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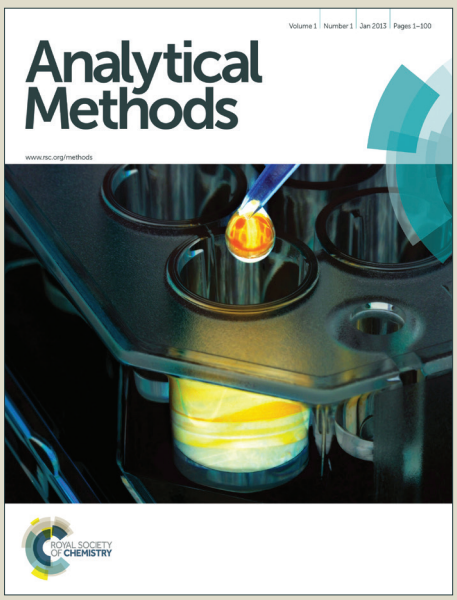

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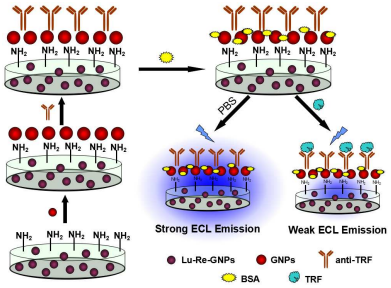

A disposable label-free electrochemiluminescent immunosensor based on luminol-reduced gold nanoparticles was designed for human transferring detection.

A disposable label-free electrochemiluminescent immunosensor for transferrin detection based on luminol-reduced gold nanoparticles-modified screen-printed carbon electrode

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Abstract:

A label-free electrochemiluminescence (ECL) immunosensor using luminol-reduced gold nanoparticles (Lu-Re-GNPs)-modified screen-printed carbon electrode (SPCE) as the sensing platform was proposed for the sensitive detection of transferrin (TRF), an anemia biomarker in human serum. To achieve the purpose of low cost and disposable use, a SPCE was used as the substrate of the immunosensor. The Lu-Re-GNPs-modified sensing platform exhibited stable and strong ECL emission, which could be employed for the recognition of target antigen with the aid of the unconjugated antibody immobilized on the electrode. With a simple label-free mode, the SPCE-based ECL assay with the advantages of low cost and facile manipulation was established successfully. In the presence of TRF, a decrease in the ECL intensity could be observed because of the immuno-binding event. This decrease in ECL intensity permitted sensitive detection of TRF in a linear range of 0.10-18 ng mL^{-1}, with a detection limit of 0.033 ng mL^{-1}. The assay results of TRF in serum samples were in a good agreement with those of ELISA. The reproducibility, the stability and the specificity of the proposed strategy were acceptable. The approach could also be further extended to facile immunoassay of other biomarker proteins.

Keywords: electrochemiluminescence; immunosensor; luminol-reduced gold nanoparticles; screen-printed carbon electrode; transferrin

Introduction

Immunoassay, based on the specific recognition between antigen and antibody, is believed to be an assay method with high selectivity. By integrating with some high sensitive detection methods such as radiometric, electrochemical, colormertric, fluorimetric, and chemiluminescent analysis, immunoassay has been extensively used in many different fields [1,2].

Currently, owing to the remarkable advantages of high sensitivity, labeling technology has been extensively applied in immunoassays. In previous reports, various signal probes, including radioisotope [3], enzyme [4], nanoparticle [5] and metal complex [6], were utilized for establishing tremendous amount of immunoassays based on labeling technology. However, in spite of the huge success achieved, traditional labeling techniques could still improve because the labeling process is always labor-intensive and time-consuming. Moreover, the conjugation chemistry involved in the labeling process increases the risk of damaging the activities of the biomolecules such as antibody and enzyme. Sometimes poor sensitivity is also obtained from the low conjugation efficiency of irrational conjugation chemistry [7]. Therefore, some label-free immunoassay strategies, such as surface plasmon resonance [8], electrochemical impedance spectroscopy [9] and quartz crystal microbalance [10], have attracted increasing interest since they can perfectly overcome these defects.

Electrochemiluminescent (ECL) immunoassay has attracted considerable attention due to its unique superiorities, such as high sensitivity and selectivity, easy controllability and simple instrumentation. Unfortunately, most of ECL immunoassays were developed by labeling techniques using such ECL probes as $Ru(bpy)_3^{2+}$ [11], luminol [12], carbon nanodot [13] and quantum dot [14]. Therefore, it is

necessary to develop facile, low cost and label-free ECL immunoassay for the purpose of point-of-care test.

Nowadays, special attention has been paid to ECL nanomaterials for their outstanding luminescence properties. In previous investigations, luminol-reduced gold nanoparticles (Lu-Re-GNPs) were reported as an ideal ECL nanomaterial for biosensors fabrication owing to their fascinating ECL characteristics [15]. Since thousands of luminol molecules were coated on the surface of gold nanoparticles (GNPs) in a convenient one-pot strategy, this nanomaterial exhibited stupendous amplification and better stability of ECL signal compared to the conventional non-nanoscaled ECL probe. However, in these reported works, it is still necessary to conjugate Lu-Re-GNPs with the recognition biomolecules such as aptamer [16] and antibody [17].

Herein, we pursue a novel strategy for disposable, simple, low-cost and sensitive immunoassay of protein. For this purpose, an ECL immunosensor was proposed by using Lu-Re-GNPs-modified screen-printed carbon electrode (SPCE) and unconjugated antibody to fabricate the sensing interface. The immuno-binding occurred on the interface was found to suppress the ECL emission of Lu-Re-GNPs. Thus a label-free ECL immunosensor was developed by using transferrin (TRF) as a model analyte. TRF is a major iron-binding monomeric glycoprotein with a typical concentration range of 2-4 mg mL^{-1} in plasma [18]. Hepatitis, anemia, and pregnancy often lead to increased level of transferrin, while nephritic syndrome, rheumatism, cirrhosis, malignant tumor, and leukemia result in concentration decrease of this protein[19]. The results of TRF detection in human plasma samples exhibited satisfactory sensitivity, stability and specificity of this method.

Experimental

Reagents and materials

Mouse monoclonal antibody for TRF (anti-TRF) was purchased from Abcam Ltd (USA). TRF, IgG, IgM, IgA and human serum albumin (HSA) was provided by Beijing Boisynthesis Biotechnology Co., Ltd (China). Luminol was purchased from Sigma-Aldrich (USA). A luminol solution (0.010 M) was prepared by dissolving 88.6 mg luminol in 50 mL 0.10 M NaOH. Chloroauric acid ($HAuCl_4 \cdot 3H_2O$) was obtained from Shenyang Research Institute of Nonferrous Metals (China). A $HAuCl_4$ stock solution (6.0 mM) was prepared by dissolving 1.0 g of $HAuCl_4 \cdot 3H_2O$ in 423 mL of ultrapure water and stored at 4 ^{o}C. Bovine serum albumin (BSA) was obtained from DingGuo Biotechnology Company (China). The chitosan solution at 1% was prepared by ultrasonically dissolving 0.25 g of chitosan (95% deacetylation, Aladdin Chemistry Co., Ltd., China) in 25 mL of 1% acetic acid. The ELISA kit for TRF was purchased from R&D Systems, Inc (China). Unfunctionalized gold nanoparticles (GNPs) with a diameter of 16 nm were home-prepared in accordance with a previous report [20]. The serum samples were provided by three healthy adult volunteers in Southwest University. All other chemicals were of analytical reagent grade and used without further purification. All aqueous solutions were prepared using the ultrapure water (18.2 MΩ) treated by ELGA PURELAB Classic system. Dilution buffer for capture antibody and antigen was 0.010 M phosphate buffer saline at pH 7.4 (PBS), and blocking buffer was PBS containing 0.1 % BSA and 0.05 % Tween-20.

Apparatus

ECL signal was detected by a MPI-A ECL analyzer (Xi'an Remax Electronic Science & Technology Co., Ltd., China) equipped with a photomultiplier biased at -1000 V and a potentiostat. ELISA was performed on a high performance Infinite M200 PRO multifunctional microplate reader (TECAN Group Ltd., Swiss) with injector option. Scanning electronmicrographs (SEMs) were obtained with an S-3000N scanning electronmicroscope (Hitachi Instrument Co., Ltd., Japan). Transmission electronmicrographs (TEMs) were obtained with a TECNA-110 transmission electron microscope (Royal Dutch Philips Electronics Ltd., Dutch).

Synthesis of Lu-Re-GNPs

The Lu-Re-GNPs with an average diameter of 20 nm were synthesized with a modified previous protocol [15]. Briefly, a 50-mL solution of $HAuCl_4$ (0.01%) was heated to boiling in an oil bath at 130 ^oC. With a vigorously stirring, 1.2 mL of 0.010 M luminol solution was added rapidly. The solution was maintained at the boiling temperature for 30 min under continuing stirring. During this period, a color change from light yellow to black, then to purple was observed before a wine-red color was finally reached. Then the oil bath was removed, and the product was cooling at room temperature (RT) for 30 min to quench the reaction. At last, the Lu-Re-GNPs was washed (centrifuged at 12500 rpm for 15 min) with ultrapure water for three times to remove unreacted reagents, and the red precipitate was dispersed in ultrapure water. The resulted Lu-Re-GNPs was stored at 4 °C until use.

Fabrication of label-free immunosensor

As seen in Fig. 1A and B, with a screen-printing technology [21], the SPCE (2.4 cm×1.0 cm) was assembled by one Ag/AgCl reference electrode, one carbon auxiliary

electrode, and one carbon working electrode. The working electrode was girdled around by the reference electrode and the auxiliary electrode. The Lu-Re-GNPs solution and the chitosan (CS) solution (1%) were thoroughly mixed at a volume ratio of 1:2. Then 5.0 μL of this mixture was coated onto the surface of the working electrode in the SPCE and dried at RT to form an amino-rich film. After that, the electrode was immersed in unfunctionalized gold colloid for 2.0 h to load the GNPs. Subsequently, 5.0 μL of the anti-TRF solution (25 μg mL^{-1}) was incubated with the GNPs film overnight. Finally, the modified SPCE was incubated in the blocking buffer for 30 min at RT to decrease the nonspecific adsorption. The resulted label-free immunosensor was washed with PBS and ready for use.

ECL immunoassay

Twenty microliter of TRF sample was dropped on the working electrode of the immunosensor, and incubated at 37 ^{o}C for 1.5 h. After thoroughly rinsing with PBS, the label-free immunosensor was immersed into an electrochemical cell filled with 6.0 mL of H_2O_2 in carbonate buffer solution (CBS, 0.050 M, pH 10.0). When a double-step potential (30 s pulse period, 1.0 s pulse time, 0.80 V pulse potential and 0 V initial potential) was applied to the working electrode, the ECL signal was generated and collected by the photomultiplier of the ECL analyzer.

Results and discussion

Strategy for label-free ECL immunosensor

Fig. 1C outlined schematically the assay protocol of the proposed label-free ECL immunosensor using Lu-Re-GNPs as the sensing platform. First, Lu-Re-GNPs were embedded in CS matrix on the surface of SPCE to form an amino-rich film, which was consisted of the highly deacetylated CS. As is known, at 22 ^{o}C, both amino group and hydrosulfide group of organisms could interact with a gold surface to generate

strongly covalent Au-S and Au-N bonds, respectively [22,23,24,25]. Therefore, the unfunctionalized GNPs could adsorbed on the Lu-Re-GNPs-coated SPCE via the covalent binding of gold and amidocyanogen. Subsequently, the antibody for TRF was directly immobilized on the GNPs monolayer through the formation of Au-S bonds. Here, a signal amplification effect could be achieved by using GNPs as the carrier of a large number of luminol molecules. In the absence of TRF, a strong ECL signal from Lu-Re-GNPs could be observed by applying suitable potential. It is known that proteins could interrupt the interfacial electron transfer and hinder the diffusion of the electrochemically active molecules due to their nonconductive properties [26]. Therefore, the immunoreaction between antibody and TRF produced a barrier for electro-transfer and influenced the electro-oxidation reaction of luminol, leading to a decrease in ECL intensity.

Characterization of immunosensor and Lu-Re-GNPs

Compared with the inhomogeneous surface of the bare SPCE (Fig. 2A), the morphology of the Lu-Re-GNPs-doped CS membrane-coated SPCE exhibited a three-dimensional porous structure (Fig. 2B). From this figure it was also observed that Lu-Re-GNPs were uniformly dispersed into the backbone of the CS membrane. The bright particles in the porous structure in Fig. 2C clearly indicated the presence of unfunctionalized GNPs, which could increase the loading amount of the capture antibody and accelerate the electron transfer. After finishing the overnight absorption of anti-TRF antibody, as seen in Fig. 2D, the porous structure was almost completely covered with a large number of aggregates of antibody, implied the consummation of the immunosensor fabrication.

Additionally, as shown in Fig. 3, the Lu-Re-GNPs were monodispersed spherical nanoparticles with an average size of approximate 20 nm.

Optimization of ECL immunoassay conditions

The factors influencing the performance of the label-free immunosensor was investigated in detail using antigens at 10 ng mL^{-1}. It is known that the ECL response of the immunosensor was related to the amount of the as-formed immunocomplex on its surface, while the latter was affected by the incubation time and the concentration of the capture antibody. As shown in Fig. 4A, the ECL response decreased sharply with an increasing incubation time and almost tended to the minimum at 1.5 h, suggesting that the immuno-binding attained the saturation at this incubation time. Additionally, the effect of the concentration of the capture antibody on the response was investigated using TRF at 10 ng mL^{-1} and PBS (as a blank) in parallel. Fig. 4B shows that the signal-to-blank ratios reached the minimum when the concentration of anti-TRF was 25 μg mL^{-1}, indicating that the ECL signal quenching ability of the sample was strongest. Thus, the incubation time of 1.5 h and the concentration of the capture antibody of 25 μg mL^{-1} were selected in the further study.

For the ECL detection system in this investigation, the pH value and the concentration of H_2O_2 were the crucial factors influencing the ECL response. As seen in Fig. 4C, the ECL response reached the maximum when the pH value was 10.0 (in 0.050 M CBS). The effect of the concentration of H_2O_2 on the ECL response was also studied. It was found that the ECL response varied with the concentration of H_2O_2 and reached the maximum when the concentration was 2.0 mM (Fig. 4D). Therefore, a H_2O_2 solution at 2.0 mM and pH 10.0 was adopted throughout this investigation.

Analytical performance of the ECL immunosensor

Under the optimal conditions, the ECL responses decreased linearly when the concentrations of TRF increased from 0.10 to 18 ng mL^{-1} (Fig. 5), since the immunoreaction occurred on the label-free immunosensor depressed the ECL

emission of the Lu-Re-GNPs. The linear regression equation was I (a. u.) = -286.3 C (ng mL^{-1}) + 7608, and the correlation coefficients (R^2) was 0.9907. The limit of detection for TRF was 0.033 ng mL^{-1} at a signal to noise ratio of 3. Furthermore, the relative standard derivations (RSDs) of five replicate determinations of TRF at 0.50 ng mL^{-1}, 5.0 ng mL^{-1} and 15 ng mL^{-1} were all less than 5.8%. Moreover, the storage stability of the immunosensor was also studied. After storage at 4 ^{o}C for 4 weeks, over 90% of the initial response still remained. Therefore, good reproducibility and acceptable durability were proved for the label-free ECL immunosensor using Lu-Re-GNPs.

Specificity of the immunosensor

In order to evaluate the specificity of the immunosensor, the interferences of various species including IgG, IgM, IgA and HSA were investigated since these proteins exist in the real serum samples. The specificity was estimated by comparing the responses to TRF and the interferent proteins. An obvious decrease of 46% in ECL intensity was observed for TRF at 10 ng mL^{-1}, while the ECL intensities for IgG, IgM, IgA and HSA at the same concentration were almost same as the blank signal obtained from PBS (data not shown). The results suggested that the specificity of the developed immunosensor for TRF was acceptable for real sample assay.

Application in serum samples

To evaluate the application potential of the proposed method, we measured the levels of TRF in three healthy human sera samples using the proposed label-free ECL immunosensor. For real samples analysis, standard curve method was adopted after the samples were diluted for 10^6 times to ensure the concentrations were within the linear range. The obtained results of the concentration of TRF in the three real samples (3.7, 3.1 and 2.9 mg mL^{-1}) showed acceptable agreement with those from the

conventional ELISA method (3.7, 3.2 and 2.8 mg mL^{-1}), with the RSDs of not more than 5.5%. For ELISA experiments, the samples were diluted for 10^3 times prior to assay. The relative errors for the measurement of the three samples using this method were -0.5%, -1.8%, -0.8%, respectively, compared with the results obtained from the reference method. Morevoer, recovery test was conducted using these samples spiked with TRF standards at different concentrations. As seen in Table 1, the recoveries from 96.3 to 106.3% and the RSDs below 6.4% demonstrated the reliability of the proposed method. All the RSDs were obtained by single addition and 5 repetitions of the measurements.

Conclusion

In summary, a novel disposable label-free ECL immunosensor based on Lu-Re-GNPs and unconjugated antibody-modified SPCE was developed for immunoassay of TRF. Compared with the classic labeling technology-based immunoassay method, this label-free strategy shows simple manipulation, low cost, and importanly, good bioactivity retaining of antibody. Furthermore, this method also showed better sensitivity than other non-labeling immunoassay methods such as immune precipitation and immune turbidity because ECL assay with high sensitive and Lu-Re-GNPs with high luminescence efficiency were adopted. This proof-of-principle work demonstrated its feasibility in clinical analysis of single protein. Our further investigation will apply it on immunosensors array for multiplexed assay of multiple proteins for point-of-care test purpose.

Acknowledgements

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References

[1] J. H. Lin, H. X. Ju, *Biosens. Bioelectron.*, 2005, **20**, 1461-1470.

[2] S. Viswanathana, C. Ranib, J. A. Ho, *Talanta*, 2012, **94**, 315-319.

[3] V. Laurent, I. Lindberg, *Anal. Biochem.*, 2002, **309**, 143-149.

[4] J. S. Kim, M. S. Jeon, K. J. Paeng, I. R. Paeng, *Anal. Chim. Acta*, 2008, **619**, 87-93.

[5] C. F. Ding, F. Zhao, R. Renb, J. M. Lin, *Talanta*, 2009, **78**, 1148-1154.

[6] Z. J. Cao, H. Li, C. W. Lau, Y. H. Zhang, *Anal. Chim. Acta*, 2011, **698**, 44-50.

[7] G. P. Anderson, R. H. Glaven, W. R. Algar, K. Susumu, M. H. Stewart, I. L. Medintz, E. R. *Anal. Chim. Acta*, 2013, **786**, 132-138.

[8] D. E. P. Souto, J. V. Silva, H. R. Martins, A. B. Reis, R. C. S. Luz, L. T. Kubota, F. S. Damos, *Biosens. Bioelectron.*, 2013, **46**, 22-29.

[9] B. Derkus, E. Emregul, C. Yucesan, K. C. Emregul, *Biosens. Bioelectron.*, 2013, **46**, 53-60.

[10] A. S. Afonso, B. F. Zanetti, A. C. Santiago, F. Henrique-Silva, L. H. C. Mattoso, R. C. Faria, *Talanta*, 2013, **104**, 193-197.

[11] Y. W. Chi, Y. Q. Dong, G. N. Chen, *Anal. Chem.*, 2007, **79**, 4521-4528.

[12] J. P. Li, Q. Xu, C. Fu, Y. Zhang, *Sens. Actuators B*, 2013, **185**, 146-153.

[13] S. W. Wang, L. Ge, Y. Zhang, X. R. Song, N. Q. Li, S. G. Ge and J. H. Yu, *Lab Chip*, 2012, **12**, 4489-4498.

[14] X. L. Fang, M. Han, G. F. Lu, W. W. Tu, Z. H. Dai, *Sens. Actuators B*, 2012, **168**, 271-276.

[15] H. Cui, W. Wang, C. F. Duan, Y. P. Dong, J. Z. Guo, *Chem-Eur. J.*, 2007, **13**, 6975-6984.

[16] F. Li, H. Cui, *Biosens. Bioelectron.*, 2013, **39**, 261-267.

[17] F. Li, Y. Q. Yu, H. Cui, D. Yang, Z. P. Bian, *Analyst*, 2013, **138**, 1844-1850.

[18] A. Leibman, P. Aisen, *Blood*, 1979, **53**, 1058-1065.

[19] A.D. Gupta, A. Abbi, *Am. J. Hematol.*, 2003, **72**, 158-161.

[20] A. Ambrosi, M. T. Castañeda, A. J. Killard, M. R. Smyth, S. Alegret, A. Merkoci, *Anal. Chem.*, 2007, **79**, 5232-5240.

[21] J. Wu, J. H. Tang, Z. Dai, F. Yan, H. X. Ju, N. E. Murrc, *Biosens. Bioelectron.*, 2006, **22**, 102-108.

[22] X. H. Xia, M. X. Yang, Y. C. Wang, Y. Q. Zheng, Q. G. Li, J. Y. Chen, Y. N. Xia, *ACS Nano*, 2012, **6**, 512-522.

[23] G. K. Ramachandran, T. J. Hopson, A. M. Rawlett, L. A. Nagahara, A. Primak, S. M. Lindsay, *Science*, 2003, **300**, 1413-1416.

[24] M. Kamenetska, M. Koentopp, A. C. Whalley, Y. S. Park, M. L. Steigerwald, C. Nuckolls, M. S. Hybertsen, L. Venkataraman, *Phys. Rev. Lett.*, 2009, **102**, 126803.

[25] M. Frei, S. V. Aradhya, M. Koentopp, M. S. Hybertsen, L. Venkataraman, *Nano Lett.*, 2011, **11**, 1518-1523.

[26] L. Yuan, W. Wei, S. Q. Liu, *Biosens. Bioelectron.*, 2012, **38**, 79-85.

Figure Captions

Fig. 1. Schematic illustrations of the immunosensor and the proposed strategy for TRF assay. (A) Photograph of immunosensors; (B) structure of SPCE: (a) polyethylene terephthalate substrate, (b) Ag conductive channel, (c) insulating layer, (d) carbon working electrode, (e) carbon auxiliary electrode, (f) Ag/AgCl reference electrode; (C) fabrication of the label-free ECL immunosensor.

Fig. 2. SEM images of (A) bare, (B) Lu-Re-GNPs/CS modified (C) Lu-Re-GNPs/CS/GNPs modified and (D) Lu-Re-GNPs/CS/GNPs/anti-TRF modified SPCE.

Fig. 3. TEM image of Lu-Re-GNPs.

Fig. 4. Effect of (A) incubation time (B) concentration of anti-TRF on the signal-to-blank ratio (C) pH of CBS (D) concentration of H_2O_2 on the ECL response. All the tests are performed under the optimal conditions, where $n = 3$ for each point.

Fig. 5. (A) ECL responses of TRF at concentrations of (a) 0.10, (b) 0.50, (c) 1.0, (d) 5.0 (e) 10, (f) 15, and (g) 18 ng mL^{-1}. Inset: calibration curves, where $n = 5$ for each point. All the tests are under the optimal conditions.

Figure 1

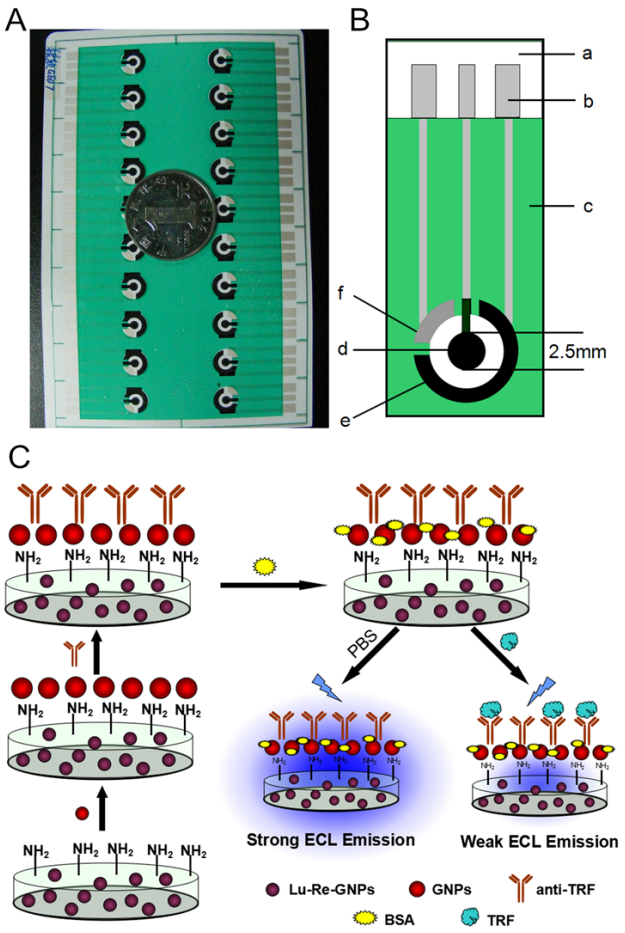

Figure 2

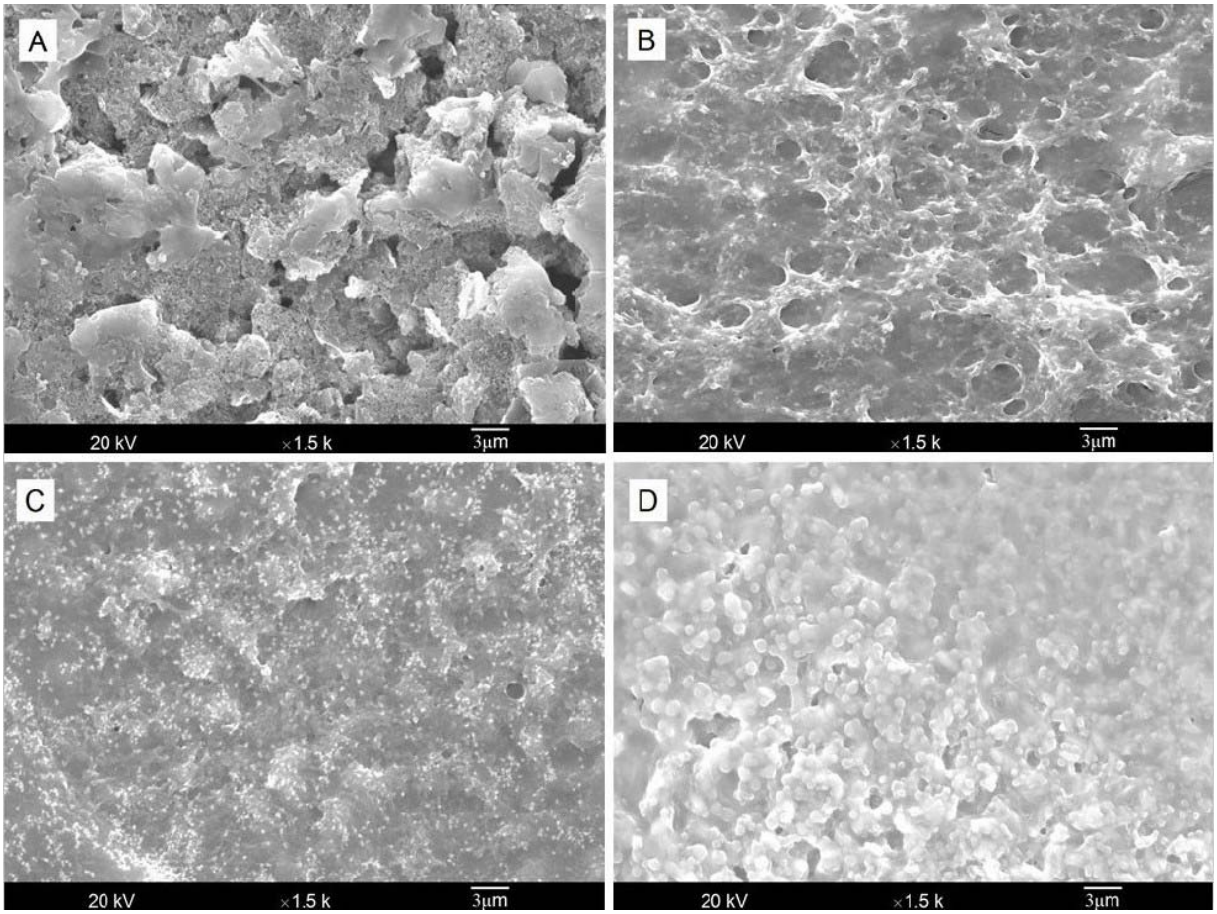

Figure 3

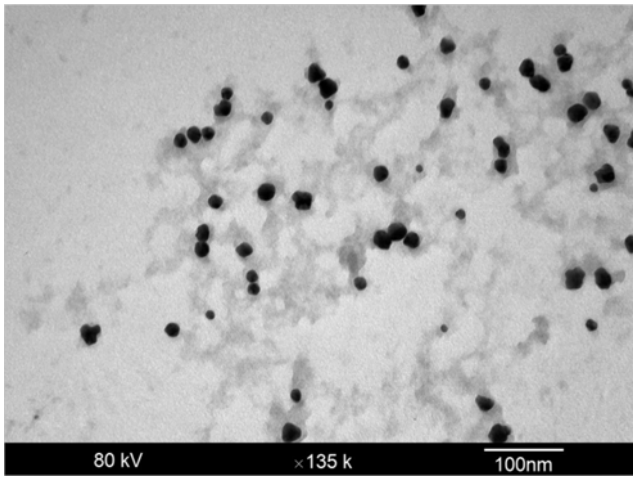

Figure 4

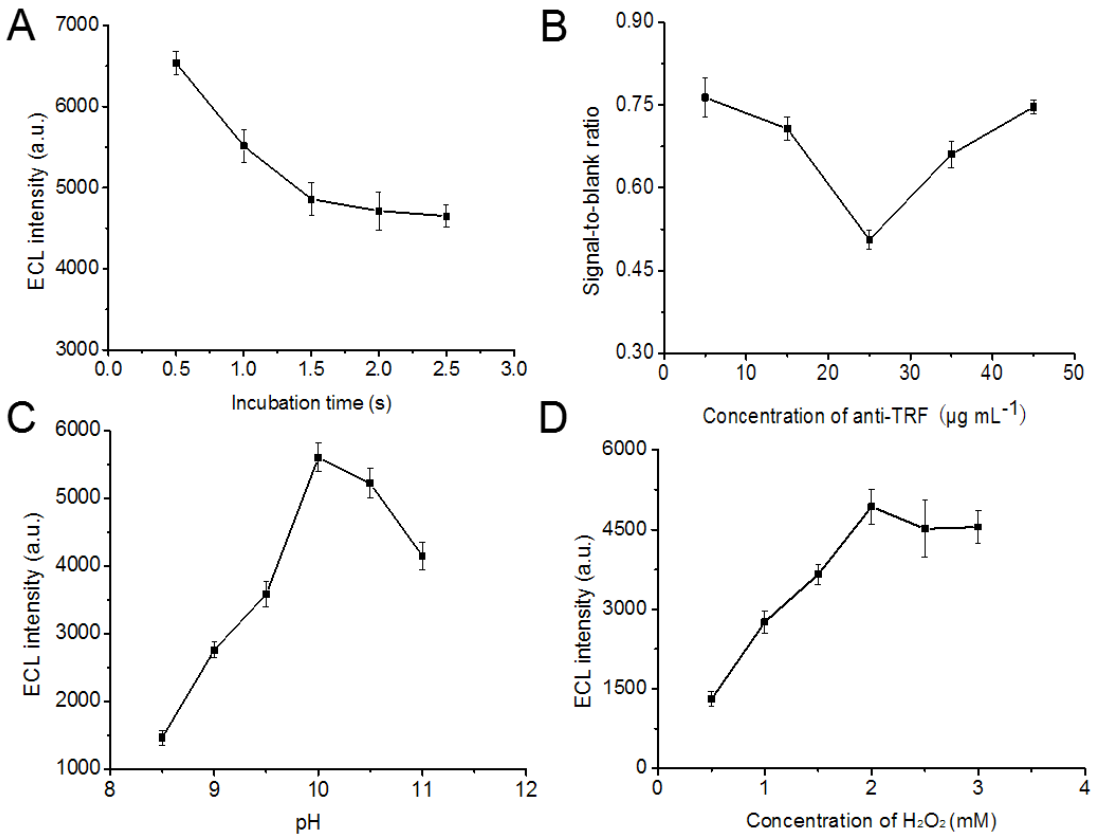

Figure 5

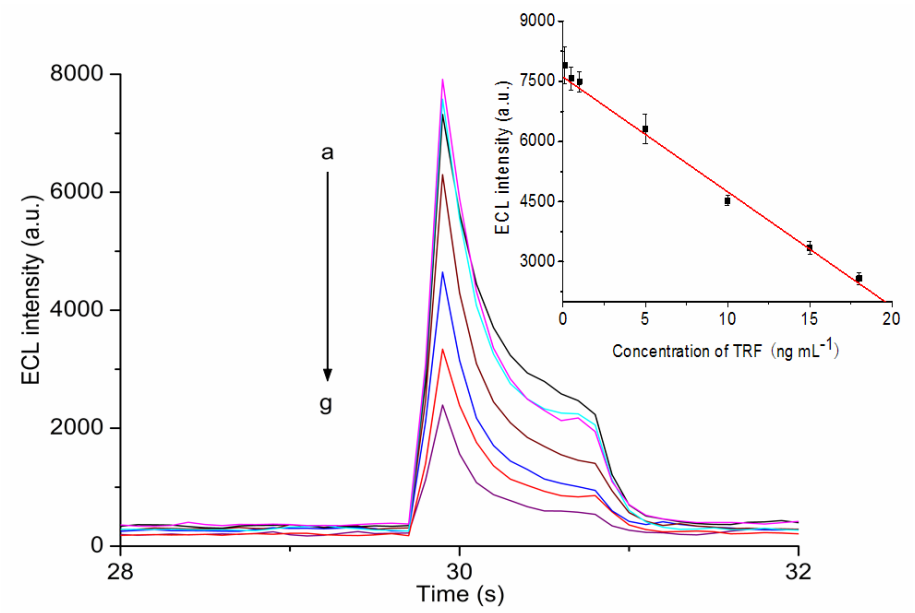

Table 1

Table 1. Recoveries of TRF spiked in the 10^6 times-diluted human serum samples obtained by the proposed method (n=5).

Serum samples number	1		2		3	
Initial (ng mL^{-1})	3.7		3.1		2.9	
Added (ng mL^{-1})	5.0	10.0	5.0	10.0	5.0	10.0
Found (ng mL^{-1})	8.4	13.6	7.8	13.8	8.4	12.9
RSD (%)	4.6	6.4	4.9	4.4	3.7	5.9
Recovery (%)	96.6	99.3	96.3	105.3	106.3	100.0