Article

Generation of Small Single Domain Nanobody Binders for Sensitive Detection of Testosterone by Electrochemical Impedance Spectroscopy

Guanghui Li, Min Zhu, Lu Ma, Junrong Yan, Xiaoling Lu, Yanfei Shen, and Yakun Wan

ACS Appl. Mater. Interfaces, **Just Accepted Manuscript** • DOI: 10.1021/acsami.6b04658 • Publication Date (Web): 19 May 2016

Generation of Small Single Domain Nanobody Binders for Sensitive Detection of Testosterone by Electrochemical Impedance Spectroscopy

Guanghui Li [1]**, Min Zhu [1]**, Lu Ma [2], Junrong Yan [2], Xiaoling Lu [5, 6, 7], Yanfei Shen [4]*, Yakun Wan [1, 3]*

[1]CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, P.R. China.

[2]Institute of Life Sciences, Southeast University, Nanjing 210018, P.R. China.

[3]Jiangsu Nanobody Engineering and Research Center, Nantong 226010, P.R. China.

[4]Medical school, Southeast University, Nanjing 210009, P.R. China.

[5]National Center for International Research of Biological Targeting Diagnosis and Therapy, Guangxi Medical University, Nanning, Guangxi 530021, P.R. China.

[6]Guangxi Key Laboratory of Biological Targeting Diagnosis and Therapy Research, Guangxi Medical University, Nanning, Guangxi 530021, P.R. China.

[7]The Department of Immunology, Guangxi Medical University, Nanning, Guangxi 530021, P.R. China.

*Corresponding authors at:

Yanfei Shen PhD, Professor, Dingjiaqiao No.87, Southeast University, Nanjing, 210009, P.R. China.

E-mail: Yanfei.Shen@seu.edu.cn (Y. Shen)

Yakun Wan PhD, Professor, 555 Zuchongzhi Road, CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, 201203, P.R. China

Tel.: +86-21-50805906.

E-mail: ykwan@simm.ac.cn (Y. Wan)

ABSTRACT

A phage display library of variable domain of the heavy chain only antibody (VHH) or nanobody (Nb) was constructed after immunizing a bactrian camel with testosterone. With the smaller molecular size (15 kD), improved solubility, good stability, high affinity, specificity and lower immunogenicity, nanobodies (Nbs) are a promising tool in the next generation of diagnosis and medical applications. Testosterone is a reproductive hormone, playing an important role in normal cardiac function and being the highly predictive marker for many diseases. Herein, a simple and sensitive immunosensor based on electrochemical impedance spectroscopy (EIS) and Nbs was successfully developed for the determination of testosterone. We have successfully isolated the anti-testosterone Nbs from an immune phage display library. Moreover, one of the Nbs was biotinylated according to *in vivo* BirA system, which showed the highest production yield and the most stable case. Further, the EIS immunosensor was set up for testosterone detection by applying the biotinylated anti-testosterone Nb. As a result, the biosensor exhibited a linear working range from 0.05 to 5 ng mL^{-1} with a detection limit of 0.045 ng mL^{-1}. In addition, the proposed immunosensor was successfully applied in determining testosterone in serum samples. In conclusion, the proposed immunosensor revealed high specificity of testosterone detection and showed as a potential approach for sensitive and accurate diagnosis of testosterone.

KEYWORDS: *nanobody; testosterone; immunosensor; glassy carbon electrode, electrochemical impedance spectroscopy*

1. INTRODUCTION

Testosterone was produced by both men and women with ten-fold higher levels of circulating in men than that in women. It is significantly correlated with several physiological processes, such as sperm production, energy, strength, sexual behavior, sleep, mood and the general well-being of men[1]. Whatever, being fundamental for the development and maintenance of male characteristics and male sexual organs, testosterone also has effects on most major organs, such as the brain, muscle, kidney, bone, liver and skin [2]. Therefore, testosterone is necessary and eventful for our bodies. Recently, several studies have also revealed that testosterone also plays an important role in normal cardiac function, and it has been opposing effect on cardiac remodeling and function compared with estrogen [3-4]. In the past few years, great efforts have been devoted to revealing the relationship between testosterone and physiological behaviors, and increasing evidences had proven its important roles in human diseases.

Because of the important relationship between testosterone and many diseases, testosterone is involved in regular six hormones diagnosis to assess the health condition of people accompanied with follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P) and Prolactin (PRL) [5]. Moreover, numerous studies have demonstrated that testosterone is a promising biomarker to predict stroke in old men [6], and it can be a better predictor to warn intermittent claudication in older men than E2 [7]. Furthermore, the low testosterone levels may be associated with earlier all-cause and cardiovascular related mortality among men [8], and basal testosterone level can be functionalized as a symbol for the ovarian response [9]. Accordingly, concentration determination of testosterone in serum is important to prognosis numerous diseases with other additional clinical features or symptoms.

Accurate and sensitive determination of testosterone becomes an important requirement to meet physical examination and disease diagnosis. Until now, a wide range of analytical methods is available for testosterone detection in biological fluids, including HPLC [10], GC-MS [11-12] and LC–MS [13-14]. However, these methods do need expensive instruments, specialized personnel and much time to get the results that limit the routine use. Meanwhile, many immunological assays were developed founded on the recognition of antigens by antibodies in clinical diagnosis. Various immunoassays have been developed to detect testosterone such as enzyme-linked immunoassay (EIA) , radioimmunoassay (RIA) [15] in plasma [16], serum [17] and human urine [18-19]. Nevertheless, the immunoassays mentioned above are often laborious, time-consuming and a major limited factor is the large requirement of high-quality testosterone-specific antibodies, which have hindered their further wide applications.

With the development of genetically engineered antibody and phage displays technology, antibody production has moved from conventional antibodies (polyclonal and monoclonal) to recombinant antibodies (Fab, Fv or scFv). Recently, an antibody fragment named VHH or nanobody is derived from heavy-chain-only antibodies that obtained in sera of camelids, such as camels, llamas and dromedaries [20]. The molecular weight of VHH domain was only about 15 KD, Nbs are potential reagents in the next generation of diagnosis and medical applications with the small molecular size, high affinity and specificity, improved solubility, good stability, well-performed acid-resistance, high yields with less cost and lower immunogenicity. In addition, because of the location of the antigen biding site is opposite to the C-terminus, Nb provides a of opportunities of modifications to make them powerful tools in research and application fields Compared with conventional antibodies, Nb was proved of obvious advantages in molecular size,

stability and specificity, which are the important properties of antibodies. These features of Nb us a promising alternative to replacing conventional antibodies in diagnosis, medicine discovery and more aggressive fields [23]. In our previous studies, Nbs were successfully applied for detection several clinical biomarkers, for example, apolipoprotein B-100 [24], NGAL [25] and procalcitonin [26].

Electrochemical immunosensors with lower sample consumption, less cost of instrumentations, good sensitivity, accurate and visible results as the main reasons, have been widely used in many markers detections. Electrochemical impedance spectroscopy (EIS) is useful for evaluating the interfacial properties of biochemical and biophysical processes with high specificity and sensitivity. Information can be collected from the signal alteration influenced by the adsorbing of target analytes to the functionalized surface of the electrodes. EIS has been widely applied for immunosensors fabrication, such as antigen–antibodies [27], DNA hybridization [28] according to the collection of charge transfer impedance variation.

A sample and sensitive immunosensor was established for testosterone determination depended on anti-testosterone Nb applying EIS. As shown in Figure 1, an immune phage display Nbs library against testosterone was constructed. After three rounds of bio-panning, three anti-testosterone Nbs have been isolated and expressed efficiently. Moreover, the highest yield and stability one of the obtained Nbs was selected to couple with biotin *in vivo*. Then, after with H_2SO_4 and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydro-xysuccinimide (NHS), the streptavidin (SA) was dropped onto the surface of glassy electrode (GCE). Subsequently, biotinylated Nb was added to SA. Eventually, the electrochemical immunosensor was successfully built up and for testosterone determination. The proposed immunosensor may offer an advanced approach to combine the classic electrochemical method

Nbs and generate a promising way for determination of testosterone.

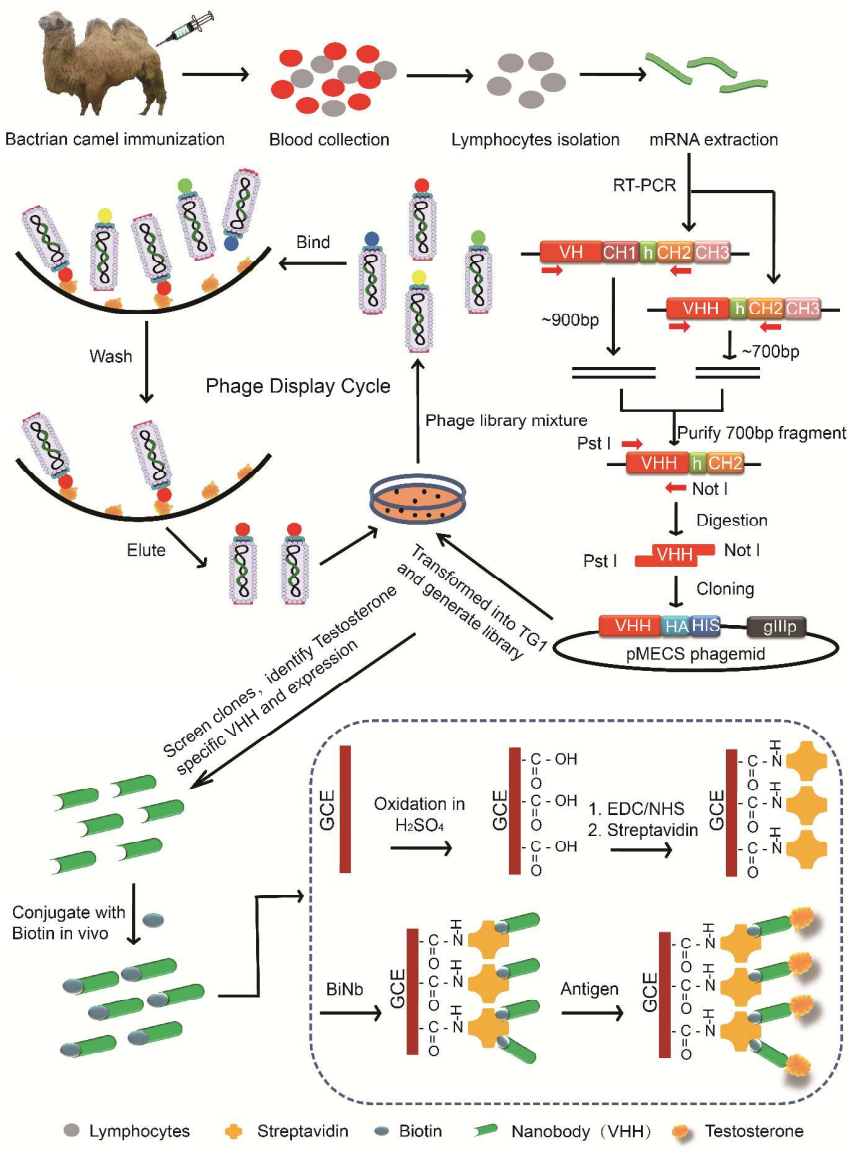


Figure 1. Workflow of the identification of anti-testosterone Nbs and Nbs-based electrochemical immunosensor development.

2. EXPERIMENTAL SCETION

2.1 Materials and Instruments. Testosterone-BSA was purchased from Fitzgerald (Acton, USA). Testosterone was achieved from Aladdin (Shanghai, China). Streptavidin (SA) was obtained from Shanghai Ruiqi Biological Technology Co., Ltd. (Shanghai, China). Freund's

complete adjuvant, Freund's incomplete adjuvant, anti-mouse IgG-alkaline phosphatase, ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), Bis (p-nitrophenyl) phosphate, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydro-xysuccinimide (NHS) and Ethanolamine-HCl were purchased from Sigma-Aldrich (St. Louis., USA). Mouse anti-HA tag antibody was obtained from Covance (Princeton, USA). Lymphocyte isolation sterile solution was provided by GE healthcare (Piscataway, USA). Oligo (dT) primer and 96-well plates were purchased from Thermo Fisher Scientific (Waltham, USA). *Pst* I, *Not* I, *Nco* I and *BstE* II were obtained from NEB (Ipswich, USA). D-biotin was purchased from Bio Basic Inc (Markham, Canada). Bovine serum albumin (BSA) was purchased from Sunshine Biotechnology Co., Ltd. (Nanjing, China). Streptavidin Mutein Matrix was purchased from Roche (Vilvoorde, Belgium). All aqueous solutions were prepared with deionized water (DI water, 18 mΩ cm^{-1}, Milli-Q, Millipore, Billerica, USA). Absorbance determination was performed on Bio-Rad iMark™ (Bio-Rad, California, USA). Concentration measurements of mRNA, DNA and protein were carried out with Nano Drop 2000 (Thermo Fisher Scientific, Waltham, USA). Affinity was measured by Biacore T200 (GE, Piscataway, USA), EIS was performed on a CHI 660 electrochemical workstation (Chenhua Instruments Co., Shanghai, China) with a three-electrode. The electrode system contained a modified GCE (3 mm in diameter), a Pt wire counter electrode, and a KCl saturated Ag / AgCl reference electrode.

2.2 Bactrian Immunization. A young male bactrian camel was immunized with 400 μg testosterone-BSA in phosphate buffered solution (PBS, 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl) with equal volume of Freund's complete adjuvant (the first time) and Freund's incomplete adjuvant (next 5 times). The injections were conducted every week, after six times

injections, lymphocytes cells were extracted from the peripheral blood of immunized camel for the VHH library construction.

2.3 Phage Display Library Construction. After immunization the library was constructed. Briefly, RNA was extracted from blood and transcribed to cDNA. We obtained the VHH regions via two-step nest PCR, which fragment about 400 bp. The PCR outcomes and the vector of pMECS phagemid were digested with *Pst* I and *Not* I, subsequently, ligated to pMECS/Nb recombinant. After ligation, the products were transformed into *Escherichia coli* (*E. coli)* TG1 cells by electroporation. Then, the transformants were enriched in growth medium, followed by planted on plates. Finally, library size we constructed was estimated by counting colonies number.

2.4 Bio-panning, Expression and Purification of Soluble Nbs. Library bio-panning was conducted to screen Nbs against testosterone after library construction. Firstly, testosterone-BSA was diluted with coating buffer (100 mM $NaHCO_3$, pH 8.2) to 100 μg mL^{-1} and coated onto microtiter plates at 4ºC overnight. BSA protein in the coating buffer was used as control. 200 μL of 0.1% casein (dissolved in 1×PBS) were added for 2 h incubation after washed by PBST (PBS with 0.05% Tween-20) to block the residual antigen binding sites. After removing the blocking buffer, the phages were added and incubated at room temperature for 1 h. The specific phages to testosterone were eluted with 100 mM trimethylamine followed by neutralized with Tris-HCl (1.0 M, pH 7.4). The rest of the phages were infected with TG1 cells, which have diluted at various concentration before. After 2 rounds of panning, the testosterone-specific phages were amplified enough to perform the following research. Afterwards, we obtained 15 positive colonies after the process of library bio-panning and PE-ELISA. Testosterone-specific VHHs were confirmed by sequencing of these positive colonies. Nbs were picked out according to the sequence. Then the

plasmids were extracted and electroporated into *E. coli* WK6 electrocompetent cells. Briefly, when the culture of WK6 cells reached an OD_{600} value of 0.6 to 0.9, 1 mM IPTG was added, followed by shaking overnight at 28ºC. Protein was released by osmotic shock. Nbs specific to testosterone containing 6×His tag, therefore they were purified with nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography. After eluting with PBS that containing 500 mM imidazole, we assessed the purity and size of the Nbs using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue.

2.5 The Thermofluor Experiment. The thermofluor experiment was used to detection the T_m value of Nb16. A 96-well microplate was used in the measurement, with the wells containing 19.5 μL of Nb16, which had diluted into 0.5 mg mL^{-1} with triplicate and 0.5 μL of SYPRO Orange. A LightCycler® 96 Real-Time PCR system (Hoffmann-La Roche Ltd., Basel, Switzerland) was chosen to control the temperature and record the fluorescence changes during T_m measurement. Samples were placed at 25ºC for 3 min, the temperature was set increased from 25ºC to 95ºC by 5ºC increments, the equilibration time of each temperature was 10 s. The fluorescent properties of the dye-protein complex with a maximal absorption at 470 nm and maximal emission at 569 nm, minimize the interference with background fluorescence from small molecules. At last, the data was analyzed by software LightCycler 96 [29-30].

2.6 Solvent Effect. We explored the tolerance to organic solvent of Nb16. Since EtOH, isopropanol and trichlormethane are used in the extraction procedures of testosterone, we chose these three organic solvents to our research. Firstly, testosterone-BSA was diluted with coating buffer (100 mM $NaHCO_3$, pH 8.2) to 5 μg mL^{-1} and planted onto 96-well plates at 4ºC overnight. BSA protein as control. Next, 200 μL of 1% milk (dissolved in 1×PBS) were added to the wells

after washed by PBST for blocking. After removed the blocking buffer, the Nb16, which diluted with different organic solvents from 5 mg mL^{-1} to 10 μg mL^{-1} was added and incubated for 1 h. After washed with PBST, antibody mouse anti-HA and anti-mouse IgG were added, respectively. The absorption of 405 nm was observed at last.

2.7 Surface Plasmon Resonance (SPR) Affinity Detection. The affinities of the obtained Nbs were tested by Biacore T200 after getting the specific Nbs against testosterone. Firstly, Nbs with the concentration of 20 μg mL^{-1} were immobilized on the CM5 sensor chip. Then five different concentrations of testosterone were added with a flow rate of 10 μL per minute [31]. Afterwards, the binding sensorgrams signals were recorded and used to calculate the k_a and k_d values which K_D.

2.8 Biotinylation of Nb *in vivo*. Vector pBAD has a biotin acceptor domain. VHH genes were sub-cloned to the vector by both digested with *Nco* I and *BstE* II. Then the recombinant was transformed into WK6 cells with pBirA plasmid. After cultured in supplemented with 50 μM D-biotin and continued to induce with 1 mM IPTG overnight to produce proteins conjugated with biotin, soluble Nbs coupled with biotin can be purified by Streptavidin Mutein Matrix. The biotinylated Nbs were eluted by 6 mM D-Biotin.

2.9 Immunoseneor Preparation. The electrodes were polished with micro-cloth polishing pads, using 0.3 and 0.05 μm alumina slurries for 5 min. Then carefully washed and sonicated in Milli-Q water for 1 min. CV measurement was performed to assess the quality of these polished electrodes. A conventional three-electrode configuration was applied: GCE as the working electrode, Ag/AgCl (3 M KCl) as the reference electrode and Pt as the counter electrode. Measuring conditions were as follows: minimum potential -0.2 V, maximum potential 0.6 V, scan

rate 100 mV s^{-1} in 0.1 M PBS containing 2 mM $K_3Fe(CN)_6$.

After getting the eligible GCEs, the electrodes were scanned in 0.5 M H_2SO_4 from -1 V to 1 V at the scan rate of 100 mV s^{-1} for 20 cycles. Finishing electrochemical pre-treatment, the surface of GCE was equipped with carboxylic acid groups and immersed in water to avoid contaminating by air.

2.10 CV and EIS Measurements. The GCE functionalized with carboxylic acid groups was next immersed into the mixture of NHS and EDC with the concentration of 50 mM and 100 mM respectively in 0.1 M PBS of pH 7.4 for 1 h. Then, the electrode was rinsed carefully by 0.1 M PBS of pH 7.4 and 20 μL of SA (100 μg mL^{-1}) in 0.1 M PBS was dropped onto the surface. After incubation at 4ºC for 12 hours, the electrodes were rinsed with PBS (0.1 M, pH 7.4) and then blocked with 0.5% BSA for 30 min. Then, 20 μL of BiNb16 (100 μg mL^{-1}) diluted in PBS were added to the surface of the electrode for incubation for 1 h. Finally, the electrode was incubated with different concentrations of testosterone (20 μL of droplet) for 1 h and rinsed carefully with PBS. All the above experiments were performed at room temperature.

To monitor each step of the electrode modification, all procedures were evaluated by CV and EIS. EIS was expected to monitor the electron transfer resistance in frequency range of 0.1 Hz–100 kHz at potential of 0.22 V and performed in PBS containing 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1). Obtained data was analyzed using the Zview software.

3. RESULTS AND DISCUSSION

3.1 Immune VHH Library Construction. VHHs can be derived from different libraries, compared with the non-immune library and synthetic phage displays, VHHs derived from the immune library, which has more advantages than the other two, especially in affinity and

specificity. We resorted to immune VHH library, expecting to obtain the single domain antibodies with high affinity and specificity. We immunized bactrian camel by injection of testosterone-BSA antigen with high purity and immunogenic weekly. All camel experiments were conducted according to guidelines approved by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica. After six times immunization, blood was collected for extracting RNA, which was reversely transcripted to cDNA. Then we began to amplify the heavy chain antibody variable region, as shown in Figure 2A, the first PCR fragments have evident bands around 700 bp. The products of first PCR were used as the template to enrich the VHH-only region via a nested PCR. From Figure 2B, the second PCR outcomes are correct with 400 bp. Then VHH fragments were ligated into pMECS vector and transformed in TG1 *E. coli* cells. The cells were cultured for library generation. Library size and insertion rate represents the quality of the library, the library size was estimated around 1×10^8 colony-forming units (CFU), which can be expected to generate enough high specific and sequence diverse Nbs. The insertion rate of the immune library against testosterone was evaluated by randomly selected 24 clones to perform PCR (Figure 2C). A correct insertion rate of 95.8% revealed that we had successfully constructed a VHHs library against testosterone. In conclusion, we achieved a high quality and diversity phage display Nbs library against testosterone.

3.2 Phage Library Screening and Anti-testosterone Nbs Identification. Bio-panning was started to isolate specific Nbs against testosterone after obtaining the good-quality phage display library. Firstly, the library should be pre-amplified and approximately 2×10^{11} phages were used in each round bio-panning. As shown in Figure 2D and Figure 2E, after three rounds of bio-panning, the enrichment fold had achieved from 75 (second round bio-panning) to 490. Based on the great

diversity and large size of the phage library, we stopped phage library screening and planned to pick out the positive colonies.

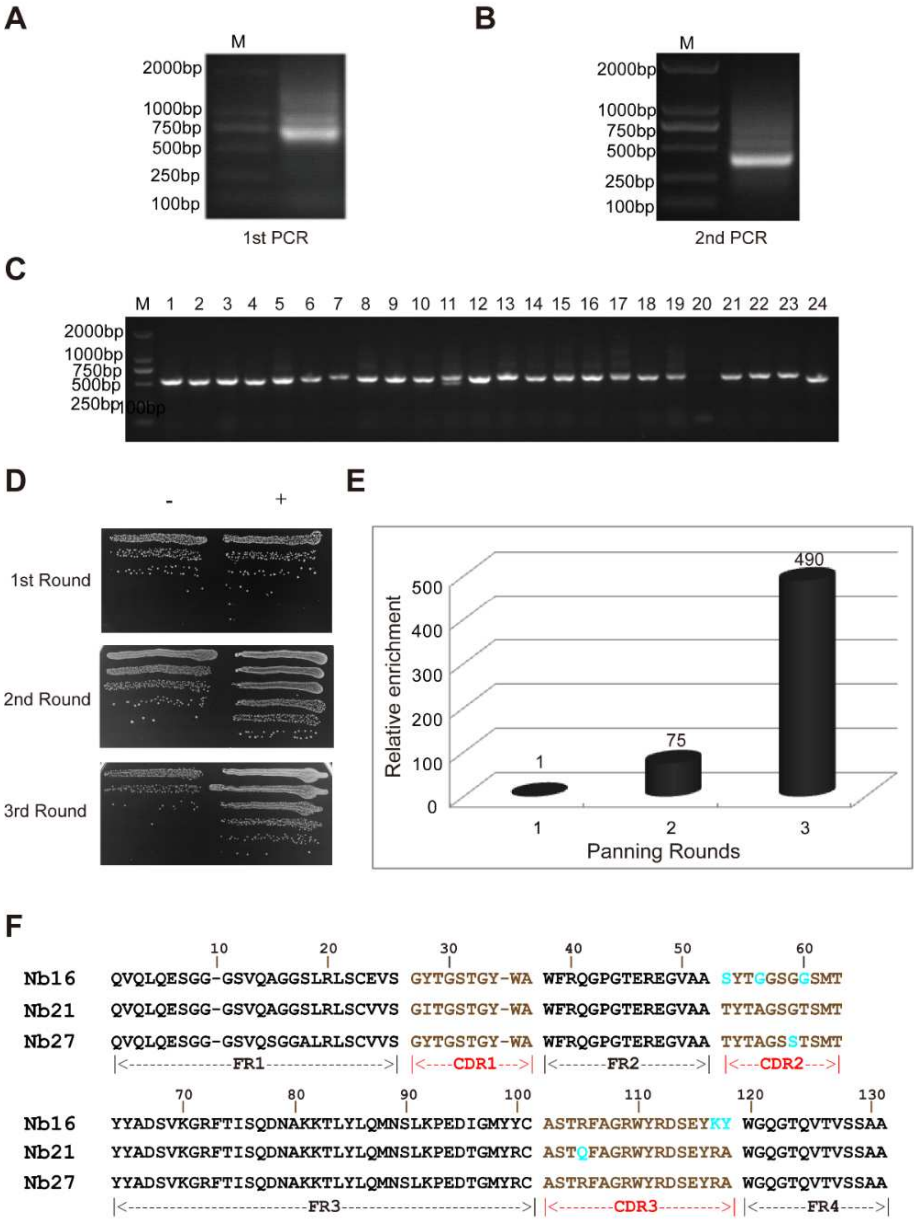


Figure 2. Library construction and testosterone specific Nbs identification. **(A)** The first PCR fragments with an event band of 700 bp. **(B)** The products of second PCR, which of an evident band of 400 bp. **(C)** 24 colonies were randomly picked to determine the insertion rate of the

anti-testosterone library. **(D)** Enrichment of each round bio-panning shown by plate. **(E)** The relative enrichment ratio of the total three consecutive rounds of bio-panning. **(F)** Different anti-testosterone Nbs was identified by sequence alignment of VHH positive colonies.

95 randomly picked colonies were subjected to periplasmic extraction enzyme-linked immunosorbent assay (PE-ELISA) aimed to acquire testosterone specific VHHs. Fortunately, 20 out of 95 were identified as the positive colonies binding to the coating testosterone antigen and the binding ratio were more than 5 relative to blank control. The plasmids of these 20 positive colonies were extracted and sequenced. According to the diversity of amino acid sequences in complementarity determining region (CDR)3, these 20 VHHs were classified into the same family which shared high similarity in sequence. Among these, we chose three Nbs whose sequences shared tiny diversity (Figure 2F) to conduct the further research, namely Nb16, Nb21 and Nb27 respectively. They share the same CDR1 region, and only a few amino acids are different in CDR2 and CDR3 regions. We deduced that the small size and tiny structure of testosterone make the Nbs unable to access more epitopes, thus the obtained Nbs are derived from the same family.

3.3 Expression and Purification of Soluble Nbs. For expression, the selected Nb plasmids were transformed into WK6, which is a non-suppressor *E. coli* strain expressing the amber stop codon between VHH and gene III. Therefore, the cells can express soluble Nbs without pIII protein. With the C-terminally hexahistidine (6×His)-tag, the obtained Nbs were purified with Ni−NTA affinity columns and detected with SDS−PAGE. As shown in Figure 3A, the size of the Nbs are about 15 kD, which is consistent with theoretical value and the results showed high quality with >90% purity. The concentration of the Nbs was determined by Bradford method. Nb16 had a yield of 3.9 mg L^{-1} bacterial culture, Nb21 was 3.5 mg L^{-1}, and Nb27 was 2.5 mg L^{-1}.

As solubility shown in Figure 2F, the characteristic substitutions in FR2 were positions 39, 46, 47 and 49, amino acids of regular antibodies in these four sites are V, G, L, W respectively, but they are F, E, R, G in Nbs. That means four hydrophobic amino acids were replaced by four hydrophilic amino acids, which leads to a significantly increase in Nbs solubility and explains why Nbs have a good solubility.

3.4 Temperature Stability and Tm Value Determination. Temperature stability of these isolated Nbs was also determined. They were incubated at 70ºC and 90ºC for 0.5, 1 and 3 h in a thermo-controlled water-bath, respectively. Each sample was used to test the activity of direct binding to the coated testosterone-BSA in a microtiter plate using ELISA (BSA protein acted as control). As shown in Figure 3C, Nb16, Nb21, Nb27 all kept nearly 80% of activity after incubated at 70ºC from 0.5 h to 3 h. Under the treatment of 90ºC (Figure 3D), compared with Nb21 and Nb27, even incubated for 3 h, Nb16 also showed about 60% activity. The results above demonstrate that Nb16 shows good thermostability. Besides, the thermofluor assay allows us to distinguish a protein's state of folded and unfolded. Based on the results, the T_m value is determined and at which temperature the hydrophobic areas of a protein become exposed. Therefore, we conducted the thermofluor assay for Nb16 to detect the T_m value. The result showed the T_m value of Nb16 was 66.18ºC, which indicated Nb16 (shows the best in temperature stability analysis) a good thermostability (Figure 3B). This advantage can broaden the functional area of the Nb, making it attractive for diagnosis, clinical application and immunoassay development especially lacking low temperature storing condition.

3.5 Solvent Effect. Nanobody with high tolerance to organic solvent is meaningful during real sample analysis, especially for lipophilic analytes. Testosterone is highly lipophilic. The use

of organic solvent, such as ethanol (EtOH), isopropanol and trichlormethane, is inescapable and necessary during extraction. We investigated the tolerance of Nb16, which is specific to testosterone in the presence of EtOH, isopropanol and trichlormethane at concentrations of 20% to 80%. Nb16 was diluted in each organic solution with the final concentration of 10 μg mL^{-1}, the activity and the binding ability of testosterone was tested by ELISA compared with that in PBS. The results were shown in Figure 3E. In general, the increase of the concentration of each organic solvent lead to the decrease of signal, particularly in trichlormethane. However, Nb16 showed good tolerance to most of organic reagents. Nb16 maintained over 80% activity in EtOH and isopropanol of concentrations from 20% to 60%, even exposed to the concentration of 80% of EtOH and isopropanol, the activity over 60%. In contrast, we can conclude that trichlormethane had the significant effect on Nb16, the activity of Nb16 was badly influenced especially in high concentration of trichlormethane. Therefore, the property of Nb16 showed us the advantages on the development of extraction of testosterone by EtOH and isopropanol, nevertheless, the property probably not available to that trichlormethane extracted samples.

3.6 SPR Binding Assay. The affinity shows the strength of binding of one epitope of antigen to an antibody, and is expressed in terms of the association constant, *Ka.* Therefore, it is important and compulsory to determine the affinity of testosterone-specific Nbs. According to the property analysis, we chose Nb16 to perform Biacore assay for the affinity determination to testosterone. Nb16 was used as ligand and captured, while testosterone was taken as analyte with 5 different concentrations. As the results in Figure 3F, Nb16 showed high affinity and strong binding to testosterone with equilibrium dissociation constant (K_D) of 0.95×10^{-6} M (k_a of 3.9×10^4 M^{-1} s^{-1} and k_d of 0.037 s^{-1}), which presented a good potential for further applications.

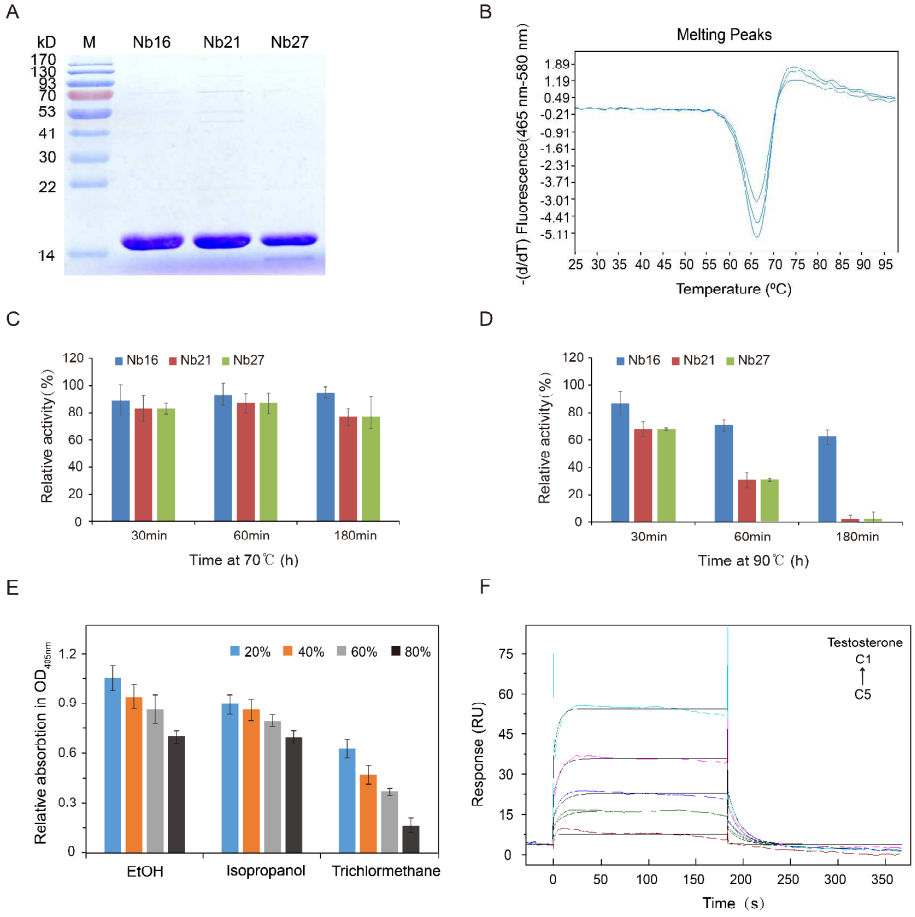


Figure 3. **(A)** Nb16 was isolated and purified by NI-NTA Superflow Sepharose column and detected by SDS-PAGE. **(B)** Sypro Orange thermal stability data. **(C)** Stability of Nb16 after incubation at 70ºC, 90ºC for 0.5 h, 1 h, 3 h. **(D)** Stability of Nb16 after incubation at 90ºC for 0.5 h, 1 h, 3 h. **(E)** The activity of Nb16 in organic solvent. **(F)** K_D data between testosterone and Nb16 was obtained using Biacore T200 instruments. As Nb16 was immobilized, testosterone was injected at concentrations of 27, 81, 243, 729 and 2187 nM (C5 to C1). The sensorgram shows testosterone interacts with Nb16 from the concentration of 27 nM (bottom curve, C5) to the concentration of 2187 nM (top curve, C1). All data were fitted to a 1:1 interaction model and represented by black curves.

3.7 Expression and Purification of Biotinylated Nbs. Electrochemical immunosensor was designed by using of biotinylated Nb (BiNb) to accomplish the orientated and efficient diagnosis of testosterone expecting to combine Nb with the traditional and classic electrochemical immunosensor. Compared with unmodified Nbs, biotinylated Nbs conquer the disadvantage of unoriginal, realizing the directional conjugation of Nbs to the surface of immunosensor. Based on the high affinity of BiNb and streptavidin, one of the strongest affinity combination in nature, we expect to develop a sample and sensitive immunosensor for the detection of testosterone.

According to the yield, stability and affinity, we chose Nb16 to conjugated with biotin *in vivo* to conduct our further research. The biotinylated Nbs was generated as above descriptions. Finally, the biotinylated Nb16 (BiNb16) were purified with Streptavidin-Mutein Matrix and with the yield of 1.2 mg L^{-1} culture.

3.8 Characterization of Immunosensor. Considering the excellent features of EIS, such as conductivity, accuracy and rapidness [32], EIS was selected to have the testosterone determination by monitoring the changes of electron transfer resistance in the surfaces of the GCEs with BiNb16. The outline of immunosensor fabrication is demonstrated in Figure 1. Firstly, the GCE was oxidated by 0.5 M H_2SO_4 to produce carboxylic acid groups on the electrode surface. Then, the electrode was immersed into the mixture of EDC and NHS. According to these amino linkages, we can combine streptavidin to the surface of GCE. BSA was applied to block the unspecific binding sites. Finally, BiNb16 was immobilized on the surface of the modified electrode by streptavatin-biotin reaction.

Corresponding cyclic voltammograms (CV) (Figure 4A) and impedance spectra (Figure 4B) were recorded in the presence of 1.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) to investigate each

immobilization and binding step. In the Nyquist plots impedance spectroscopy, Z' and -Z'' represents the real variable and the negative value of imaginary variable of impedance, respectively [33]. An equivalent circuit model (Inset in Figure 4C), including the electron transfer resistance (R_{et}), the ohmic resistance of the electrolyte solution (R_1) between the working electrode and the reference electrode, constant phase element (*Cdl)* and the Warburg impedance resulting from the diffusion of ions (W_1), was applied to simulate the complex impedance plots obtained from the EIS measurements. The curve (solid line in Figure 4C) fits well with the experimental impedance data (solid dots in Figure 4C), indicating that the equivalent circuit used is accurate. Typically, the diameter of the semicircle represents the electron-transfer resistance (R_{et}). For the bare GCEs, there are no obvious obstacles affecting electron transfer, which results in peak separation (ΔE_p) of 69 mV in CV (Figure 4A, curve a). EIS in this procedure showed an almost straight line (Figure 4B, curve a), suggesting the electrode reaction was only controlled by diffusion. The immobilization of SA obviously increases the electron transfer resistance R_{et} (Figure 4B, curve b) and the peak separation ΔE_p was increased to 182 mV (Figure 4A, curve b). Subsequently, the deposition of BSA for blocking of remaining free COOH groups on the electrode increased the R_{et} (Figure 4B, curve c) as well as the peak separation ΔE_p to 330 mV (Figure 4A, curve c). BiNb16 immobilization by streptavidin-biotin reaction with SA caused the increase of peak separation to 400 mV in CV (Figure 4A, curve d) and impedance spectrum (Figure 4B, curve d). After the testosterone immobilized, the value of R_{et} occurred a sharp increase (Figure 4B, curve e) and the CV peak separation evident increased to 440 mV (Figure 4A, curve e). The immobilized of the protein layer resulting in the frequency increase of the diameter of the semicircle in the impedance spectroscopy, which caused the increase of R_{et} value [24, 34]. Thus, we

can find the EIS is a more sensitive technique than CV for the electrochemical detection. Therefore, EIS detection model is more suitable for this work to achieve a high sensitivity.

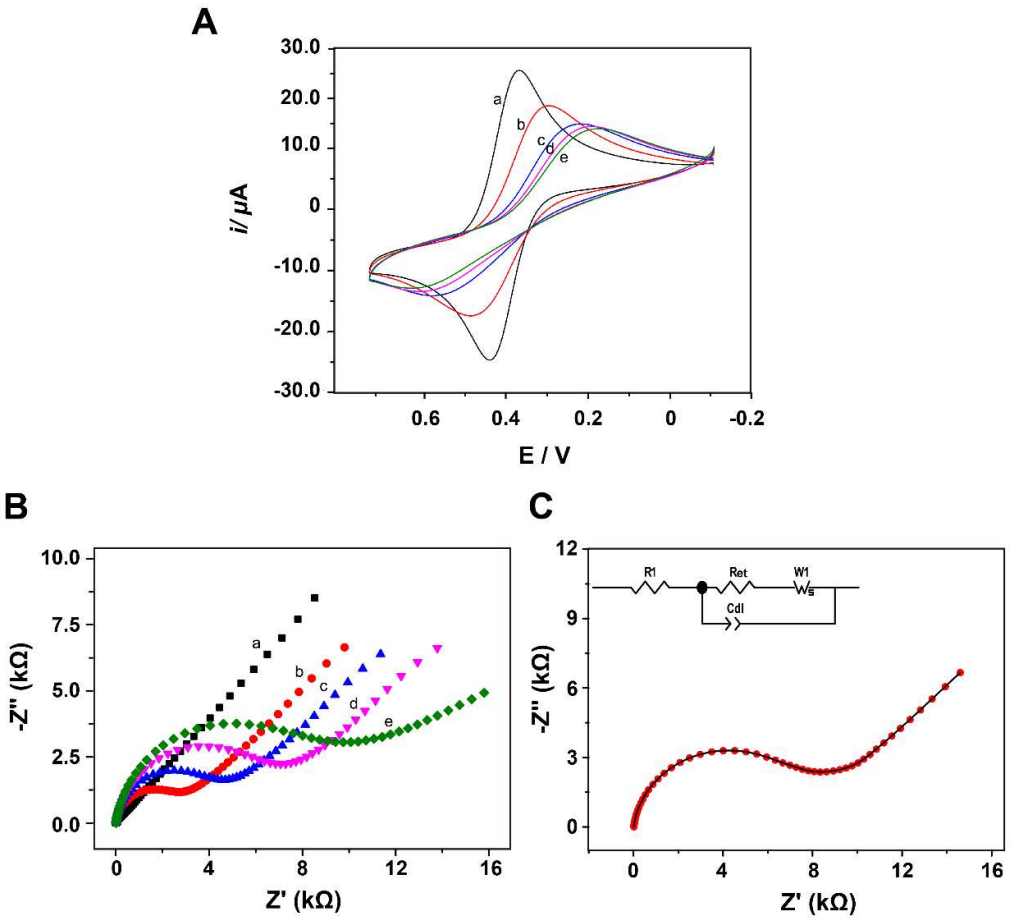


Figure 4. (A) Cyclic voltammograms of: (a) bare GCE; (b) SA/GCE; (c) BSA/SA/GCE; (d) BiNb16/BSA/SA/GCE; (e) Testosterone/BiNb16/BSA/SA/GCE (Testosterone concentration: 0.5 ng mL^{-1}). **(B)** Nyquist plots of impedance spectra obtained in PBS (0.1 M, pH 7.4) containing 1 mM $[Fe(CN)_6]^{3-/4-}$. **(C)** The equivalent circuit model applied to simulate the complex impedance plots obtained from the EIS measurements. Nyquist plot was demonstrated both in fitted (solid line) and experimental (scattered line) impedance spectra.

3.9 Determination of Testosterone. The SA/BSA/BiNb16 GCEs was exposed to various concentrations of testosterone to evaluate the reaction between Nbs and testosterone. The

corresponding Nyquist plots of impedance spectra are represented in Figure 5A, where the diameter of the Nyquist circle increased with the increasing concentration of testosterone. The reason is the higher concentrations of antigens leading to more testosterone bound to the deposited Nbs, which hindered the definite kinetic for the electron transfer. The change in R_{et} could be defined as: ΔR_{et} = R_{et} (testosterone) - R_{et} (Blank). Therefore, ΔR_{et} was used to evaluate the response to different concentrations of testosterone. ΔR_{et} was of the value of 1.57, 3.78, 8.76, 13.73 and 20.6 kΩ, while the corresponding concentration of testosterone was 0.05, 0.1, 0.5, 1 and 5 ng mL^{-1}. As shown in Figure 5B, the R_{et} responses showed a good linear correlation with the concentration of testosterone from 0.05 to 5.0 ng mL^{-1}. The linear regression equation could express as ΔR_{et} = 13330 + 9568 log $C_{testosterone}$ (ng mL^{-1}) with a correlation coefficient of 0.9839 (n=3). The limit of detection (LOD) is 0.045 ng mL^{-1}, according to the equations: LOD = 3.3 σ/S (where σ is the standard deviation of the response and S is the slope of the calibration curve) [35]. Therefore, we could detect testosterone quantitatively and efficiently accordingly. The EIS immunosensor presented here displayed better sensitivity compared with LC-MS/MS method (0.280 ng dL^{-1}) [36], gold nanowires-doped sol-gel film (0.1 ng mL^{-1}) [37], screen-printed electrode system with Fab (90 pg mL^{-1}) [38], polyelectrolyte immunoassays (0.1 ng mL^{-1}) [39], liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry method (LC/APPI-MS/MS) (10 pg mL^{-1}) [40], offering an alternative way based on Nb to determinate the testosterone.

The characters of Nbs encompass small molecular size, high solubility and thermostability, good acid resistance, lower immunogenicity. In addition, they can recognize hinder epitope because of the concave-shaped epitopes recognition[41]. The above advantages make them

promising in many fields [42]. Besides, as the easy detection of slight changes in antigen binding, EIS was used to represent the electrode surface modifications only by impedance curves [43]. Therefore, it has been widely used in the detection of antibody-antigen, DNA-DNA recognition and so on [44]. To sum up, Nb and EIS were used to testosterone determination, which offering alternative methods for detection of testosterone.

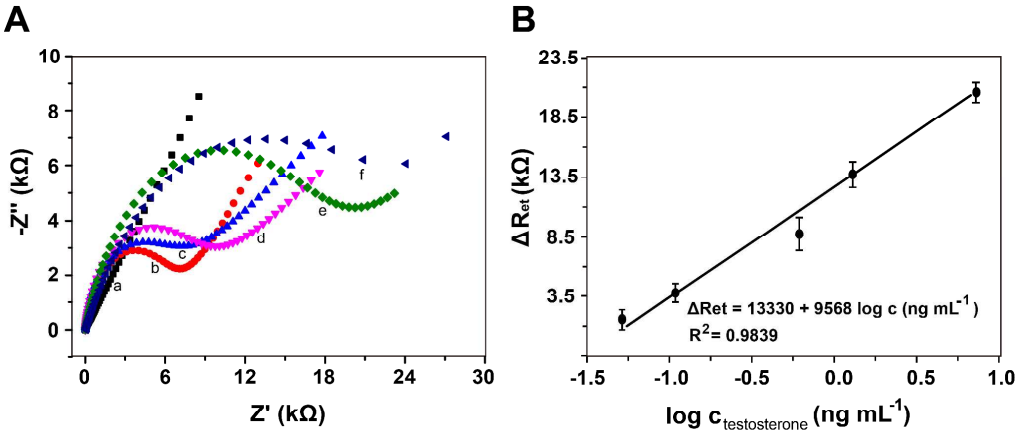


Figure 5. **(A)** Nyquist plots of EIS response of BiNb16/BSA/SA/GCE when the immobilization of series concentrations of testosterone antigen: (a) 0; (b) 0.05; (c) 0.1; (d) 0.5; (e) 1.0; (f) 5 ng mL^{-1}. **(B)** Linear relationship between EIS response and the concentrations of testosterone.

3.10 Specificity, Reproducibility, Stability and Real Sample Analysis. Nonspecific adsorption is a major problem in label-free immunosensor. To confirm the observed impedance changes and test the binding selectivity, control experiments were conducted. The electrode was exposed to 10 ng mL^{-1} metandienone, which is an important testosterone derivative that is 10-fold to testosterone. As shown in Figure 6, because of the structure and sequence similarity, it showed there was little variation on the impedance with the determination of metandienone. In addition, it is acceptable for such slight changes for nonspecific adsorption. The results indicated EIS immunosensor also has good selectivity.

The reproducibility of the immunosensor was evaluated by determining the electro transfer resistance of the same testosterone concentration (1 ng mL^{-1}) at six different GCEs. The interassay RSD of the six immunosensor is 6.8%, indicating an acceptable fabrication reproducibility. The repeated results demonstrated the value of intraassay RSD was 5.2%, suggesting the system has a good reproducibility. The stability of the modified electrodes was also estimated by storing (BiNb16/BSA/SA) in PBS (10 mM, pH 7.4) at 4ºC for four weeks. About 90% of the initial signal was deserved, indicating a good stability of the system. To develop the potential practical application, the proposed biosensor was used to the determination of testosterone in human serum samples. As Table 1 showed, the recoveries of testosterone was from 90% to 112% in serum samples, suggesting the developed method was reliable and accurate.

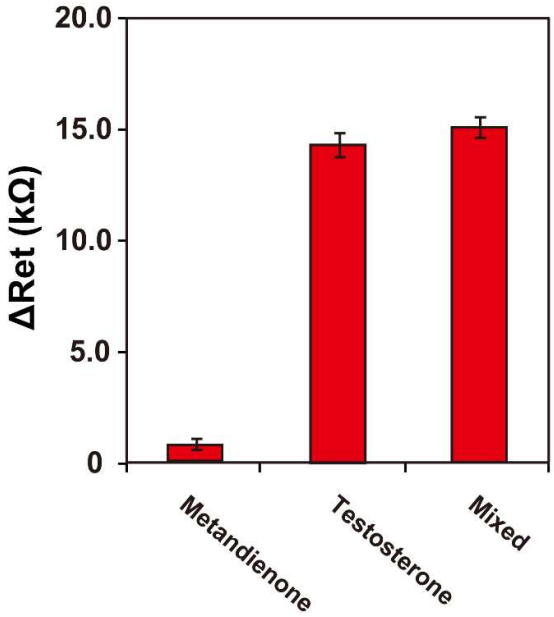


Figure 6. Investigation of the immunosensor specificity. Testosterone with 1 ng mL^{-1} was selected to the proposed immunosensor by comparing with 10 ng mL^{-1} of derivative in the mixed sample.

Sample	Testosterone concentration (ng mL^{-1})		Recovery (%)	RSD (%)
	Added	Found		
1	0.1	0.09	90	2.78
2	0.5	0.51	102	3.58
3	1	1.08	108	4.15
4	2	2.24	112	5.18
5	4	4.28	107	3.34

Table 1. EIS determination of testosterone in human serum samples.

4. CONCLUSIONS

A label-free electrochemical impedance immunosensor based on SA/BSA/BiNb and modified GCEs was set up for the detection of testosterone. Firstly, an immune testosterone Nbs library was constructed, after bio-panning, three anti-testosterone Nbs were obtained. Due to the best stability as well as yield, Nb16 was selected to couple with biotin *in vivo*. Then, BiNb16 was used in EIS immunosensor for determination of testosterone. The proposed EIS immunosensor displayed high sensitivity and good precision with the limit of detection of 0.045 ng mL^{-1}. The proposed method provided a potential application in the determination of testosterone for clinical medical diagnosis.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: ykwan@simm.ac.cn (Y.K.W.).

*E-mail: Yanfei.Shen@seu.edu.cn (Y.F.S.).

Author Contributions

Guanghui Li and Min Zhu contributed equally. Guanghui Li and Min Zhu performed the experiments and wrote the manuscript. Lu Ma analyzed the data. Junrong Yan helped with manuscript writing and language revision. Xiaoling Lu wrote parts of the manuscript and helped with data analysis. Yanfei Shen and Yakun Wan supervised the experiments, reviewed the manuscript and provided the funding.

Notes

The authors declare there are no competing interests.

ACKNOWLEDGMENTS

This work was supported by grants from grants from Chinese Academy of Sciences (XDA12020332), Jiangsu Nanobody Engineering and Research Center of China (2015-03), Program for New Century Excellent Talents in University (NCET-20130127), National Natural Science Foundation of China (grant number 31271365, 31471216 and 21305065), Key Program from the Natural Science Foundation of Jiangsu province (BK20130788), the Fundamental Research Funds for the Central Universities. This work was supported, in part, by grants from the National Natural Scientific Foundation of China (No. 81430055); International Cooperation Project of the Ministry of Science and Technology of China (No. 2015DFA31320); Project of Science and Technology of Guangxi (No. 14125008-2-12).

REFERENCES

1. Nansunga, M.; Manabe, Y. C.; Alele, P. E.; Kasolo, J., Association of Testosterone Levels with Socio-Demographic Characteristics in a Sample of Ugandan Men. *Afr. Health Sci.* **2014,** *14* (2), 348-355.

2. Grober, E. D., Testosterone Deficiency and Replacement: Myths and Realities. *Can. Urol. Assoc. J.* **2014,** *8* (7-8 Suppl 5), S145-147.

3. Prabhavathi, K.; Selvi, K. T.; Poornima, K. N.; Sarvanan, A., Role of Biological Sex in Normal Cardiac Function and in Its Disease Outcome - a Review. *J. Clin. Diagn. Res.* **2014,** *8* (8), BE01-04.

4. Cavasin, M. A.; Tao, Z. Y.; Yu, A. L.; Yang, X. P., Testosterone Enhances Early Cardiac Remodeling after Myocardial Infarction, Causing Rupture and Degrading Cardiac Function. *Am. J. Physiol. Heart Circ. Physiol.* **2006,** *290* (5), H2043-2050.

5. Chapin, R. E.; Creasy, D. M., Assessment of Circulating Hormones in Regulatory Toxicity Studies Ii. Male Reproductive Hormones. *Toxicol Pathol.* **2012,** *40* (7), 1063-1078.

6. Yeap, B. B.; Alfonso, H.; Paul Chubb, S. A.; Hankey, G. J.; Handelsman, D. J.; Golledge, J.; Almeida, O. P.; Flicker, L.; Norman, P. E., In Older Men, Higher Plasma Testosterone or Dihydrotestosterone Are Independent Predictors for Reduced Incidence of Stroke but Not Myocardial Infarction. *J. Clin. Endocrinol. Metab.* **2014**, jc20142664.

7. Yeap, B. B.; Alfonso, H.; Chubb, S. A.; Handelsman, D. J.; Hankey, G. J.; Golledge, J.; Flicker, L.; Norman, P. E., Lower Plasma Testosterone or Dihydrotestosterone, but Not Estradiol, Is Associated with Symptoms of Intermittent Claudication in Older Men. *Clin. Endocrinol. (Oxf)*

2013, *79* (5), 725-732.

8. Zarotsky, V.; Huang, M. Y.; Carman, W.; Morgentaler, A.; Singhal, P. K.; Coffin, D.; Jones, T. H., Systematic Literature Review of the Risk Factors, Comorbidities, and Consequences of Hypogonadism in Men. *Andrology* **2014,** *2(6)*,819-834.

9. Sun, B.; Wang, F.; Sun, J.; Yu, W.; Sun, Y., Basal Serum Testosterone Levels Correlate with Ovarian Response but Do Not Predict Pregnancy Outcome in Non-Pcos Women Undergoing Ivf. *J. Assist. Reprod. Genet.* **2014,** *31* (7), 829-835.

10. Navajas, R.; Imaz, C.; Carreras, D.; Garcia, M.; Perez, M.; Rodriguez, C.; Rodriguez, A. F.; Cortes, R., Determination of Epitestosterone and Testosterone in Urine by High-Performance Liquid Chromatography. *J. Chromatogr. B. Biomed. Appl.* **1995,** *673* (2), 159-164.

11. Saudan, C.; Baume, N.; Mangin, P.; Saugy, M., Urinary Analysis of 16(5alpha)-Androsten-3alpha-Ol by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry: Implications in Anti-Doping Analysis. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2004,** *810* (1), 157-164.

12. Amaral, C.; Cunha, S. C.; Fernandes, J. O.; Tavares da Silva, E.; Roleira, F. M.; Teixeira, N.; Correia-da-Silva, G., Development of a New Gas Chromatography-Mass Spectrometry (Gc-Ms) Methodology for the Evaluation of 5alpha-Reductase Activity. *Talanta* **2013,** *107*, 154-161.

13. Ke, Y.; Bertin, J.; Gonthier, R.; Simard, J. N.; Labrie, F., A Sensitive, Simple and Robust Lc-Ms/Ms Method for the Simultaneous Quantification of Seven Androgen- and Estrogen-Related Steroids in Postmenopausal Serum. *J. Steroid. Biochem. Mol. Biol.* **2014,** *144 Pt B*, 523-534.

14. Leinonen, A.; Kuuranne, T.; Kotiaho, T.; Kostiainen, R., Screening of Free 17-Alkyl-Substituted Anabolic Steroids in Human Urine by Liquid Chromatography-Electrospray

Ionization Tandem Mass Spectrometry. *Steroids* **2004,** *69* (2), 101-109.

15. Halmenschlager, G.; Rhoden, E. L.; Riedner, C. E., Calculated Free Testosterone and Radioimmunoassay Free Testosterone as a Predictor of Subnormal Levels of Total Testosterone. *Int. Urol. Nephrol.* **2012,** *44* (3), 673-681.

16. Lewis, J. G.; Longley, N. J.; Elder, P. A., Monoclonal Antibodies to Human Sex Hormone-Binding Globulin (Shbg): Characterization and Use in a Simple Enzyme-Linked Immunosorbent Assay (Elisa) of Shbg in Plasma. *Steroids* **1999,** *64* (4), 259-265.

17. Shrivastav, T. G.; Chaube, S. K.; Prasad, P. K.; Kariya, K. P.; Kumar, D., Heterologous Enzyme Linked Immunosorbent Assay for Measurement of Testosterone in Serum. *J. Immunoassay Immunochem.* **2012,** *33* (3), 252-268.

18. Al-Dujaili, E. A., Development and Validation of a Simple and Direct Elisa Method for the Determination of Conjugated (Glucuronide) and Non-Conjugated Testosterone Excretion in Urine. *Clin. Chim. Acta* **2006,** *364* (1-2), 172-179.

19. Stopforth, A.; Grobbelaar, C. J.; Crouch, A. M.; Sandra, P., Quantification of Testosterone and Epitestosterone in Human Urine Samples by Stir Bar Sorptive Extraction--Thermal Desorption--Gas Chromatography/Mass Spectrometry: Application to Hiv-Positive Urine Samples. *J. Sep. Sci.* **2007,** *30* (2), 257-265.

20. De Meyer, T.; Muyldermans, S.; Depicker, A., Nanobody-Based Products as Research and Diagnostic Tools. *Trends Biotechnol.* **2014,** *32* (5), 263-270.

21. Ma, L.; Sun, Y.; Kang, X.; Wan, Y., Development of Nanobody-Based Flow Injection Chemiluminescence Immunoassay for Sensitive Detection of Human Prealbumin. *Biosens. Bioelectron.* **2014,** *61*, 165-171.

22. Campuzano, S.; Salema, V.; Moreno-Guzman, M.; Gamella, M.; Yanez-Sedeno, P.; Fernandez, L. A.; Pingarron, J. M., Disposable Amperometric Magnetoimmunosensors Using Nanobodies as Biorecognition Element. Determination of Fibrinogen in Plasma. *Biosens. Bioelectron.* **2014,** *52*, 255-260.

23. Shu, M.; Xu, Y.; Wang, D.; Liu, X.; Li, Y.; He, Q.; Tu, Z.; Qiu, Y.; Ji, Y.; Wang, X., Anti-Idiotypic Nanobody: A Strategy for Development of Sensitive and Green Immunoassay for Fumonisin B(1). *Talanta* **2015,** *143*, 388-393.

24. Li, H.; Yan, J.; Ou, W.; Liu, H.; Liu, S.; Wan, Y., Construction of a Biotinylated Cameloid-Like Antibody for Lable-Free Detection of Apolipoprotein B-100. *Biosens. Bioelectron.* **2014,** *64C*, 111-118.

25. Li, H.; Mu, Y.; Yan, J.; Cui, D.; Ou, W.; Wan, Y.; Liu, S., A Label-Free Photoelectrochemical Immunosensor for Neutrophil Gelatinase-Associated Lipocalin Based on the Novel Use of Nanobodies. *Anal. Chem.* **2015,** *87(3)*, 2007-2015.

26. Li, H.; Sun, Y.; Elseviers, J.; Muyldermans, S.; Liu, S.; Wan, Y., A Nanobody-Based Electrochemiluminescent Immunosensor for Sensitive Detection of Human Procalcitonin. *Analyst* **2014,** *139* (15), 3718-3721.

27. Elshafey, R.; Tavares, A. C.; Siaj, M.; Zourob, M., Electrochemical Impedance Immunosensor Based on Gold Nanoparticles-Protein G for the Detection of Cancer Marker Epidermal Growth Factor Receptor in Human Plasma and Brain Tissue. *Biosens. Bioelectron.* **2013,** *50*, 143-149.

28. Xu, H.; Wang, L.; Ye, H.; Yu, L.; Zhu, X.; Lin, Z.; Wu, G.; Li, X.; Liu, X.; Chen, G., An Ultrasensitive Electrochemical Impedance Sensor for a Special Brca1 Breast Cancer Gene

Sequence Based on Lambda Exonuclease Assisted Target Recycling Amplification. *Chem. Commun. (Camb)* **2012,** *48*, 6390-6392.

29. King, A. C.; Woods, M.; Liu, W.; Lu, Z. J.; Gill, D.; Krebs, M. R. H., High-Throughput Measurement, Correlation Analysis, and Machine-Learning Predictions for Ph and Thermal Stabilities of Pfizer-Generated Antibodies. *Protein Science* **2011,** *20* (9), 1546-1557.

30. Alexandrov, A. I.; Mileni, M.; Chien, E. Y. T.; Hanson, M. A.; Stevens, R. C., Microscale Fluorescent Thermal Stability Assay for Membrane Proteins. *Structure* **2008,** *16* (3), 351-359.

31. Li, H.; Mu, Y.; Yan, J.; Cui, D.; Ou, W.; Wan, Y.; Liu, S., Label-Free Photoelectrochemical Immunosensor for Neutrophil Gelatinase-Associated Lipocalin Based on the Use of Nanobodies. *Anal. Chem.* **2015,** *87* (3), 2007-2015.

32. Ruan, C.; Yang, L.; Li, Y., Immunobiosensor Chips for Detection of Escherichia Coil O157:H7 Using Electrochemical Impedance Spectroscopy. *Anal. Chem.* **2002,** *74* (18), 4814-4820.

33. Dong, J.; Zhao, H.; Xu, M.; Ma, Q.; Ai, S., A Label-Free Electrochemical Impedance Immunosensor Based on Aunps/Pamam-Mwcnt-Chi Nanocomposite Modified Glassy Carbon Electrode for Detection of Salmonella Typhimurium in Milk. *Food Chem.* **2013,** *141* (3), 1980-1986.

34. Wang, G. L.; Xu, J. J.; Chen, H. Y.; Fu, S. Z., Label-Free Photoelectrochemical Immunoassay for Alpha-Fetoprotein Detection Based on Tio(2)/Cds Hybrid. *Biosens. Bioelectron.* **2009,** *25* (4), 791-796.

35. Jarocka, U.; Sawicka, R.; Gora-Sochacka, A.; Sirko, A.; Zagorski-Ostoja, W.; Radecki, J.; Radecka, H., Electrochemical Immunosensor for Detection of Antibodies against Influenza a Virus H5n1 in Hen Serum. *Biosens. Bioelectron.* **2014,** *55*, 301-306.

36. Wang, Y.; Gay, G. D.; Botelho, J. C.; Caudill, S. P.; Vesper, H. W., Total Testosterone Quantitative Measurement in Serum by Lc-Ms/Ms. *Clin. Chim. Acta* **2014,** *436*, 263-267.

37. Liang, K. Z.; Qi, J. S.; Mu, W. J.; Chen, Z. G., Biomolecules/Gold Nanowires-Doped Sol-Gel Film for Label-Free Electrochemical Immunoassay of Testosterone. *J. Biochem. Biophys. Methods* **2008,** *70* (6), 1156-1162.

38. Lu, H.; Kreuzer, M. P.; Takkinen, K.; Guilbault, G. G., A Recombinant Fab Fragment-Based Electrochemical Immunosensor for the Determination of Testosterone in Bovine Urine. *Biosens. Bioelectron.* **2007,** *22* (8), 1756-1763.

39. Zherdev, A. V.; Byzova, N. A.; Izumrudov, V. A.; Dzantiev, B. B., Rapid Polyelectrolyte-Based Immunofiltration Technique for Testosterone Detection in Serum Samples. *Analyst* **2003,** *128* (10), 1275-1280.

40. Zhang, F.; Rick, D. L.; Kan, L. H.; Perala, A. W.; Geter, D. R.; LeBaron, M. J.; Bartels, M. J., Simultaneous Quantitation of Testosterone and Estradiol in Human Cell Line (H295r) by Liquid Chromatography/Positive Atmospheric Pressure Photoionization Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2011,** *25* (20), 3123-3130.

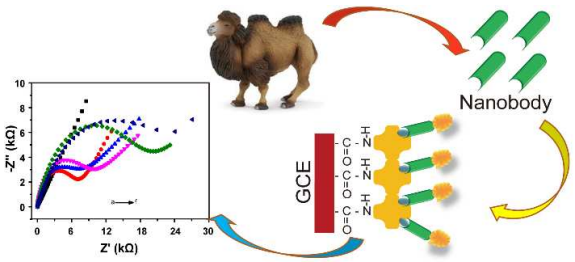
41. Kazemi-Lomedasht, F.; Behdani, M.; Bagheri, K. P.; Habibi-Anbouhi, M.; Abolhassani, M.; Arezumand, R.; Shahbazzadeh, D.; Mirzahoseini, H., Inhibition of Angiogenesis in Human Endothelial Cell Using Vegf Specific Nanobody. *Mol. Immunol.* **2015,** *65* (1), 58-67.

42. De Genst, E.; Dobson, C. M., Nanobodies as Structural Probes of Protein Misfolding and Fibril Formation. *Methods Mol. Biol.* **2012,** *911*, 533-558.

43. Guan, J. G.; Miao, Y. Q.; Zhang, Q. J., Impedimetric Biosensors. *J. Biosci. Bioeng.* **2004,** *97* (4), 219-226.

44. Park, J. Y.; Kwon, S. H.; Park, J. W.; Park, S. M., Label-Free Detection of DNA Molecules on the Dendron Based Self-Assembled Monolayer by Electrochemical Impedance Spectroscopy. *Anal. Chim. Acta* **2008,** *619* (1), 37-42.

For TOC only



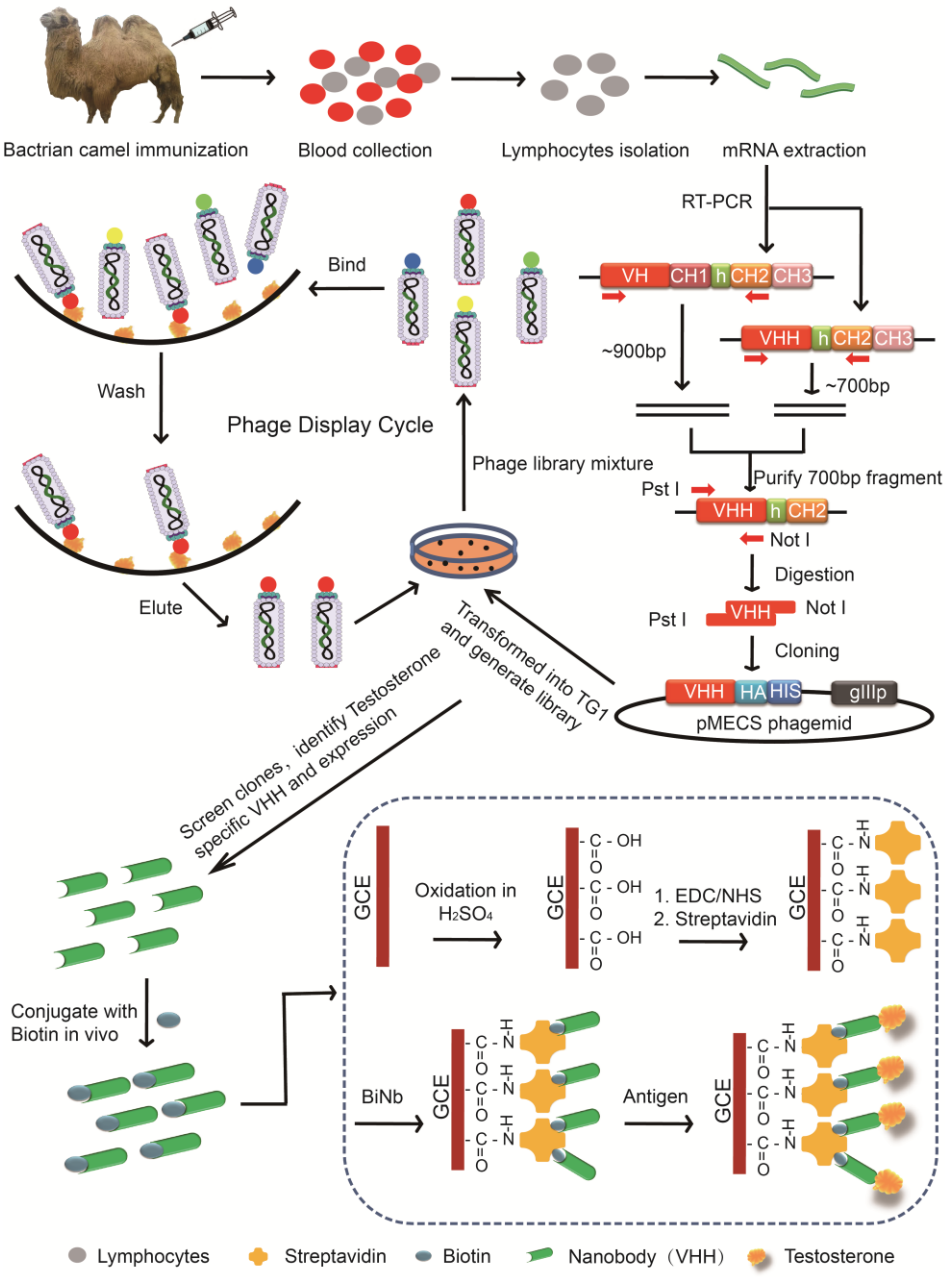


Figure 1. Workflow of the identification of anti-testosterone Nbs and Nbs-based electrochemical immunosensor development.

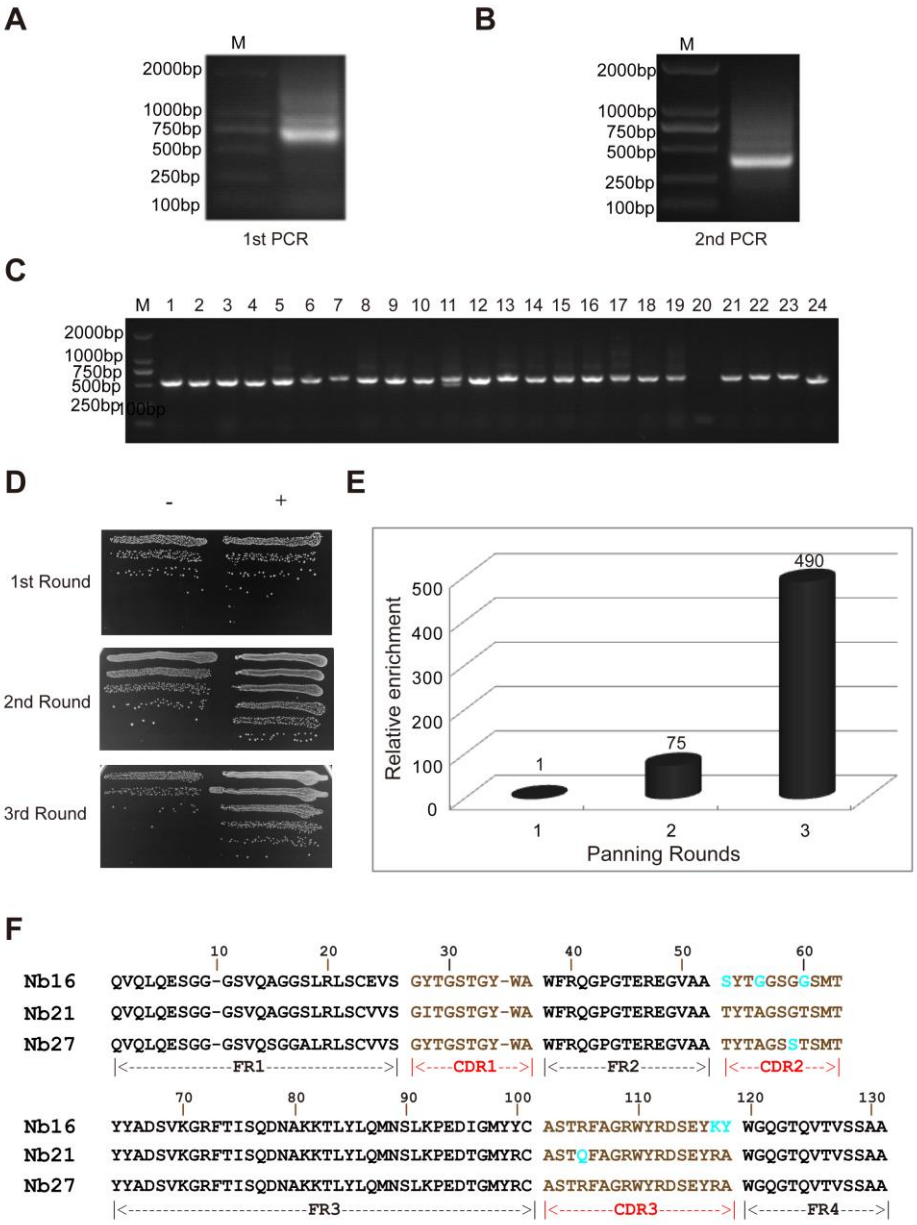


Figure 2. Library construction and testosterone specific Nbs identification. **(A)** The first PCR fragments with an event band of 700 bp. **(B)** The products of second PCR, which of an evident band of 400 bp. **(C)** 24 colonies were randomly picked to determine the insertion rate of the anti-testosterone library. **(D)** Enrichment of each round bio-panning shown by plate. **(E)** The relative enrichment ratio of the total three consecutive rounds of bio-panning. **(F)** Different anti-testosterone Nbs was identified by sequence alignment of VHH positive colonies.

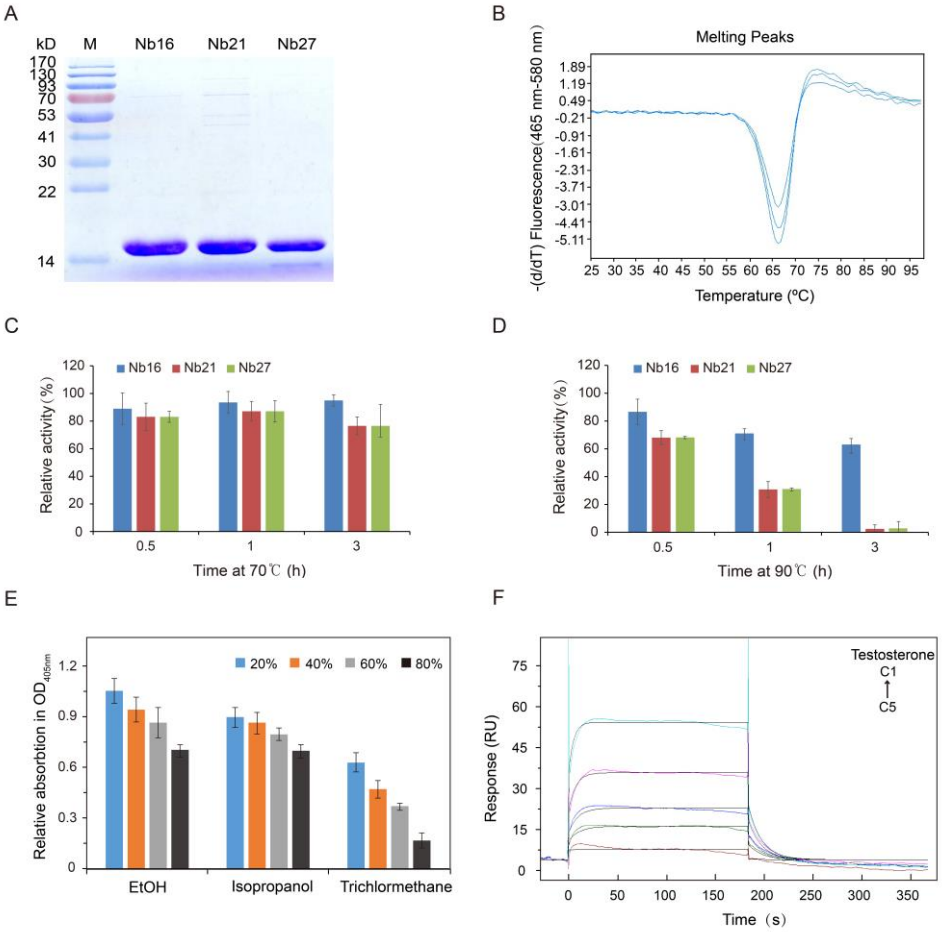


Figure 3. (A) Nb16 was isolated and purified by NI-NTA Superflow Sepharose column and detected by SDS-PAGE. **(B)** Sypro Orange thermal stability data. **(C)** Stability of Nb16 after incubation at 70ºC, 90ºC for 0.5 h, 1 h, 3 h. **(D)** Stability of Nb16 after incubation at 90ºC for 0.5 h, 1 h, 3 h. **(E)** The activity of Nb16 in organic solvent. **(F)** K_D data between testosterone and Nb16 was obtained using Biacore T200 instruments. As Nb16 was immobilized, testosterone was injected at concentrations of 27, 81, 243, 729 and 2187 nM (C5 to C1). The sensorgram shows testosterone interacts with Nb16 from the concentration of 27 nM (bottom curve, C5) to the concentration of 2187 nM (top curve, C1). All data were fitted to a 1:1 interaction model and represented by black curves.

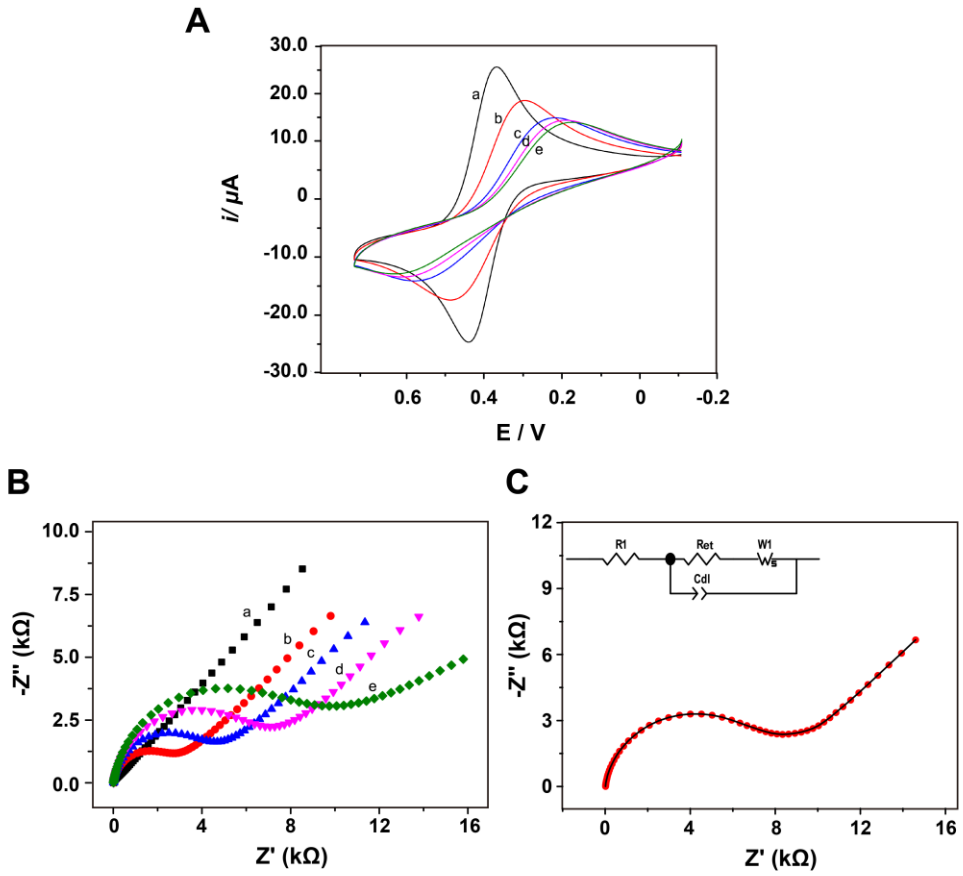


Figure 4. (A) Cyclic voltammograms of: (a) bare GCE; (b) SA/GCE; (c) BSA/SA/GCE; (d) BiNb16/BSA/SA/GCE; (e) Testosterone/BiNb16/BSA/SA/GCE (Testosterone concentration: 0.5 ng mL^{-1}). **(B)** Nyquist plots of impedance spectra obtained in PBS (0.1 M, pH 7.4) containing 1 mM $[Fe(CN)_6]^{3-/4-}$. **(C)** The equivalent circuit model applied to simulate the complex impedance plots obtained from the EIS measurements. Nyquist plot was demonstrated both in fitted (solid line) and experimental (scattered line) impedance spectra.

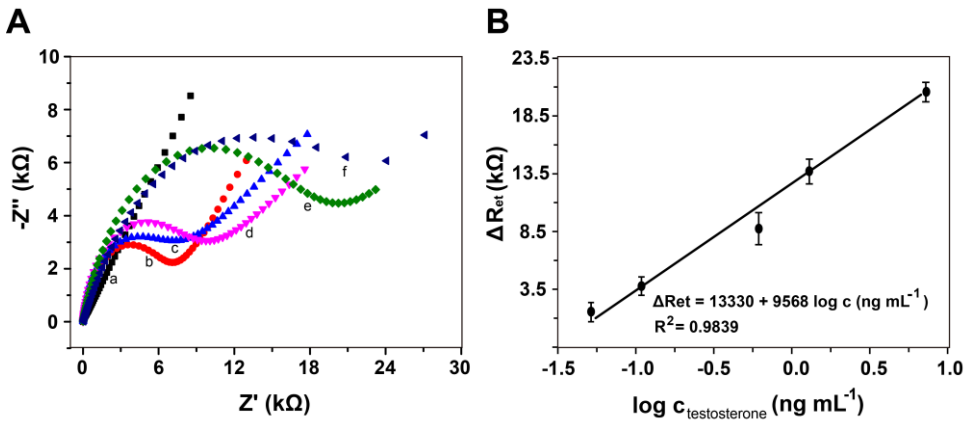


Figure 5. (A) Nyquist plots of EIS response of BiNb16/BSA/SA/GCE when the immobilization of series concentrations of testosterone antigen: (a) 0; (b) 0.05; (c) 0.1; (d) 0.5; (e) 1.0; (f) 5 ng mL^{-1}. **(B)** Linear relationship between EIS response and the concentrations of testosterone.

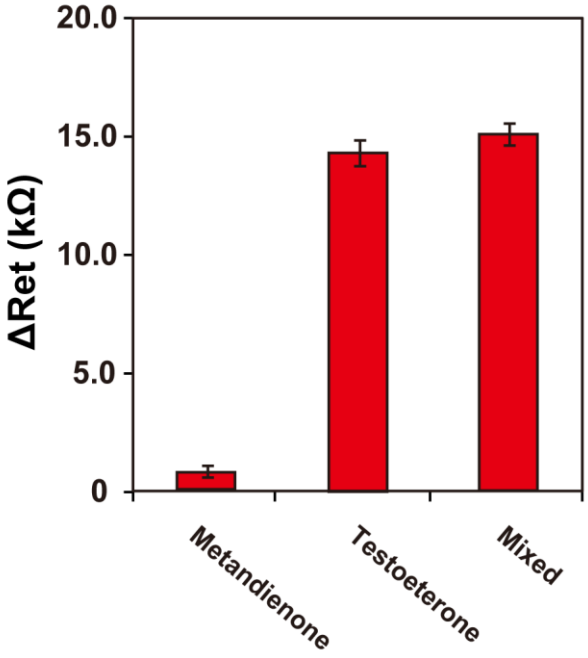


Figure 6. Investigation of the immunosensor specificity. Testosterone with 1 ng mL^{-1} was selected to the proposed immunosensor by comparing with 10 ng mL^{-1} of derivative in the mixed sample.

TOC