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Sensitive Immunosensor for N-Terminal Pro-brain Natriuretic Peptide Based on N-(Aminobutyl)-N-(ethylisoluminol)-Functionalized Gold Nanodots/Multiwalled Carbon Nanotube Electrochemiluminescence Nanointerface

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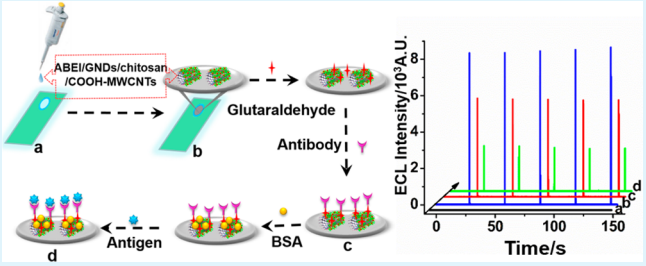
Supporting Information

ABSTRACT: A novel electrochemiluminescence (ECL) immunosensor was developed for the determination of N-terminal pro-brain natriuretic peptide (NT-proBNP) by using N-(aminobutyl)-N-(ethylisoluminol) (ABEI)-functionalized gold nanodots/chitosan/multiwalled carbon nanotubes (ABEI/GNDs/chitosan/COOH-MWCNTs) hybrid as nanointerface. First, ABEI/GNDs/chitosan/COOH-MWCNTs hybrid nanomaterials were grafted onto the surface of ITO electrode via the film-forming property of hybrid nanomaterials. The anti-NT-proBNP antibody was connected to the surface of modified electrode by virtue of amide reaction via glutaraldehyde. The obtained sensing platform showed strong and stable ECL signal. When NT-proBNP was captured by its antibody immobilized on the sensing platform via immunoreaction, the ECL intensity decreased. Direct ECL signal changes were used for the determination of NT-proBNP. The present ECL immunosensor demonstrated a quite wide linear range of 0.01−100 pg/mL. The achieved low detection limit of 3.86 fg/mL was about 3 orders of magnitude lower than that obtained with electrochemistry method reported previously. Because of the simple and fast analysis, high sensitivity and selectivity, and stable and reliable response, the present immunosensor has been successfully applied to quantify NT-proBNP in practical plasma samples. The success of the sensor in this work also confirms that ABEI/GNDs/chitosan/COOH-MWCNTs hybrid is an ideal nanointerface to fabricate a sensing platform. Furthermore, the proposed strategy could be applied in the detection of other clinically important biomarkers.



KEYWORDS: *N-terminal pro-brain natriuretic peptide, label-free immunosensor, electrochemiluminescence, N-(aminobutyl)-N-(ethylisoluminol)-functionalized gold nanodot, carbon nanotube*

INTRODUCTION

Heart failure (HF) has been a global health problem and more than 23 million people are suffering from HF around the world. In recent years, brain natriuretic peptide (BNP) as well as the N-terminal part of its prohormone (NT-proBNP) have been identified as two very important biochemical markers of cardiac function.[1] They are produced from ventricular myocytes and respond to volume expansion and pressure overload, and plasma concentrations. Because of the longer half-life, NT-proBNP tends to be more accurate for the identification of lower-degree HF.[2] Nowadays, the determination of NT-proBNP for diagnosis of HF is clinically accepted by the major HF guidelines.

Until now, NT-proBNP has been determined by an electrochemical method based on sandwich-type immunoassays using labeling technology.[3,4] With the purpose of improving the sensitivity of immunosensor, signal amplification using enzymes and nanomaterials for designing biosensors are commonly needed.[5−7] However, such labeling methods involve multiple steps of incubation and washing, which are laborious and time-consuming. Thus, a simple, fast, sensitive, and low-cost immunoassay for quick diagnosis is in high demand.[8] Recently, label-free techniques have attracted significant attention.[9−13] They are based on changes in physical signals when antigens interact with antibody and do not need the secondary antibody.[14−17] They are simple, fast, economical, and are very hopeful for the design of portable point-of-care testing devices for clinical diagnosis.

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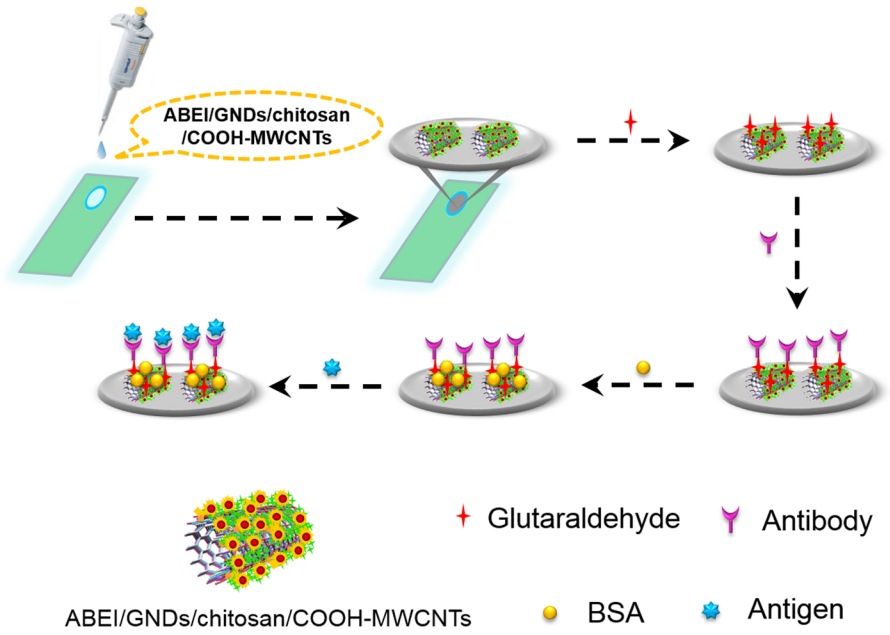

Figure 1. Schematic description for label-free NT-proBNP immunosensor based on ABEI/GNDs/chitosan/COOH-MWCNTs.

Recently, chemiluminescence (CL) functionalized nanomaterials have attracted much attention in bioassays because of their excellent signal amplification, easy assembly and good biocompatibility.[18−24] In our previous work, a high density of gold nanodots (GNDs) coated by CL molecules N-(aminobutyl)-N-(ethylisoluminol) (ABEI) was successfully assembled on the surface of chitosan-modified carboxylated multiwall carbon nanotubes (COOH-MWCNTs) to form hybrid nanomaterials (ABEI/GNDs/chitosan/COOH-MWCNTs). As stabilizers, thousands of ABEI molecules were directly attached to the hybrid nanomaterials during the synthetic process,[25] and therefore the resulting hybrid nanomaterials exhibited excellent CL/electrochemiluminescence (ECL) properties.

In the present study, a label-free immunoassay with ECL detection was developed for the determination of NT-proBNP by utilizing ABEI/GNDs/chitosan/COOH-MWCNTs as nanointerface for the fabrication of sensing platform. ABEI/GNDs/chitosan/COOH-MWCNTs hybrid nanomaterials were first grafted onto the surface of ITO electrode via the film-forming property of hybrid nanomaterials. Then the anti-NT-proBNP antibodies were assembled on the surface of the ABEI/GNDs/chitosan/COOH-MWCNTs-modified electrode through an amide reaction. When NT-proBNP was captured by its antibody via antigen−antibody immunoreaction, the ECL intensity decreased. Direct ECL signal changes enabled NT-proBNP to be determined quantitatively. The fabrication, ECL detection conditions, analytical performance and specificity of the ECL sensor were studied. In addition, the application possibility of the present ECL sensor for the determination of NT-proBNP in practical samples human plasma was examined.

EXPERIMENTAL SECTION

Chemicals and Materials. The carboxylated multiwalls carbon nanotubes were provided by Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). $HAuCl_4{\cdot}4H_2O$ was purchased from Shanghai Reagent, China. Two percent stock solution of $HAuCl_4$ (w/w) was obtained by directly dissolving 1.0 g of $HAuCl_4{\cdot}4H_2O$ (1 package) in 412 mL of ultrapure water and keeping it in storage in a refrigerator at 4 °C. ABEI (TCI, Japan) without further purification was dissolved in 0.1 M NaOH solution to prepare a 4 mM stock solution of ABEI. Working solutions of ABEI were prepared by diluting the stock solution. Chitosan (M_v > 1000 kDa, degree of deacytylation >90%) and glutaraldehyde (GA) were provided by Shanghai Chemical Reagent (Shanghai, China). 30% (v/v) H_2O_2 was purchased from Xinke Electrochemical Reagent Factory, Bengbu, China, which was used to prepare a fresh working solution of H_2O_2 daily. Human immunoglobulin G (IgG), lysozyme, and bovine serum albumins (BSA) were provided by Sigma (St. Louis, MO). Practical human plasma samples, cardiac troponin I (cTnI), NT-proBNP, and anti-NT-proBNP antibody were obtained by the First Affiliated Hospital of Nanjing Medical University. Ultrapure water obtained with a Milli-Q system (Millipore, France) was used for all the solutions and samples. The indium tin oxide (ITO)-coated glass (1.1 mm thickness, <10 Ω cm^{-2} resistance) was provided by CSG Holding Co., Ltd. (Shenzhen, China).

Apparatus. The morphology of ABEI/GNDs/chitosan/COOH-MWCNTs was characterized by transmission electron microscope (TEM, JEM-2010, Hitachi, Japan). Electrochemiluminescence measurements were performed with a home-built electrochemiluminescence system as described previously.[18] Electrochemical impedance spectroscopy (EIS) was measured with an electrochemistry workstation (CHI 760B, Chenhua, China).

Synthesis of ABEI/GNDs/chitosan/COOH-MWCNTs. The ABEI/GNDs/chitosan/COOH-MWCNTs were synthesized as described previously.[25] First, COOH-MWCNTs reacted with chitosan in acetic acid aqueous solution at 98 °C for 24 h to form chitosan-modified COOH-MWCNTs. 200 μL of the as-prepared chitosan/COOH-MWCNTs solution was dispersed in 45 mL of ultrapure water. Second, 7 mL of $HAuCl_4$ (6 mM) solution was pipetted into the mixture and stirred vigorously for 2 h. Third, 5 mL of ABEI (4 mM) was mixed to the solution above and was stirred for 12 h. Finally, a high density of ABEI-functionalized GNDs (average diameter: 1.5 ± 0.4 nm) was assembled on the surface of chitosan/COOH-MWCNTs to form ABEI/GNDs/chitosan/COOH-MWCNTs hybrids. After centrifugation, the resulting ABEI/GNDs/chitosan/COOH-MWCNTs were characterized by TEM (Figure S1 in Supporting Information) and stored in a refrigerator at 4 °C.

Preparation of ABEI/GNDs/chitosan/COOH-MWCNTs-Modified ITO Electrode. ITO-coated glass slides were treated as described previously.[25] The ABEI/GNDs/chitosan/COOH-MWCNTs- modified ITO electrode was prepared as follows. Typically, 1 mL of ABEI/GNDs/chitosan/COOH-MWCNTs colloid was centrifuged at 8000

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rpm for 10 min to remove the inorganic or organic impurities completely, and then redispersed in 100 μL of ultrapure water. Ten microliters of hybrid nanomaterial dispersion was drop-casted onto the surface of ITO electrode and dried under an infrared lamp. Thus, the ABEI/GNDs/chitosan/COOH-MWCNTs-functionalized ITO surface was obtained and ready for further experiments.

Construction of Label-Free Immunosensor with ECL Detection. Fifty microliters of 5% (v/v) GA solution was dropped with a micropipette on the ABEI/GNDs/chitosan/COOH-MWCNTs-modified ITO electrode and incubated at 37 °C for 2 h. The modified electrode was then cleaned with 0.01 M PBS in order to get rid of physically adsorbed GA. Then, 50 μL of 1.0×10^{-6} M anti-NT-proBNP antibody was dropped on the modified electrode and incubated at 37 °C for 40 min, and the modified electrode was washed several times with 0.01 M PBS in order to remove unbound anti-NT-proBNP antibody. After that, 5% BSA solution was dropped on the modified electrode and incubated at 37 °C for 40 min. Finally, 50 μL aliquots of NT-proBNP or human plasma or spiked human plasma were dropped on the modified electrode, incubated at 37 °C for 40 min and rinsed several times with 0.01 M PBS. Eventually, the NT-proBNP/anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs-modified ITO electrode, i.e., the targeted immunosensor, was successfully fabricated.

ECL Detection. ECL was measured using a three-electrode system in an ECL cell composed of a working compartment and an auxiliary compartment as described previously.[18] 3.0 mL of H_2O_2 solution containing 0.02 M carbonate buffer solution (CBS) was added into the working compartment. 3.0 mL of blank solution (0.02 M CBS, without H_2O_2) were added to the auxiliary compartment. An electrochemiluminescence signal was produced with a potential applied to the working electrode. In our previous work, the ECL behaviors of ABEI/GNDs/chitosan/COOH-MWCNTs modified on an ITO electrode have been investigated by cyclic voltammetry (CV) and pulse potential.[25] These results indicated that ECL signals (around 6000 A.U.) generated by double-step potential were much stronger than those (around 800 A.U.) generated by CV. Moreover, ECL signals obtained with double-step potential were reproducible when several pulse potentials were applied to electrode, which is well-suitable for repeated measurement of sensor. Thus, double-step potential was chosen to produce the ECL signals for the quantitative determination in this work.

RESULTS AND DISCUSSION

Construction and Sensing Strategy of NT-proBNP ECL Immunosensor. The strategy proposed for the fabrication of NT-proBNP immunosensor based on ABEI/GNDs/chitosan/COOH-MWCNTs hybrid nanomaterials as nanointerfaces is illustrated in Figure 1. First, as-prepared ABEI/GNDs/chitosan/COOH-MWCNTs were dropped on the surface of ITO electrode and dried under infrared lamp to form an ABEI/GND/chitosan/COOH-MWCNT-modified ITO electrode based on the good film-forming property of the hybrid nanomaterials. Then, 5% GA solution was immobilized onto the ABEI/GNDs/chitosan/COOH-MWCNTs-modified electrode through an amide reaction between the amino group of ABEI/GNDs/chitosan/COOH-MWCNTs and the aldehyde group of GA. The anti-NT-proBNP antibody was subsequently connected to the surface of the modified electrode via an amide reaction between the aldehyde group of GA and the amino group of antibody. After blocking with BSA, the label-free immunosensor was obtained. In the presence of target antigen NT-proBNP, NT-proBNP was captured onto the modified electrode through antigen−antibody interaction, causing a change in ECL intensity. According to the change of ECL signal, NT-proBNP concentration could be determined.

The ECL signals at different assembly stages of electrode were studied (Figure 2). No ECL response was obtained on a bare ITO electrode as shown in curve a. After the assembly of ABEI/GNDs/chitosan/COOH-MWCNTs on the surface of ITO electrode, strong ECL signals were produced (curve b), indicating that ABEI/GNDs/chitosan/COOH-MWCNTs were attached to the surface of electrode and the hybrid nanomaterial modified electrodes exhibited excellent ECL properties. In this case, ECL was originated from GNDs-catalyzed the reaction of H_2O_2 with ABEI radicals that were generated from the oxidation of ABEI.[17] After the introduction of anti-NT-proBNP antibody, the ECL signals (curve c) decreased because of their nonconductive properties, revealing successful assembly of antibody onto the surface of ABEI/GNDs/chitosan/COOH-MWCNTs modified electrode. In the presence of NT-proBNP, NT-proBNP was captured onto the modified electrode through antigen−antibody interaction, and the ECL intensity further decreased (curve d). NT-proBNP is one type of protein and is nonconductive. Therefore, it could block the interfacial electro-transfer and impede the diffusion of the electrochemically active molecules toward the matrix.[16] The decreased ECL intensity in the presence of NT-proBNP as observed was due to the formation of a nonconductive immunocomplex. Furthermore, the change in ECL intensity correlated to the concentration of NT-proBNP, laying the foundation for quantitative determination of NT-proBNP.

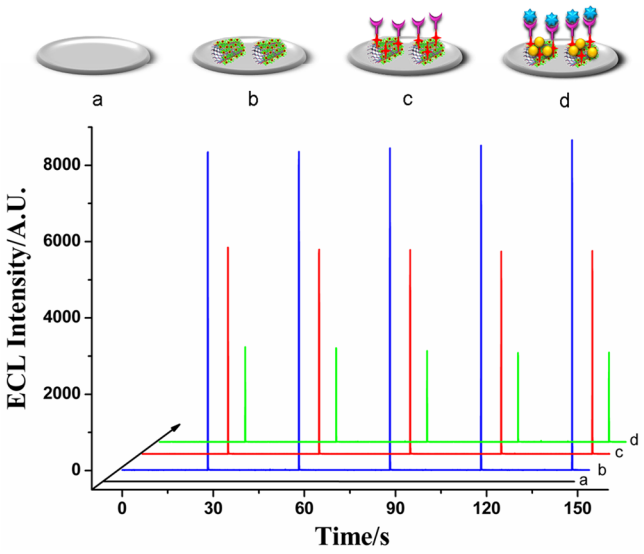

Figure 2. ECL responses under double-step potential produced on (a) bare ITO electrode, (b) ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode, (c) anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode, (d) NT-proBNP/anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode. Other conditions: 0 V Initial potential, 0.8 V pulse potential, 0.1 s pulse time, 30 s pulse period, 1 mM H_2O_2 containing CBS (0.02 M, pH 10.18) working solution.

EIS Characterization of the Modified Electrode during Stepwise Fabrication. EIS is an effective method to monitor the assemble process of the modified electrode, i.e., immunosensor. Figure 3 demonstrates EIS results at different modification stages in 1 mM $Fe(CN)_6^{4-/3-}$ solution ($Fe(CN)_6^{4-}$:$Fe(CN)_6^{3-}$ = 1:1) containing 0.1 M PBS (pH 7.0). It was observed that ABEI/GNDs/chitosan/COOH-MWCNTs modified ITO electrode (curve b) had lower impedance than bare ITO electrode (curve a), implying the good electron transfer property of ABEI/GNDs/chitosan/COOH-MWCNTs that could promote the surface electro-transfer of electrode.

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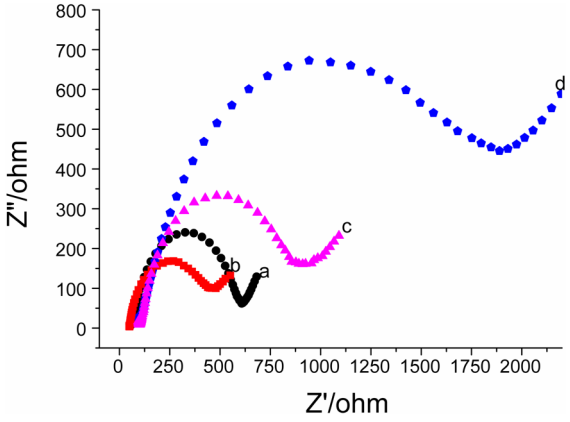

Figure 3. EIS of the electrode after different assembly stages on (a) bare ITO electrode, (b) ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode, (c) anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode, (d) NT-proBNP/anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode in 1 mM $Fe(CN)_6^{4-/3-}$ solution ($Fe(CN)_6^{4-}$:$Fe(CN)_6^{3-}$ = 1:1) containing 0.1 M PBS (pH 7.0). $C_{NT\text{-}proBNP}$ = 1.0 × 10^{-11} g/mL.

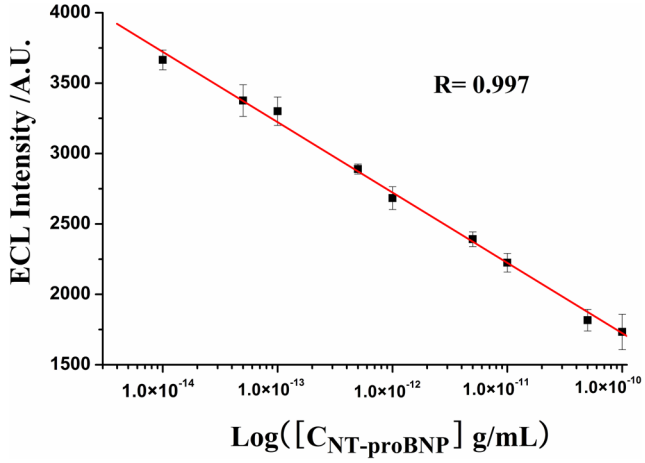

Figure 4. Calibration curve of developed immunosensor. ECL detection condition: same as Table 1.

The further assembly of anti-NT-proBNP antibody and capture of NT-proBNP increased the electron transfer resistance remarkably (curves c, d), attributed to the resistance of the interfacial electro-transfer from the proteins NT-proBNP and anti-NT-proBNP antibody. The results confirm that the resulted electrode was modified as expected.

Optimization of ECL Reaction and Immunoreaction Conditions. When a series of double-step potential was applied to the NT-proBNP/anti-NT-proBNP antibody/GA/ABEI/GNDs/chitosan/COOH-MWCNTs/ITO electrode, the pulse ECL signal could reach a stable value with several periods. Five stable signals were then averaged for the quantification of NT-proBNP. The ECL measurement parameters, such as pH and concentration of H_2O_2 solution, initial potential, pulse potential, pulse period, and pulse time, were optimized. Meanwhile, the immunochemical incubation time is a key experiment parameter for the immunosensor and 40 min was chosen according to the previous report.[4] As shown in Figures S2 and S3 (Supporting Information), the optimal ECL reaction conditions are obtained as listed in Table 1.

Analytical Performance of the Immunosensor. Under optimized conditions, the calibration curve for the response of immunosensor is obtained as shown in Figure 4. The results showed that the electrochemiluminescence intensity decreased linearly with the logarithm of concentration of NT-proBNP in the range of 0.01−100 pg/mL. The regression equation was I= −3269.7−499.4 log$C$ with a correlation coefficient of 0.997, where I and C referred to ECL intensity and NT-proBNP concentration, respectively. The detection limit was determined to be 3.86 fg/mL. The relative standard derivation (RSD) for the determinations of NT-proBNP at 1 pg/mL within a day (intraday precision, n = 5) and 5 days (interday precision, n = 5) was 4.02% and 9.62%, respectively, showing acceptable reproducibility. The results indicated that the developed sensor could be used for the ultrasensitive determination of NT-proBNP. The comparison of the proposed immunosensor with previously reported immunosensors for the determination of NT-proBNP is listed in Table 2.[3,4] Clearly, the ECL immunosensor presented in this work exhibits much better performance with wider linear range and lower detection limit, especially the detection limit, which is about 3 orders of magnitude lower than that obtained with electrochemistry method reported previously.[4] Besides, the construction process is much simpler and faster. Thus, this strategy is promising for the further development of portable point-of-care testing.

Table 2. Comparison of Developed NT-proBNP ECL Immunosensor Based on ABEI/GNDs/chitosan/COOH-MWCNTs/ITO Hybrids with Other NT-proBNP Immunosensors from the Literature

method	detection range	detection limit	ref
ECL	0.01−100 pg/mL	3.86 fg/mL	this work
microfluidic immunoassay	0.005−4 ng/mL	3 pg/mL	Liang et al. (2012)[3]
cyclic voltammetry	0.02−100 ng/mL	6 pg/mL	Zhuo et al. (2011)[4]

Selectivity of Label-Free NT-proBNP Immunosensor. With the aim of studying the selectivity of the proposed immunosensor, various interfering species including cTnI, IgG, lysozyme, BSA instead of NT-proBNP instead of NT-proBNP were used for the immunosensor. ΔI is used to assess the selectivity of the immunosensor and it is the relative ECL intensity calculated by $I_0 - I$, where I_0 and I are the ECL intensity produced by the blank solution and the ECL intensity produced by NT-proBNP or various interfering species, respectively. As shown in Figure 5, NT-proBNP exhibited the largest ΔI, whereas interfering species with ten times higher concentration of NT-proBNP showed much smaller ΔI, demonstrating that the immunosensor could distinguish NT-

Table 1. Optimal Conditions for ECL Reaction

parameter	pH of H_2O_2 solution	H_2O_2 concentration (mM)	initial potential (V)	pulse potential (V)	pulse period (s)	pulse time (s)
optimal condition	10.18	0.5	0	0.8	25	0.05

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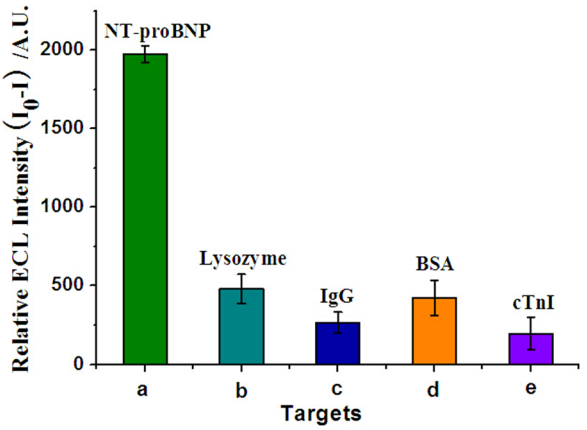

Figure 5. A comparison of ECL responses of NT-proBNP with ten times higher concentration of various interfering species. (a) NT-proBNP, (b) lysozyme, (c) IgG, (d) BSA, (e) cTnI. The relative ECL intensity $\Delta I = I_0 - I$, I_0 is ECL intensity produced by the blank solution and I is ECL intensity produced by NT-proBNP or various interfering species. NT-proBNP, 1 pg/mL; various interfering species, 10 pg/mL. ECL detection condition: same as Table 1.

proBNP from other investigated interfering species, and therefore, it has high selectivity. Ten times higher concentration of all the interfering species was used on purpose for a noticeable ΔI and easy comparison.

Determination of NT-proBNP in Human Plasma. The application possibility of the developed immunosensor in practical samples was explored by detecting NT-proBNP in human plasma samples. 100 times dilution of the human plasma samples with 0.1 M PBS (pH 7.4) was applied for quantitative determination. As seen in Table 3, the results determined by this immunosensor are comparable with those obtained by ELISA (RSD 3.3−5.9%) with high recoveries (102.40−95.80%). Accordingly, the present immunosensor could be used for the quantitative analysis of NT-proBNP in real samples of human plasma.

CONCLUSION

In the present study, a label-free ECL immunosensor based on ABEI/GNDs/chitosan/COOH-MWCNTs hybrid nanomaterials as nanointerface was established for highly sensitive detection of HF biomarker NT-proBNP. The measurement was based on the direct ECL signal changes when NT-proBNP was captured by its antibody. The as-proposed ECL immunosensor offers good selectivity and extremely high sensitivity for the determination of NT-proBNP with detection limit down to 3.86 fg/mL and wide linear range of 0.01−100 pg/mL. Furthermore, the present immunosensor has been successfully used for analysis of NT-proBNP in practical human plasma samples with satisfactory recoveries, holding great potential for further application in complicated biological samples. Compared with ABEI functionalized gold nanoparticles based immunesensor,[19] ABEI/GNDs/chitosan/COOH-MWCNTs hybrid-based label-free immunosensor avoid multiple steps of incubation and washing and labeling procedure, thus is more sensitive, faster and simpler. This work demonstrates that ABEI/GNDs/chitosan/COOH-MWCNTs hybrid nanomaterials are ideal nanointerfaces for the construction of sensing platform. Taking advantage of disposable screen-printed electrodes, it is promising to further develop portable point-of-care tests. In addition, the strategy is probably generalized for the detection of other clinically important biomarkers.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text, including TEM image of ABEI/GNDs/chitosan/COOH-MWCNTs and optimization of ECL reaction conditions for NT-proBNP immunosensor. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table 3. Quantitative Determination of NT-proBNP in Practical Human Plasma Samples

plasma sample	ELISA (pg/mL)	ECL sensor[a] (pg/mL)	relative deviation (%)	added amount (pg/ml)	detected amount (pg/mL)	recovery (%)	RSD (%) $n = 5$
1	17.97	18.25 ± 0.03	1.09	5	23.19 ± 0.04	98.80	5.9
2	3.23	3.41 ± 0.04	3.83	5	8.23 ± 0.03	96.40	4.2
3	1.39	1.26 ± 0.03	6.93	5	6.38 ± 0.06	102.40	3.3
4	0.787	0.93 ± 0.05	11.27	5	5.72 ± 0.05	95.80	5.1

[a]Mean value ± SD of five independent experiments, $n = 5$.

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