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Ultrasensitive Prostate Specific Antigen Immunosensor Based on Gold Nanoparticles Functionalized Polypyrrole@Carbon Nanotubes

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In this paper, an ultrasensitive electrochemical immunosensor for the detection of prostate specific antigen (PSA) based on gold nanoparticles functionalized polypyrrole@carbon nanotubes nanocomposite was reported. Pyrrole monomer was polymerized on MWNTs substrate in acid solution. Negatively charged gold nanoparticles (AuNPs) were assembled on the PPy@MWNTs by electrostatic interactions. With the primary antibody (Ab_1) immobilized on the AuNPs/PPy@MWNTs nanocomposite, a sandwich electrochemical immunosensor for the detection of prostate specific antigen was constructed. The electrochemical signal was amplified substantially by the nanocomposite. A wide linear range from 0.002 to 20 ng mL^{-1} with a detection limit of 0.001 ng mL^{-1} (the ratio of signal to noise, S/N = 3:1) was obtained, which is comparable or even better than most of the prostate specific antigen immunosensor. Furthermore, the prepared immunosensor showed excellent selectivity and reproducibility.

Keywords: Gold nanoparticles, MWNT, Polypyrrole, Prostate specific antigen, Electrochemical immunosensor.

INTRODUCTION

Sensitive detection of protein biomarkers in complex biological matrices is a major challenge in recent years. Due to the high specific binding of antibody against its antigen, immunosensors based on the two type of proteins' interaction are one of the most widely used techniques in the quantitative analysis of biomarkers[1-4]. In most cases, immunosensors are performed in muti-well-plates in well-equipped labs, which is time consuming and expensive[5,6]. Therefore, it is great interest in transitioning immunoanalysis from the lab to point-of-care and diagnoses diseases in an efficient way. Electrochemical immunosensors are particularly convenient platform and possesses a number of advantages compared with conventional immunoanalysis, including low cost, high sensitivity and easy of miniaturization. Hence, electrochemical immunosensors have been intensively studied and became the major analytical technique for the detection of biological species[7-9].

In immunoassay applications, antibodies are immobilized onto biointerface to capture specific biomarkers. To improve the sensitivity and reduce the size of immunosensor, immobilizing antibodies on the substrate surface at high density is essential. High surface area nanoparticles offer unprecedented opportunities for high protein loading, which have attracted widespread interest in their use as labels. In recent years, various types of nanomaterials have been enlisted as labels to construct electrochemical immunosensors, including carbon-based nanomaterials[10], conducting polymers[11] and metal nanoparticles[12]. Due to good biocompatibility, excellent electronic conductivity and relatively large surface area, gold nanoparticles (AuNPs) have been widely used in modification of various electrodes and fabrication of different kinds of biosensors. Recent reports showed that AuNPs provide an environment similar to the native system of immobilized biomolecules to effectively retain their biological function[13]. However, the AuNPs are unstable and easy to aggregate in the solution or on the solid surface[14]. Decoration of AuNPs on the nanomaterials surface or embedding AuNPs in the polymer substrate is considered to be an effective way to solve this problem. Lu *et al.*[15] dotted AuNPs on carbon nanotube-graphene composite and the resulted immunosensor showed improved performance. Chen *et al.* co-electropolymerized AuNPs and polypyrrole (PPy) and a porous, conductive, stable nanocompsite were obtained[1]. Though AuNPs can be assembled on carbon nanotubes directly, most of these methods involve a boring assembly process and usually require multistep reactions because of the poor solubility and processibility of carbon nanotubes[16]. Furthermore, the electronic properties of the carbon nanotubes may be weakened if too many interlinker reagents were used[17]. Therefore, it has been realized that it is necessary to introduce surface anchoring on the carbon nanotubes surface to provide more binding sites.

In the present paper, An AuNPs functionalized the Ppy@MWNTs composite was developed. High density AuNPs were decorated on PPy@MWNTs composite by electrostatic adsorption. By using AuNPs/PPy@MWNTs nanocomposite as substrate to immobilize Ab_1, an ultrasensitive sandwich electrochemical immunosensor for detection of Prostate-specific antigen (PSA) was constructed. The experimental results showed that the immunosensor exhibited high sensitivity, wide linear range and excellent analytical performance.

EXPERIMENTAL

Prostate specific antigen, monoclonal anti-prostate specific antigen antibody (Ab_1) and horseradish peroxidase (HRP) conjuaged anti-human prostate specific antigen monoclonal antibody (Ab_2) were purchased from Yes Biotech Laboratories Ltd (Canada). L-cysteine, lyophilized 99 % bovine serum albumin (BSA), Tween-20, MWNTs, pyrrole monomer and ammonium peroxydisulphate (APS) were from Sigma Aldrich (St. Louis, MO). The MWNTs (95 % 20-60 nm) were treated with nitric acid during purification process and then filtered, rinsed thoroughly with water and dried. All other chemicals were of analytical grade and used as received. The immunoreagents were dissolved in phosphate saline (PBS) buffer (pH 7.4, 0.01 M phosphate, 0.14 M NaCl, 2.7 mM KCl). The rinsing buffer was composed of 0.01 M PBS and 0.1 % Tween (pH 7.4).

All electrochemical measurements were performed on an IM6e electrochemical workstation (Zahner-Elektrik, Kronach, Germany) in a conventional three electrode cell comprised of a platinum wire auxiliary, An Ag/AgCl reference and glass carbon (GC) working electrode. The electrochemical behaviour of the immunosensor was recorded in pH 7 PBS containing 1 mM H_2O_2 and 0.5 mM 2-hydroxy-1,4-naphthoquinone (HNQ) by differential pulse voltammetry (DPV) from -0.55 to 0.05 V with a pulse amplitude of 50 mV and a pulse width of 20 ms. Transmission electron microscope (TEM) images were obtained from a JEM-2000EX microscope (Japan).

Gold nanoparticles were prepared in aqueous solution according to the reference[18]. Gold nanoparticles have negatively charged surface by preparing in this way. Polypyrrole coated MWNTs were synthesized by in situ polymerization of pyrrole on MWNTs. Briefly, 0.1 g treated carbon nanotubes were dispersed in the mixed solvent of 0.01 M HCl and 0.01 mM sodium dodecylsulfonate solutions at room temperature for 2 h using an ultrasonic homogenizer. Thereafter, pyrrole monomer was added into the solution and stirred for 0.5 h, an APS solution (2 g mL^{-1}) was added dropwise (1 mL min^{-1}) to the above solution with constant sonication at ambient temperature. The reaction mixture was stirred for 24 h. The black suspension was collected by filtration and washed with deionized water and ethanol thoroughly. Finally the powder was dried under vacuum at 60 °C for 3 h. The PPy@MWNTs composite was dispersed in water at a concentration of 30 mg mL^{-1} with the aid of utrasonication for 30 min. AuNPs assembled PPy@MWNTs was prepared by adding 50 mL AuNPs solution into 10 mL PPy@MWNTs suspension. Due to PPy is positive charged in oxidized state, the AuNPs absorped on the PPy@MWNTs surface. The precipitated AuNPs/PPy@MWNTs was centrifuged and washed with water several times. Then, the composite was dried in vacuum at 60 °C for 12 h before use.

The preparation of the immunosensor was illustrated in Fig. 1. In detail, 2 mg of AuNPs/PPy@MWNTs was dispersed in 5 mL water with the aid of ultrasonic bath to give a 0.4 mg mL^{-1} black suspension. The GC electrode (3 mm in diameter) was polished carefully with 1, 0.3 and 0.05 μm alumina slurry and washed with ultrasonication, respectively. After dried in the air, a suspension (10 μL) of the AuNPs/PPy@MWNTs was dropped on the cleaned GC electrode and dried at room temperature. Then the electrode soaked in 0.1 mol L^{-1} L-cysteine solution for 2 h. Subsequently, 5 μL of 50 μg mL^{-1} monoclonal anti-prostate specific antigen antibody (Ab_1) solution was added onto the modified electrode surface and incubated for 3 h. The assembly was washed successively with rinsing buffer and then incubated in 1 % BSA solution for 1 h to eliminate nonspecific binding, followed by washing with rising buffer for 3 min. All resulting electrodes were stored at 4 °C when not in use.

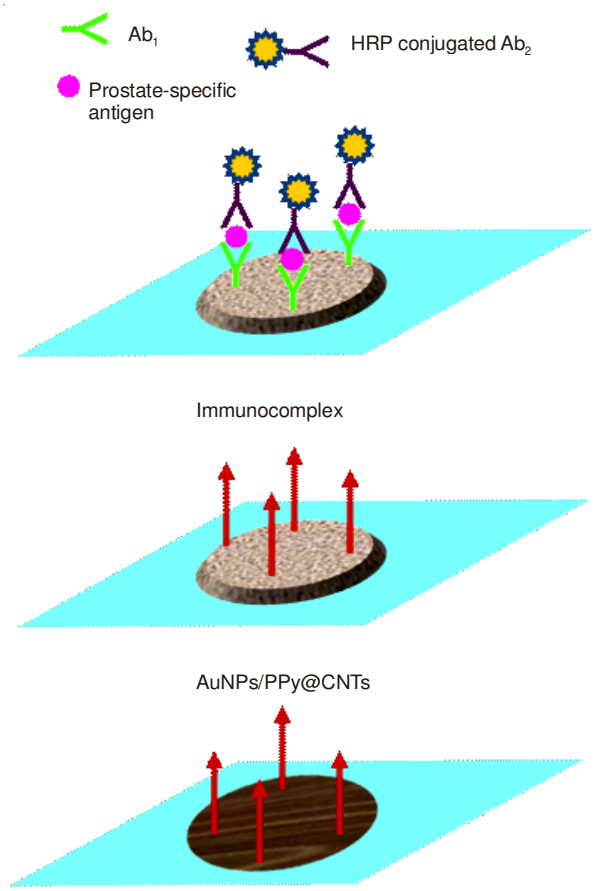

Fig. 1. Schematic of immunosensor based on AuNPs/PPy@MWNTs composite

Detection method: For detection of prostate specific antigen, The Ab_1/AuNPs/PPy@MWNTs/GC immunosensor constructed as described above was incubated for 1 h with a 10 μL drop of prostate specific antigen solution with varying concentration, followed by washing with rinsing buffer for 3 min to eliminate nonspecific binding. Sequentially, 20 μL HRP-Ab_2 (0.5 mg mL^{-1}) was casted on the electrode surface and

then rinse with rising buffer. After washed with rinsing buffer, the electrode was ready for electrochemical measurement.

RESULTS AND DISCUSSION

Characterization of AuNPs/PPy@MWNTs nanocomposite: Fig. 2 illustrates the preparation of AuNPs/PPy@MWNTs nanocomposite and TEM images of AuNPs/PPy@MWNTs. It was clear that the individual MWNTs could be coated with polypyrrole. For an oxidative polymerization, the PPy networks are formed on the surface of the MWNTs. The AuNPs with high density were well dispersed on the surface of the PPy@MWNTs. PPy is characterized by high surface energy and hydrophilicity, which is important for the biomolecular and nanoparticles adhesion. While AuNPs are well known to reduce the insulating effect of the protein shell and thus enhance electron transfer in the reaction processes. In this paper, AuNPs/PPy@MWNTs nanocomposite was firstly prepared, which potentially provide a good biocompatible interface and excellent microenvironment for the conjugation of proteins.

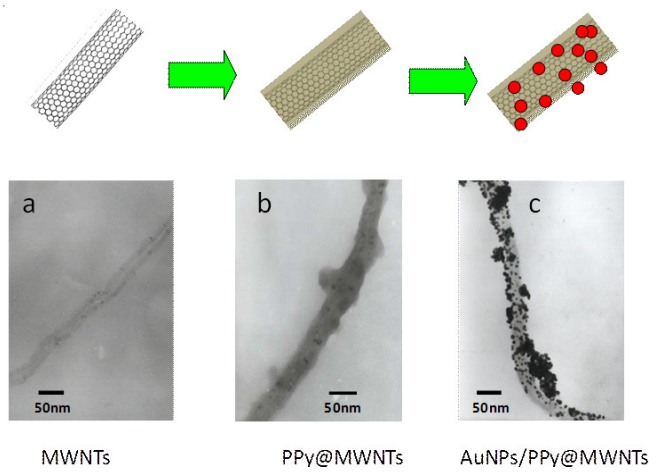

Fig. 2. TEM images of individual (a) MWNTs, (b) PPy@MWNTs, (c) AuNPs/PPy@MWNTs

Characterization of immunosensor: Fig. 3(A) shows the cyclic voltammetry (CV) curves at GC, AuNPs/PPy@MWNTs/GC and Ab_1/AuNPs/PPy@MWNTs/GC electrodes in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl solution. After modified GC electrode with AuNPs/PPy@MWNTs, the anodic peak and cathodic peak are increased greatly (curve b), indicating AuNPs/PPy@MWNTs composite improves the electroactive area of electrode obviously. The AuNPs/PPy@MWNTs modified GC electrode gave a quasi reversible electrochemistry with a peak separation of 68 mV at 100 mV/s. A decrease of the redox peaks and increase of peak separation were observed when Ab_1 was immobilized on AuNPs/PPy@MWNTs electrode (curve c), resulting from less conductivity of protein Ab_1, which indicated that Ab1 was successfully immobilized on the surface of the AuNPs/PPy@MWNTs /GC electrode[4].

To examine the sensitivity of the immunosensor, the electrochemical immunosensor Ab_1/AuNPs/PPy@MWNTs/GC was prepared with the established procedure. After immobilized with 10 ng mL^{-1} prostate specific antigen and HRP-Ab_2, the electrochemical performance of the immunosensors were investigated by CV in the presence of 0.5 mM HNQ. As shown in Fig. 3(B), a couple of well-defined redox peaks were observed for the modified electrode (curve a), which indicated a fast electron transfer reaction. After the addition of 1 mM H_2O_2 in the solution, the shape of the CV curve changed dramatically (curve d). The reduction peak current increases greatly, while the oxidation peak current diminished. As controls, two other immunosensors Ab_1/AuNPs/GC and Ab_1/PPy@MWNTs/GC were also prepared with same procedures. Cuve c and d in Fig. 3(B) show the current response of the two electrodes, respectively. However, the reduction peak currents were much lower than that of Ab_1/AuNPs/PPy@MWNTs/GC, indicating AuNPs/PPy@MWNTs composite exhibits enhanced performance compared with each component.

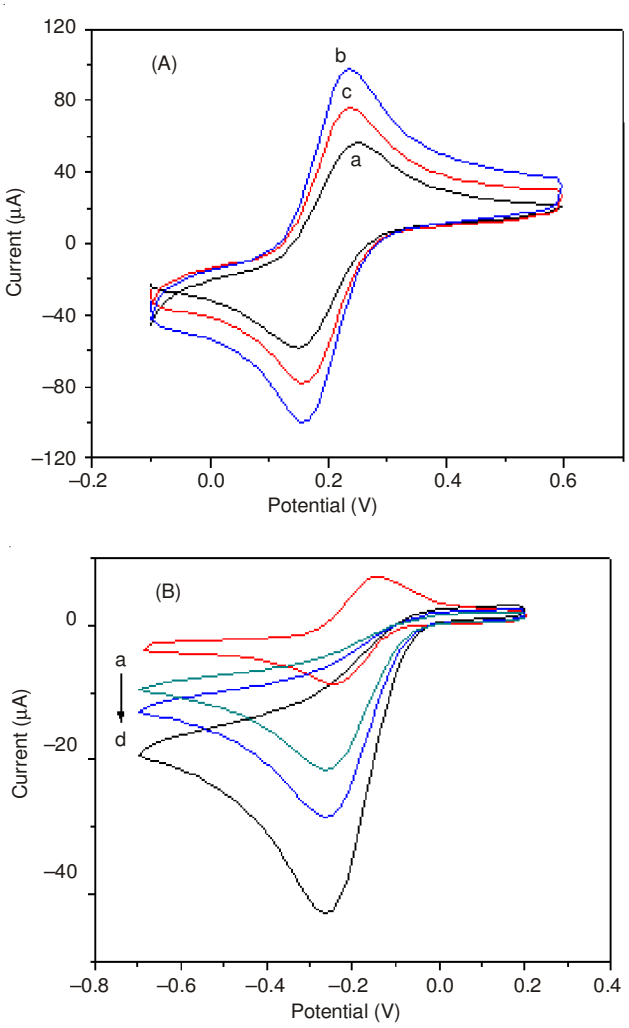

Fig. 3. (A) CVs at: (a) GC, (b) AuNPs/PPy@MWNTs/GC, (c) Ab_1/AuNPs/PPy@MWNTs/GC modified electrode. Supporting electrolyte, 0.1 M KCl and 5 mM $Fe(CN)_6^{3-/4-}$; scan rate, 100 mV/s; (B) CV of the immunosensors toward 10 ng mL^{-1} PSA in PBS solution: (a) Ab_1/AuNPs/PPy@MWNTs/GC in the absence of H_2O_2, (b) Ab_1/AuNPs/GC, (c) Ab_1/ PPy@MWNTs/GC, (d) Ab_1/AuNPs/PPy@MWNTs; supporting electrolyte, 1 mM H_2O_2 (unless otherwise noted) and 0.5 mM HNQ; scan rate, 100 mV/s

Effect of AuNPs/PPy@MWNTs nanocomposite loading on the current response: In order to achieve maximum electro-chemical response, the amount of the AuNPs/PPy@MWNTs nanocomposite modified on the GC electrode

was investigated. The amount was controlled by varying the volume of the suspension dropped on the electrode. As shown in Fig. 4, the sensitivity of the prepared immunosensor toward 1 ng mL^{-1} prostate specific antigen increase with the AuNPs/PPy@MWNTs nanocomposite loading from 3 to 10 µL. With futher increase the nanocomposite loading, the current response reached a platform. Therefore, the amount of AuNPs/PPy@MWNTs nanocomposite 10 µL was selected in this paper.

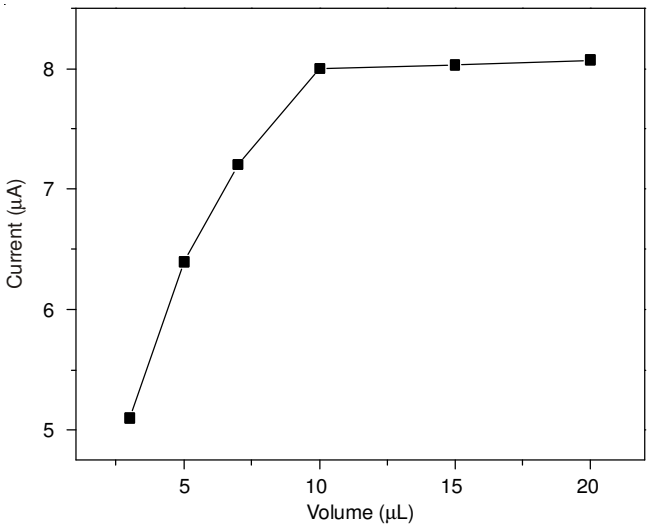

Fig. 4. Effect of AuNPs/PPy@MWNTs nanocomposite amount on the sensitivity of the immunosensor

Performance of the immunosensor: Fig. 5 shows the relationship between the current response and prostate specific antigen concentration. The DPV peak currents increase with the increase of the concentrations and display a linear relationship between the currents and the logarithm of prostate specific antigen concentration within the range of 0.002-20 ng mL^{-1} with a correlation coefficient of 0.996. The electrode has a sensitivity of 24.8 µA cm^{-2} (ng mL^{-1})$^{-1}$ and a detection limit of 0.001 ng mL^{-1} (S/N = 3).The linear range is much

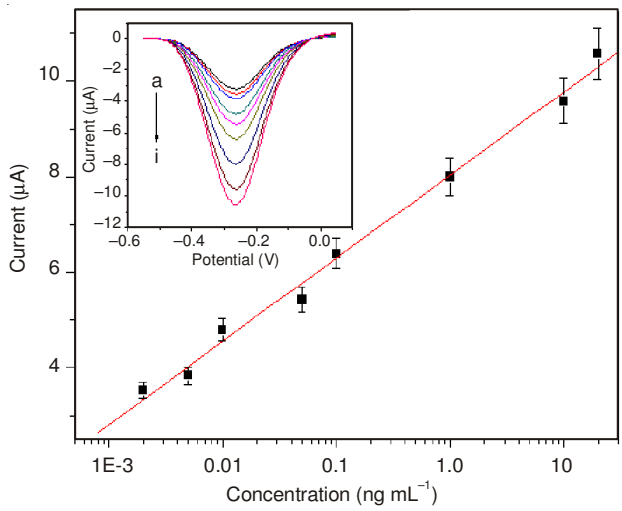

Fig. 5. Calibration curves of the electrochemical immunosensor toward PSA standards in PBS solution containing 1 mM H_2O_2 and 0.5 mM HNQ. Insert, DPV curves with varying PSA concentration from a to i, 0, 0.002, 0.005, 0.01, 0.05, 1, 10, 20 ng mL^{-1}

wider than that of 0.05-4 ng/mL with a disposable electrochemical immunosensor[19] and 0.05-5 ng/mL based on grapheme/methylene blue nanocomposite[20]. The detection limit of the immunosensor is better than those of previously reported 0.01 ng mL^{-1} [21], 0.004 ng mL^{-1} [22]. The high sensitivity and low detection limit may be attributed to the high surface area of the nanocomposite, which substantially increases the loading of Ab_1 due to its high surface-to-volume ratio. Moreover, AuNPs decorated PPy@MWNTs with good biocompatibility is favorable to maintain the active configuration of the immunocomplex.

Selectivity, reproducibility and stability: To check the selectivity of the immunosensor, the amperometric response of the immunosensor toward human IgG, BSA, IL-6 and glucose were studied. 1 ng mL^{-1} of prostate specific antigen solution containing 100 ng mL^{-1} of interfering substances was tested. As shown in Fig. 6, the current response of the immunosensor toward the interfering agents was less than 7.5 % of the current response measured without the interfering agents, which indicated the proposed immunosensor possessed high selectivity.

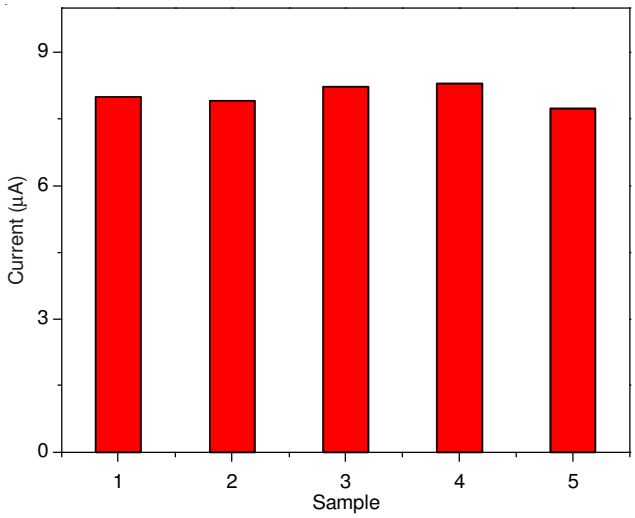

Fig. 6. Current response of the immunosensor to 1 ng mL^{-1} PSA in the presence of 100 ng mL^{-1} interference: (1) no interference, (2) human IgG, (3) BSA, (4) IL-6, (5) glucose

To examine the reproducibility of the immunosensor, a batch of five electrodes were prepared for detecting 1 ng mL^{-1} of prostate specific antigen. The relative standard deviation (RSD) of the measurements for the five electrodes was 4.7 %, suggesting the precision and reproducibility of the immunosensor was acceptable. The good reproducibility could be ascribed to the simplicity of the label and the immunosensor preparation methods.

The immunosensor also exhibited satisfactory stability, the peak current decreased by 8 % after storage of the immunosensor and labels at 4 °C for 15 days. This may be due to the nanocomposite has strong affinity for the immunoreagent assembling.

Real sample analysis: To evaluate the potential application of the immunosensor for real sample analysis, the immunosensor results of clinical serum samples using the proposed method were compared with standard ELISA

methods. The serum samples were diluted with phosphate buffer when the levels of biomarker were over the calibration range. Five samples were analyzed. The relative standard deviations (RSD) for all the samples were below 8 %, which indicates the immunosensor is reliable for practical using.

Conclusion

In this paper, AuNPs assembled PPy@ MWNTs composite was prepared by noncovalent strategy and used for primary antibody immobilization and immunosensor construction. AuNPs/PPy@MWNTs nanocomposites enhanced the current responses substantially. The proposed immunosensor showed high selectivity, sensitivity and reproduciability for prostate specific antigen detection. The method for immunosensor does not need complicated fabrication procedure and is promising for clinical applications.

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