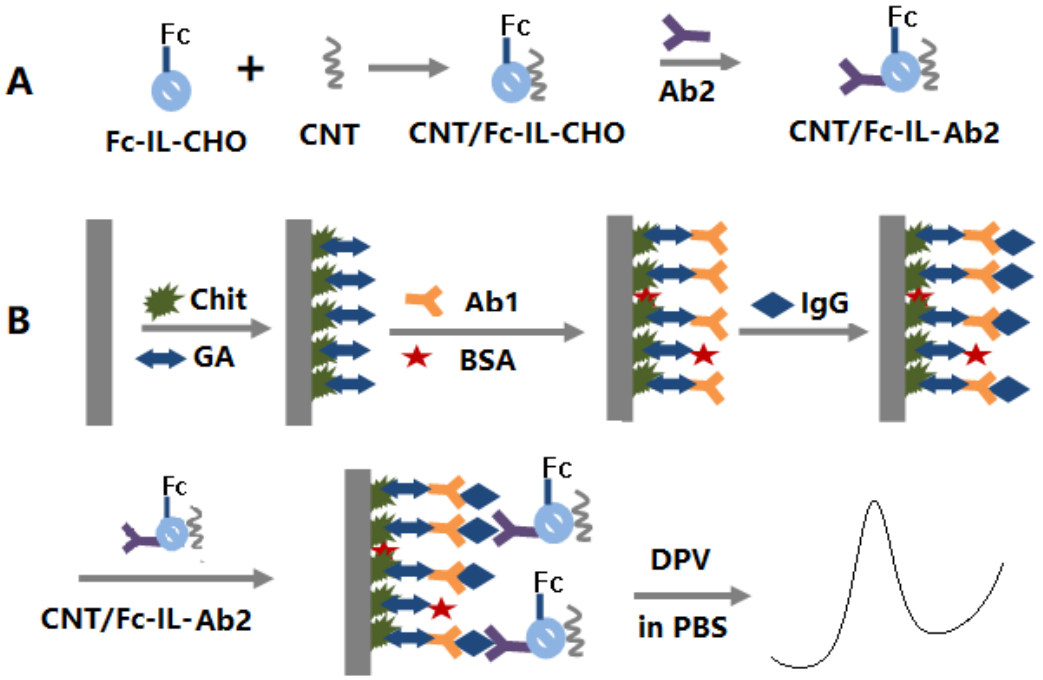

Figure 1. (A) Preparation route for CNT/Fc-IL-Ab_2 labels. (B) Schematic diagrams of fabrication of the electrochemical immunosensor.

2.5. ELISA Assay

In order to investigate the practicability of the proposed immunosensor, the enzyme-linked immunosorbent assay (ELISA) was performed using commercial kits according to the manufacturer's instructions. Before ELISA assays, the blood serum samples were diluted with PBS.

3. RESULTS AND DISCUSSION

3.1. Characterization of CNT/Fc-IL-CHO

The formation of nanocomposites of CNT/Fc-IL-CHO was characterized with UV–vis spectroscopy. As can be seen from Fig. 2, there was no absorption peaks of UV–vis spectrogram of

CNT (curve a), which was consistent with the result reported by Tahir and co-workers [27]. Fc-IL-CHO has two absorption peaks at 227 and 248 nm (curve b). The absorption peaks of CNT/Fc-IL-CHO nanocomposite were at 211 and 252 nm (curve c), the shifts of absorption peaks demonstrated Fc-IL-CHO was successfully bound to CNT.

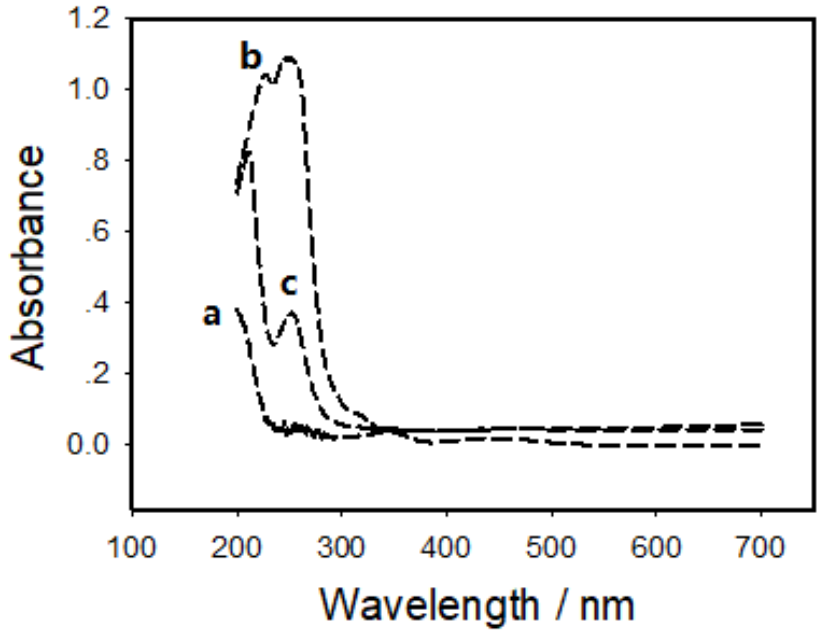

Figure 2. UV–vis spectra of CNT (a), Fc-IL-CHO (b) and CNT/Fc-IL-CHO (c).

3.2. Electrochemical characterization of the electrode modification

CV is a very convenient tool for investigating the barrier changes of the electrode surface during the stepwise fabrication [28,29]. In this work, the CV profiles were measured in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 100 mV s^{-1}. Fig. 3A showed the CV profiles of electrode at different states. The CV profiles of bare Au electrode had a couple of redox peaks (a). After the Chit film was formed on the electrode, the current response decreased obviously due to the insulating behavior of Chit (b). Similarly, the current response further decreased when GA was immobilized on the chit film (c). Following this, when primary antibodies (Ab1) were immobilized on GA via the covalent interaction between the aldehyde groups of GA and the amino groups of antibody, the redox peak current decreased (curve d), demonstrating that antibody had been successfully immobilized on the electrode surface. Herein, BSA was used to prevent the nonspecific binding. When BSA was bound to nonspecific sites, the redox peak current of CV decreased (curve e). After the IgG molecules were bound to antibodies, the decrease of anodic and cathodic peak was observed (curve f). Those results were consistent with the result reported [23]. However, when CNT/Fc-IL-CHO-Ab2 nanocomposite was modified on the electrode through antibody-antigen reaction, the peak current greatly increased (curve g), which can be ascribed to the high conductivity of IL and CNT. In addition, two oxidation peaks were observed (Fig. 3A, inset), indicating Fc was introduced to the electrode surface.

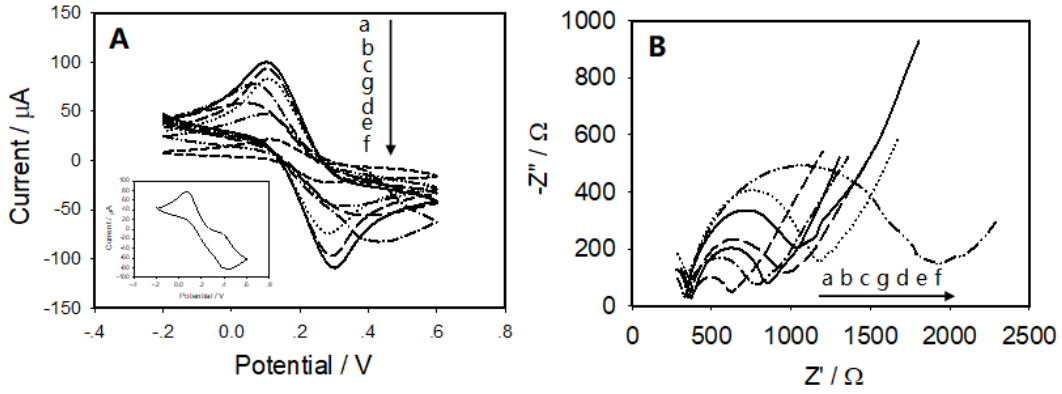

Figure 3. CV (A) and EIS (B) profiles of the stepwise modifying of the electrode: (a) Au bare electrode, (b) Chit/Au, (c) GA-Chit/Au, (d) Ab1-GA-Chit/Au, (e) BSA-Ab1-GA-Chit/Au, (f) IgG-BSA-Ab1-GA-Chit/Au, (g) CNT/Fc-IL-Ab_2/IgG-BSA-Ab1-GA-Chit/Au. The inset of (A) was the CV profile of CNT/Fc-IL-Ab_2/IgG-BSA-Ab1-GA-Chit–modified electrode. The concentration of IgG is 10 ng mL^{-1}.

EIS is an effective and convenient tool to monitor modifications on the electrode surface [30,31]. The EIS was carried out in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution. The semicircle diameter of the impedance spectrum reflects the situation of electron-transfer kinetics of the redox probe. The size of the diameter equals the electron transfer resistance (Ret) [32]. As shown in Figure 3B, bare electrode displayed a small semicircle (curve a). When bare electrode surface was stepwise modified with Chit, GA, Ab1, BSA, and IgG, the Ret values increased gradually (curves b, c, d, e, f, respectively). Finally, when the electrode was modified with CNT/Fc-IL-CHO-Ab2 nanocomposite, the Ret values decreased obviously (g).

3.3. Optimization of the experimental conditions

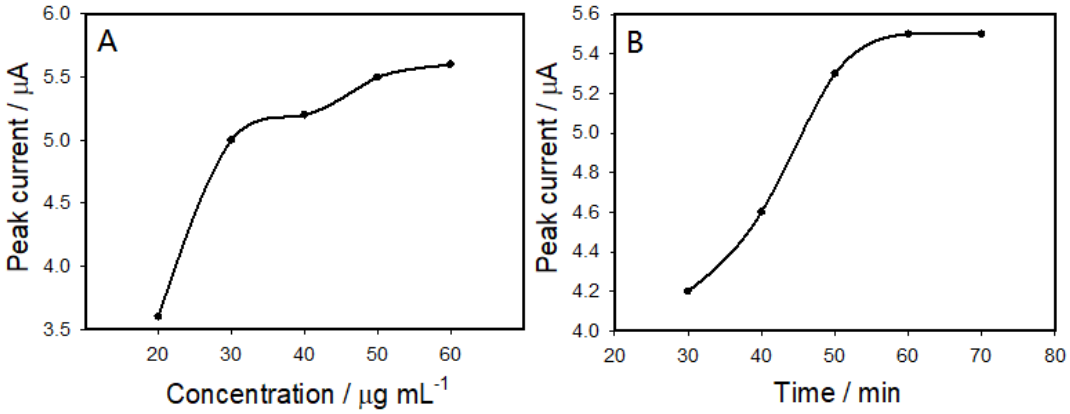

Figure 4. (A) Effect of the concentration of antibody on the peak current of DPV of the immunosensor. (B) Effect of immobilization time of CNT/Fc-IL-Ab2 on the peak current of DPV of the immunosensor. The concentration of IgG is 10 ng mL^{-1}.

In order to obtain a good performance of immunosensor, experimental conditions including the concentration of antibody, the immobilization time of CNT/Fc-IL-CHO-Ab2 nanocomposite were optimized. As can be seen from Fig. 4A, the antibody concentration of 50 μg mL^{-1} can be selected for this study. The influence of reaction time between antigen and CNT/Fc-IL-CHO-Ab2 on the current value was investigated in a range from 20 to 70 min. As can be seen from Fig. 4B, the peak current of DPV increased with increasing time up to 60 min. Thus, a reaction time of 60 min was selected for this work.

3.4. The detection of IgG

Under optimal experimental conditions, IgG was detected based on the peak currents of DPV corresponding to each concentration of IgG. Fig. 5A showed that the peak current increased with the increasing concentrations of IgG. Furthermore, the concentrations of IgG and the peak currents possesse a good linear relation in the range from 0.05 to 30 ng mL^{-1} with a detection limit of 0.01 ng mL^{-1} (S/N=3). The proposed immunosensor was compared with other IgG immunosensors. As shown in Table 1, the fabricated immunosensor by us was comparable with other immunosensors. More importantly, the reported IgG immunosensor based on Fc as labels had a linear relation in the range from 1 to 300 ng mL^{-1} with a detection limit of 0.4 ng mL^{-1} (S/N=3) [7]. Although the proposed immunosensor had a narrower linear range compared with the reported IgG immunosensor, the detection limit is 40 times higher of the proposed immunosensor's.

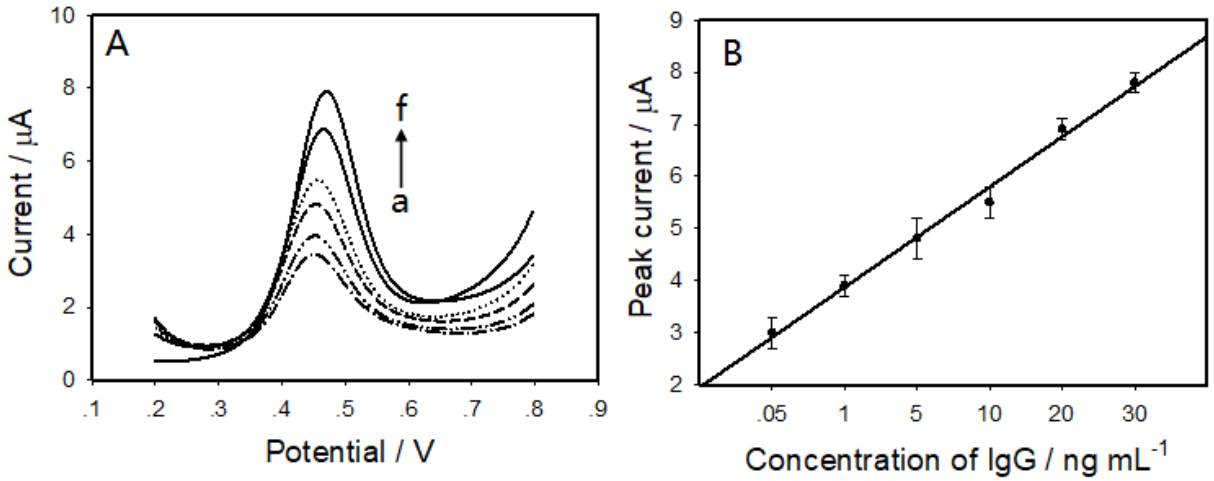

Figure 5. (A) DPV responses of the immunosensor to different concentrations of IgG. From a to f: 0.05, 1, 5, 10, 20, 30 ng mL^{-1}. (B) Calibration curve of the immunosensor to different concentrations of IgG. Error bars represent standard deviation, n=3.

Table 1. Comparison of the proposed immunosensor and other IgG immunosensors.

Modifying materials	Linear range ($ng.mL^{-1}$)	detection limit ($ng.mL^{-1}$)	References
Ionic liquid	0.1–10	0.03	[23]
Cellulose	0.5–45	0.3	[33]
Graphene oxide	2–100	1.70	[34]
Fc as label	1–300	0.4	[7]
CNT/Fc-IL-CHO as label	0.05–30	0.01	This work

3.5. Specificity, reproducibility, stability of the immunosensor

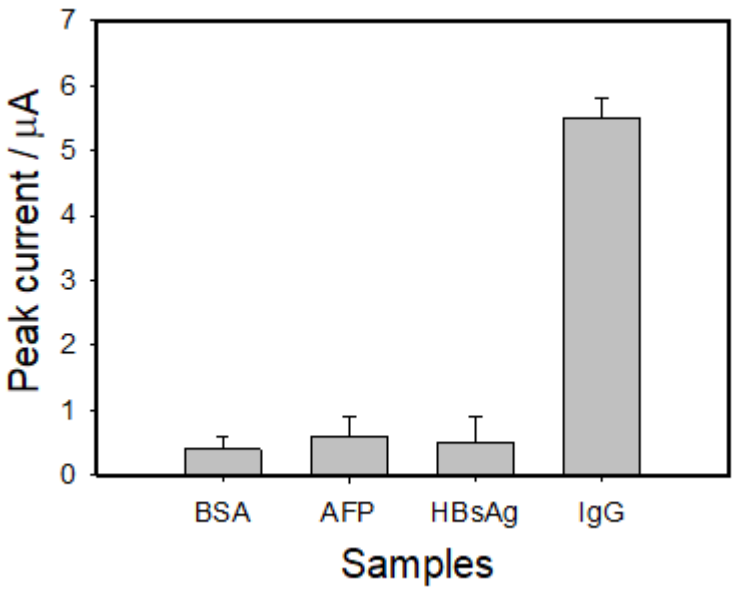

Figure 6. Specificity of the immunosensor. The concentration of nonspecific materials is 100 ng mL^{-1} and the concentration of IgG is 10 ng mL^{-1}, Error bars represent standard deviation, n=3.

The specificity of proposed immunosensor was examined by bovine serum albumin (BSA), α-fetoprotein (AFP), hepatitis B surface antigen (HBsAg). Under the same experimental conditions, the proposed immunosensor was incubated with the solution of BSA, AFP, and HBsAg, respectively. Fig. 6 showed that the peak current of DPV resulted from these nonspecific species was lower. However, the peak current corresponding to 10 ng mL^{-1} of IgG was the highest. The obtained results indicated that the specificity of the prepared immunosensor was acceptable.

Reproducibility of the proposed immunosensor was estimated by intra-assay and inter-assay at 10 ng mL^{-1} IgG (n = 5). The coefficient of variation (CV) for intra-assay and inter-assay was 9.7 and 6.6%, respectively, indicating that the immunosensor for IgG was reproducible.

The stability of the fabricated immunosensor was evaluated by measuring the DPV every one week. The modified electrode was kept at 4 ^{o}C when it wasn't in use. After two weeks, the peak current still retained 92% of initial value, indicating the immunosensor had a good stability.

3.6. Real sample analysis

The feasibility of the proposed immunosensor was investigated by detecting IgG in human serum samples. These immunoassay results were compared with those obtained by ELISA method. Table 2 showed that the proposed immunosensor was acceptable.

Table 2. Real sample analysis and comparison with ELISA method (n=3).

Sample	ELISA (ng mL^{-1})	This method (ng mL^{-1})	Relative deviation (%)
1	16.65	16.12	-3.2
2	5.39	5.76	6.7
3	20.17	22.03	9.2
4	4.54	4.74	4.4

4. CONCLUSIONS

In this work, we fabricated a sandwich-type electrochemical immunosensor for IgG using nanocomposite of carbon nanotube and multifunctional ionic liquid containing Fc as labels. The high sensitivity of the immunosensor was due to (1) the large surface area of CNT for increased-loading of Fc; (2) the high conductivity of CNT and IL. In addition, the developed immunosensor possessed good reproducibility, stability and specificity. This strategy provided a novel and simple technique for preparing labels. We anticipate it can be applied for the detection of other biomarkers.

ACKNOWLEDGMENTS
This work was supported by Hunan Provincial Natural Science Foundation of China (2016JJ6105).

References

1. U. Bilitewski, *Anal. Chem.,* 72 (2000) 692.
2. Z. P. Aguilar, W. R. Vandaveer and I. Fritsch, *Anal. Chem.,* 74 (2002) 3321.
3. J. Wu, L. Wang, H. Yu, Z. Abdin, R. U. Khan and M. Haroon, *J. Organomet. Chem*., 828 (2017) 38.
4. X. Yang, Y. Lu, Y. Ma, Y. Li, F. Du and Y. Chen, *Chem. Phys. Lett.*, 420 (2006) 416.
5. D. Sung and S. Yang, *Electrochim. Acta*, 133 (2014) 40.
6. L.B. Crepaldi, S. Aquino Neto, F.P. Cardoso, P. Ciancaglini and A.R. De Andrade, *Electrochim. Acta*, 136 (2014) 52.

7. G. Wang, X. Gang, X. Zhou, G. Zhang, H. Huang, X. Zhang and L. Wang, *Talanta*, 103 (2013) 75.
8. M. Senel, C. Nergiz and E. Cevik, *Sens. Actuators* B, 176 (2013) 299.
9. J. Qiu, M. Deng, R. Liang and M. Xiong, *Sens. Actuators B*, 135 (2008) 181.
10. M. Zhou, Z. Sun, C. Shen, Z. Li, Y. Zhang and M. Yang, *Biosens. Bioelectron.*, 49 (2013) 243.
11. P. Kanyong, S. Rawlinson and J. Davis, *J. Electroanal. Chem*., 766 (2016) 147.
12. R. Liang, L. Fan, D. Huang and *J. Qiu, Electroanal*., 23 (2011) 719.
13. W. Li and Z. Ma, *Sens. Actuators* B, 248 (2017) 545.
14. A. L. Goff, F. Moggia, N. Debou, P. Jegou, V. Artero, M. Fontecave, B. Jousselme and S. Palacin, *J. Electroanal. Chem.*, 641 (2010) 57–63.
15. G. Liu, M. Qi, Y. Zhang, C. Cao and E. M. Goldys, *Anal. Chim. Acta*, 909 (2016) 1.
16. K. Shang, X. Wang, B. Sun, Z. Cheng and S. Ai, *Biosens. Bioelectron*., 45 (2013) 40.
17. T. Feng, X. Qiao, H. Wang, Z. Sun and C. Hong, *Biosens. Bioelectron*.,79 (2016) 48.
18. M.A. Hussein, K. A. Alamry, F.M.A. Shehry and A.M. Asiri, *Talanta*, 150 (2016) 71.
19. S. Kim and H. Choi, *Sens. Actuators* B, 207 (2015) 424.
20. J. Wang, G.D. Liu and M. Rasul Jan, J. *Am. Chem. Soc*.,126 (2004) 3010.
21. M. J. A. Shiddiky, and A. A. J. Torriero, *Biosens. Bioelectron*., 26 (2011) 1775.
22. B. Kavosi, A. Salimi, R. Hallaj and K. Amani, *Biosens. Bioelectron*., 52 (2014) 20.
23. S. Dong, M. Tong, D. Zhang and T. Huang, *Sens. Actuators* B, 251 (2017) 650.
24. M. Roushani and A. Valipour, *Sens. Actuators* B, 222 (2016) 1103.
25. L, Zhou, J. Wang, L. Gai, D. Li and Y. Li, *Sens. Actuators* B, 181 (2013) 65.
26. Y. Shen, G. Shen and Y. Zhang, *Anal. Methods*, 10 (2018) 1612.
27. M.B. Tahira, G. Nabi, T. Iqbal, M. Sagir and M. Rafique, *Ceram. Int.*, 44 (2018) 6686.
28. S. MansouriMajd, H. Teymourian, A. Salimi and R. Hallaj, *Electrochim. Acta*, 108 (2013) 707.
29. Q. Zhu, Y. Chai, R. Yuan, Y. Zhuo, J. Han, Y. Li and N. Liao, *Biosens. Bioelectron.* 43 (2013) 440.
30. G. Lai, H. Cheng, D. Xin, H. Zhang and A. Yu, *Analy. Chim. Acta*, 902 (2016) 189.
31. H. Chen, J.H. Jiang, Y. Huang, T. Deng, J.S. Li, G.L. Shen and R.Q. Yu, *Sens. Actuators B*, 117 (2006) 211
32. E. Arkan, R. Saber, Z. Karimi, A. Mostafaie and M. Shamsipur, *J. Pharmaceut. Biomed.*, 92 (2014) 74.
33. X. Zhang, G. Shen, S. Sun, Y. Shen, C. Zhang and A. Xiao, *Sens. Actuators* B, 200 (2014) 304.
34. C. Thunkhamrak, P. Reanpang, K. Ounnunkad and J. Jakmunee, *Talanta*, 171 (2017) 53.