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Streptavidin-Functionalized Silver-Nanoparticle-Enriched Carbon Nanotube Tag for Ultrasensitive Multiplexed Detection of Tumor Markers

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A streptavidin-functionalized silver-nanoparticle-enriched carbon nanotube (CNT/Ag NP) is designed as trace tag for ultrasensitive multiplexed measurements of tumor markers using a disposable immunosensor array. The CNT/Ag NP nanohybrid is prepared by one-pot in situ deposition of Ag NPs on carboxylated CNTs. The nanohybrid is functionalized with streptavidin via the inherent interaction between the protein and Ag NPs for further linkage of biotinylated signal antibodies to obtain tagged antibodies. The functionalization process greatly improves the dispersibility of the nanohybrid in water. The immunosensor array is prepared by covalently immobilizing capture antibodies on chitosan-modified screen-printed carbon electrodes. Through a sandwich-type immunoreaction on the immunosensor array, numerous Ag NPs are captured onto every single immunocomplex and are further amplified by a subsequent Ag NP-promoted deposition of silver from a silver enhancer solution to obtain the sensitive electrochemical-stripping signal of the Ag NPs. Using carcinoembryonic antigen and α-fetoprotein as model analytes, this proposed multiplexed immunoassay method shows acceptable precision and wide linear ranges over four orders of magnitude with detection limits down to 0.093 and 0.061 pg mL^{-1}, respectively. The assay results of serum samples with the proposed method are in acceptable agreement with the reference values. The newly designed strategy and the functionalized tag avoid cross-talk and the requirement of deoxygenation for electrochemical immunoassay, and thus provide a promising potential in clinical application.

1. Introduction

The levels of tumor markers in blood or tissue provide essential information for clinical cancer screening and disease diagnosis.[1,2] Electrochemical immunoassay has shown promise in fast, selective, and sensitive detection of tumor markers with simple instrumentation in recent years.[3–6] Moreover, to accurately determine biomarkers at ultralow levels during the early stage of diseases, numerous signal amplification strategies based on various bionanocomposites with a high content of detection tags for signal tracing[7–12] have been employed to enhance the electrochemical signal transduction of recognition events, which has led to a series of sensitive electrochemical immunoassay methods. Meanwhile, due to the limited specificity of tumor markers to a particular disease, multiplexed immunoassay methods for simultaneous measurement of panel of biomarkers have also been developed for cancer screening.[13–15] These methods commonly use enzymes such as horseradish peroxidase to produce an electrochemical signal during a cathodic scan. Thus the reduction of dissolved oxygen[16–18] and cross-talk, which occurs due to the diffusion of electroactive product generated at one electrode to a neighboring electrode, interfere with the amperometric response,[19–21] which limits the clinic application of the electrochemical immunoassay and further miniaturization of electrochemical immunosensor arrays. To overcome these limits, glucose-oxidase-functionalized carbon nanotubes (CNTs) have been designed for signal tracing coupled with a Prussia-blue-mediated enzymatic cycle,[15] and gold (Au NPs)[22,23] and silver nanoparticles (Ag NPs)[24] have been used as labels to develop new reagentless immunosensing strategies. Compared with the enzyme tags, metal nanoparticles have shown great promise for sensitive bioelectrochemical tracing through stripping detection of the tagged metal nanoparticles in the dissolved oxygen interference-free potential range.

Au NPs possess good biocompatibility and have been extensively applied in immunoassays. The convenient functionalization of Au NPs with biomacromolecules such as proteins makes them easy to use as a trace tag to combine with different detection technologies or as carrier for loading numerous single tags, including enzymes, for signal amplification. In a deoxygenation-free electrochemical immunoassay, the functionalized Au NPs as the tag are firstly chemically oxidized with a harmful dissolution reagent[22,25] or electrochemically oxidized at a relatively positive potential in HCl,[23,26] and then reduced to produce

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DOI: 10.1002/adfm.201100396

a detectable signal. Compared with Au NPs, Ag NPs can be directly detected using an electrochemical-stripping detection due to their lower oxidation potential and facile stripping conditions. However, few works using Ag NPs as the tag have been reported due to the limited stability of Ag NPs in saline buffer and the tedious synthetic procedure.[27,28] The reported Ag NPs are usually stabilized by functionalizing their surface with oligonucleotides,[27,28] silica,[29] or polymer.[24,30] The coverage on the Ag NP leads to poor anodic stripping peak of Ag NPs.[28] In this work we used silver-nanoparticle-enriched carbon nanotubes (CNT/Ag NPs) to synthesize streptavidin-functionalized Ag NP nanohybrids. The functionalization process greatly improved the stability and dispersibility of the nanohybrid in water. Thus the hybrid could be conveniently further bound to biotin-labeled antibody for preparation of tagged antibody.

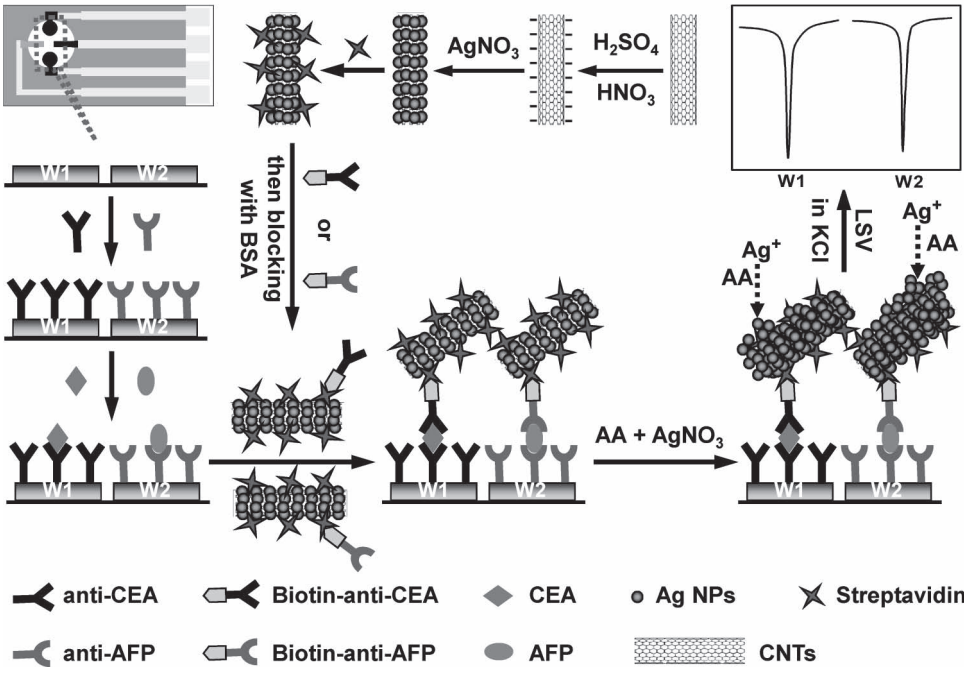
The CNT/Ag NPs can be prepared by electrochemical and chemical methods.[31–34] The electrochemical method can produce size and distribution-controllable CNT/Ag NPs with very high purity.[31] However, it is very difficult to produce gram quantities of nanohybrids. After a reductant is adsorbed onto the CNTs, Ag NPs can be deposited on their surface by chemical reduction.[32] However, this method suffers from impurities due to the added reducing agent[34] and the challenge of synthesis controllability.[33] Thus an efficient approach to prepare the CNT/Ag NPs is presented by the direct reduction of Ag^+ ions on carboxylated CNTs.[33] Herein, we further modify this approach by adding citrate in Ag^+ solution to neutralize the solution to improve the deposition of Ag NPs onto the CNTs. The obtained Ag NP-enriched CNTs could thus be easily functionalized with streptavidin. By combining the novel functional tag with a disposable immunosensor array, a multiplexed electrochemical immunoassay method was developed.

To further improve the sensitivity of the proposed method, an Ag NP-promoted silver deposition was further performed by the formation of sandwich immunocomplex labeled with CNT/Ag NPs (**Scheme 1**). The amplified Ag NPs showed well-defined anodic stripping peak in 1.0 M KCl solution. Both the high content of Ag NPs on the synthesized tag and the following silver enhancement greatly amplified the detection signal, which led to a detection limit down to sub-picogram per milliliter level. The proposed assay approach excluded completely cross-talk and the interference of dissolved oxygen. The functional nanohybrid showed potential application as a biotag for determination of low-abundance biomarkers in clinical diagnostics.

2. Results and Discussion

2.1. Characterization of CNT/Ag NPs

The CNT/Ag NPs were characterized by using high-resolution transmission electron microscopy (TEM). Compared with the bare carboxylated CNTs, numerous uniformly distributed nanoparticles with a size of about 2 nm were observed on the surface of nanotubes after an in situ chemical deposition of Ag NPs (**Figure 1**), which indicates formation of the CNT/Ag NP nanohybrid. X-ray photoelectron spectroscopy (XPS) was further used to characterize the formation of CNT/Ag NPs nanohybrids. Compared with the spectrum of the bare carboxylated CNTs (**Figure 2**, curve a), the spectrum of CNT/Ag NPs showed three new strong peaks at 369.1, 571.7, and 602.0 eV (Figure 2, curve b), which correspond to Ag3d, $Ag3p_{3/2}$, and



Scheme 1. Schematic representation of preparation of immunosensor array and trace tag, and detection strategy by linear-sweep stripping voltammetric analysis of Ag NPs on the immunosensor surface.

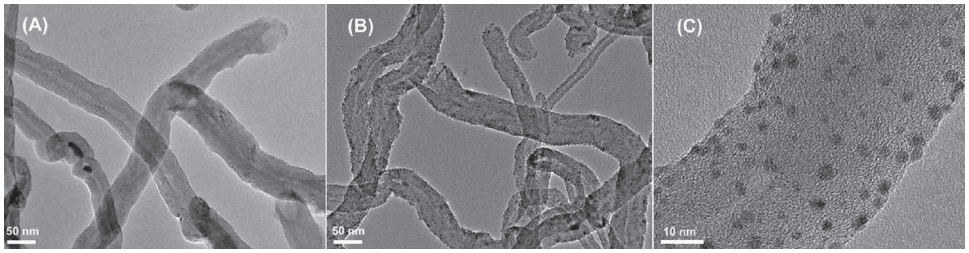


Figure 1. TEM images of A) carboxylated CNTs, B) CNT/Ag NPs, and C) the magnified image of CNT/Ag NPs.

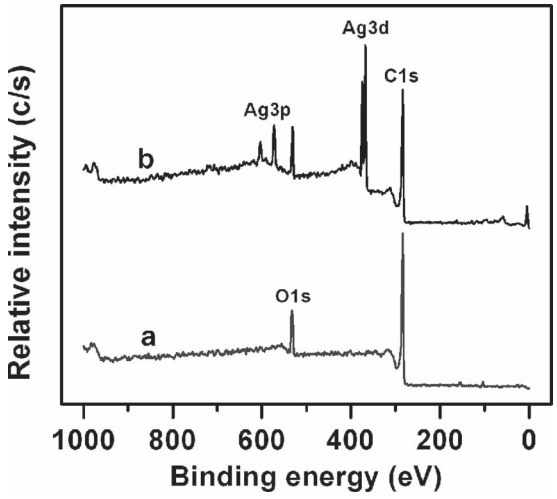


Figure 2. XPS spectra of a) carboxylated CNTs and b) CNT/Ag NPs.

$Ag3p_{1/2}$, respectively. This result confirmed that the Ag NPs were deposited successfully on the CNTs' surface. The deposition should be attributed to the inherent properties of the carboxylated CNTs; that they are rich in electrons and have numerous carboxylic acid groups, which allows carboxylated CNTs to serve as catalyst, silver catcher, and support.[33,35] In this case, Ag NPs were generated in situ from the $AgNO_3$ solution at room temperature and attached to the surface of CNTs, to form the CNT/Ag NP nanohybrid.

2.2. Characterization of Streptavidin-Functionalized CNT/Ag NPs

Through the inherent interaction between noble-metal NPs and the mercapto and amino groups of proteins,[36,37] streptavidin could be easily attached to the surface of Ag NPs to form streptavidin functionalized CNT/Ag NPs. The FTIR spectrum of CNT/Ag NPs displayed an obvious peak at 1530 cm^{-1} (**Figure 3**A, curve a), which was assigned to the carbonyl stretch mode of the carboxylated CNTs.[38] In comparison with CNT/Ag NPs, the functionalized CNT/Ag NPs displayed obvious absorption peaks corresponding to the amide bands I (1641 cm^{-1}) and II (1544 cm^{-1}) of streptavidin (Figure 3A, curve b), which indicated that the streptavidin molecules were successfully attached onto the CNT/Ag NPs. In addition, the XPS spectrum of the functionalized CNT/Ag NPs showed an obvious N1s peak at 399.6 eV, while no N1s peak was observed in the XPS spectrum of CNT/Ag NPs (Figure 3B). This result further confirmed the successful functionalization of CNT/Ag NPs with streptavidin. Because of the negatively charged character of streptavidin, the streptavidin-functionalized CNT/Ag NPs could be well dispersed in a physiological buffer (Figure 3C), which is very favorable for their further application in bioassays.

2.3. Characterization of Immunocomplex and Subsequent Silver Deposition

Scanning electron microscopy (SEM) was used to characterize the sandwich-type immunoreaction process. Different from the uniform chitosan film (**Figure 4**A), when the captured antibody was immobilized on the chitosan-modified screen-printed carbon electrode (SPCE) by glutaraldehyde cross-linking, an obvious aggregation of the trapped biomolecules could be observed on the electrode surface (Figure 4B). After a sandwich-type immunoreaction, numerous functional CNT-based tags could be clearly observed on the electrode surface (Figure 4C), which indicates an efficient capture of the antigen and the functionalized CNT/Ag NP labeled signal antibody. Upon the further silver deposition, obvious aggregation of Ag NPs was observed on the surface of the CNTs,

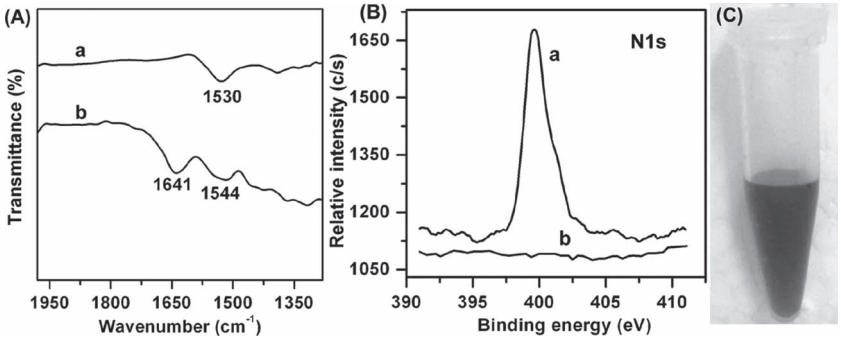


Figure 3. A) FTIR spectra of CNT/Ag NPs (curve a) and CNT/Ag NP/streptavidin (curve b), B) XPS spectra of N1s peak from CNT/Ag NP/streptavidin (curve a) and CNT/Ag NPs (curve b), and C) photograph of CNT/Ag NP/streptavidin suspension.

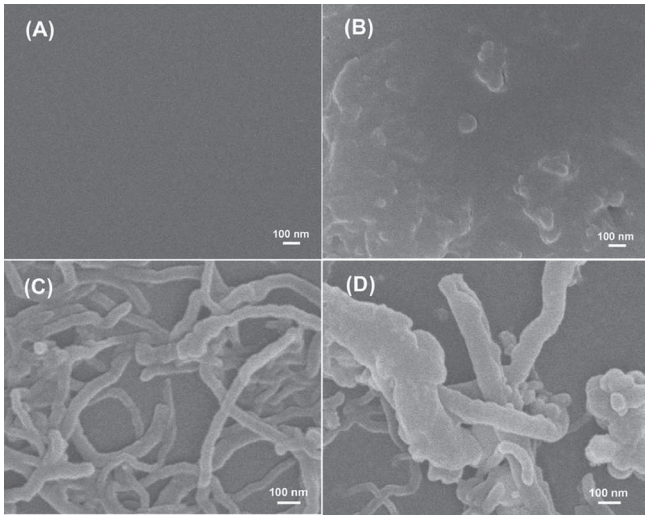


Figure 4. SEM images of A) chitosan-modified SPCE, B) immunosensor, C) immunocomplexes on immunosensor, and D) silver-deposition product formed on the immunocomplexes.

which resulted in an increase of diameter and an enrichment of the texture of the CNTs (Figure 4D). This phenomenon should be attributed to seed-mediated nucleation growth, caused by the captured Ag NPs, of the trace tag on the immunosensing surface.[24]

2.4. Optimization of Detection Conditions

During a linear potential sweep, the silver-deposition-amplified Ag NPs showed a well-defined anodic stripping peak at a peak potential of 0.05 V (as shown below), which was more negative than that reported previously[28] due to the presence of Cl^-. The peak current increased with the increasing concentration of antigen used in the sandwich immunoreaction, and could thus be used for immunoassaying.

The effect of the concentration of silver-deposition solution on the stripping-peak current was studied at a 2:1 concentration ratio of $AgNO_3$ to ascorbic acid (AA). When no silver-deposition enhancement was adopted, only limited current and a low signal–noise ratio were obtained. The stripping current increased greatly with an increasing concentration of silver-deposition solution. However, higher concentrations of silver-deposition solution also produced a higher background current, which led to a decrease of the signal–noise ratio after the silver concentration was over 0.5 mM (**Figure 5**). Thus, a silver-deposition solution containing 0.5 mM $AgNO_3$ and 0.25 mM AA was adopted in this work.

The incubation time is an important parameter affecting the analytical performance of immunoassays. With an increasing incubation time, the stripping voltammetric responses for carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) increased and trended to constant values after an incubation time of 40 min (Figure S1), which indicated the saturated formation of the sandwich immunocomplex. Thus, an incubation time of 40 min was selected for the sandwich-type immunoassay.

2.5. Evaluation of Cross-Reactivity and Cross-Talk

The cross-reactivity between analytes and noncognate antibodies was investigated. On the immunosensor array two different capture antibodies for CEA and AFP were immobilized on W1 and W2 separately. The cross-reactivity was evaluated by comparing the silver-stripping currents on the immunosensor array incubated with blank solution, target proteins, and a tenfold-higher concentration of noncognate proteins. As expected, both the CEA and AFP immunosensor showed obvious stripping responses only towards their corresponding proteins (Figure S2). Obviously, the cross-reactivity between the two antibodies towards noncognate proteins was negligible. In addition, no substrate or mediator was required in the detection procedure, and the silver-deposition enhancement and stripping analysis occurred only on the corresponding immunosensing surface. Therefore, no electrochemical cross-talk occurred among neighboring electrodes, and simultaneous multianalyte immunoassay could be performed in a single run using the disposable immunosensor array.

2.6. Analytical Performance

With a sandwich-type immunoassay format, the quantitatively captured and deposited Ag NPs on the immunosensors could be easily detected by anodic-stripping voltammetric analysis, and the well-defined sharp silver stripping peak was favorable for high detection sensitivity due to the Ag/AgCl solid-state voltammetric process in KCl.[24] Under optimal conditions, the stripping peak currents for simultaneous detection of CEA and AFP on the immunosensor array increased with increasing concentrations of analytes. Both calibration plots showed good linear relationships between the peak currents and the logarithm values of the analyte concentrations in the range from 0.1 pg mL^{-1} to 5.0 ng mL^{-1} (**Figure 6**). The correlation coefficients were 0.9988 and 0.9942 for CEA and AFP, respectively. The limits

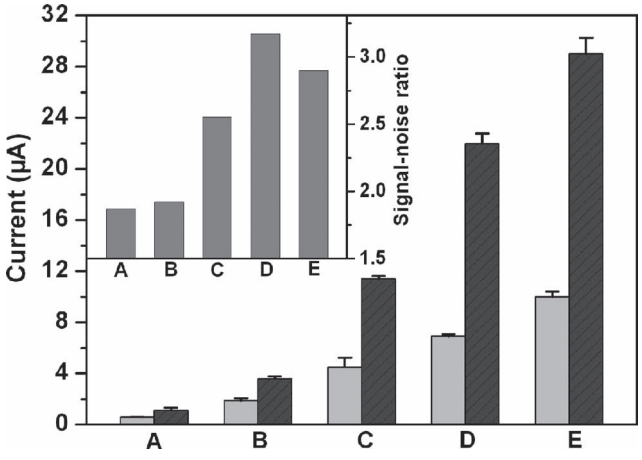


Figure 5. Effect of concentration of silver-deposition solution on stripping-current response of Ag NPs to 0.5 ng mL^{-1} CEA (in black) and blank control (in gray) in 1.0 M KCl. $AgNO_3$ concentration was A) 0, B) 0.1, C) 0.2, D) 0.5, and E) 1.0 mM, and AA concentration was half of the $AgNO_3$ concentration. Inset: ratios of signal–noise at different concentrations.

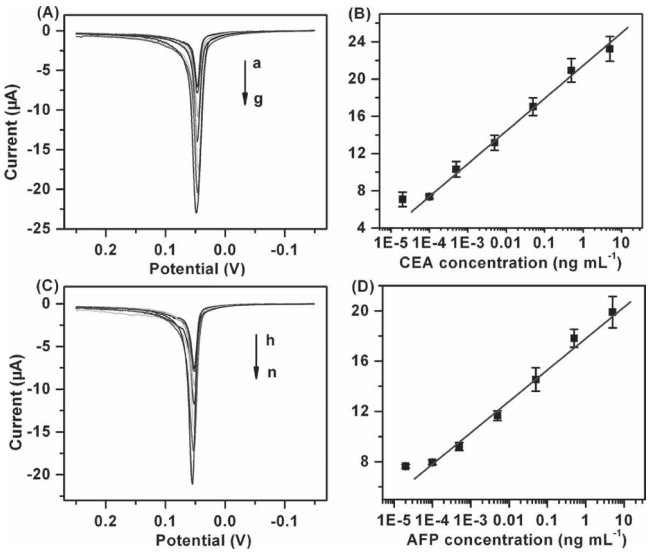


Figure 6. Linear-sweep stripping voltammetric curves of Ag NPs (A, C) and calibration curves (B, D) for simultaneous multiplexed detection of CEA (A, B) and AFP (C, D) using the proposed strategy. Curves a–g and h–n are for CEA and AFP at concentrations from 0.02 pg mL^{-1} to 5.0 ng mL^{-1} at W1 and W2, respectively.

of detection for CEA and AFP were 0.093 and 0.061 pg mL^{-1} at a signal–noise ratio of 3, respectively, which were much lower than those reported in the previous studies.[10,12,15,19,20] Compared with traditional enzyme labels, the functionalized CNT/Ag NP-labeled signal antibody can be easily prepared. The detection step can be performed with a one-step stripping analysis in KCl solution without any pretreatment or addition of substrate. The excellent analytical performance, including very low detection limits and wide linear ranges over four orders of magnitude for multiplexed analysis of two analytes, was very significant for practical applications.

The interassay precision of the immunosensor array was examined with two panels of proteins at different concentrations. Each panel was measured five times using five arrays. The coefficients of variation were 4.6% and 5.0% for 0.05 ng mL^{-1} CEA and AFP, and 4.5% and 3.8% for 0.5 ng mL^{-1} CEA and AFP, respectively. In addition, the immunosensor array could be stored in the dry at 4 °C. In this way, over 90% of the initial responses remained after two weeks storage for both CEA and AFP. These results indicated that the immunosensor array has acceptable stability and reproducibility.

2.7. Application in Analysis of Serum Samples

To evaluate the analytical reliability and application potential of the designed immunosensing method, the assay results of CEA and AFP in human serum samples using the proposed method were compared with the reference values obtained by commercial electrochemiluminescent single-analyte tests. The results are listed in Table S1. Acceptable results with relative errors less than 10.4% for both CEA and AFP detection were achieved, which indicates good accuracy of the proposed method for clinical sample detections.

3. Conclusions

A novel functional CNT/Ag NP nanohybrid has been prepared as a trace tag by using a one-pot in situ deposition of Ag NPs on carboxylated CNTs and their subsequent functionalization with streptavidin. The functionalized CNT/Ag NPs can easily be linked to a biotinylated signal antibody for multiplexed immunoassay of tumor markers. Through a sandwich-type immunoreaction on a disposable immunosensor array, the high-content Ag NPs can be captured on the immunosensor surface to further induce the silver deposition, which greatly amplifies the detection signal. Based on the electrochemical stripping detection of the Ag NPs on the immunosensor surface, the proposed simultaneous multianalyte immunoassay method shows an ultrahigh sensitivity with a wide detection range. This method completely excludes cross-talk and interference from dissolved oxygen. The functionalized CNT/Ag NPs as a trace tag possesses a promising potential in ultrasensitive detection.

4. Experimental Section

Materials and Reagents: Multiwalled carbon nanotubes (CNTs, CVD method, purity ≥98%, diameter 60–100 nm, and length 1–2 μm) were purchased from Nanoport Co. Ltd. (Shenzhen, China). Chitosan (≥85% deacetylation) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Glutaraldehyde (25% aqueous solution) was purchased from Alfa Aesar China Ltd. Streptavidin was purchased from Promega Co. (USA). Ascorbic acid (AA), $AgNO_3$, and trisodium citrate were obtained from Shanghai Reagent Company (Shanghai, China). Polyclonal anti-CEA and anti-AFP capture antibodies were purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). Biotinylated anti-CEA and anti-AFP mouse monoclonal signal antibodies, and CEA and AFP standard solutions were from enzyme-linked immunosorption assay (ELISA) kits of CEA and AFP, respectively, which were supplied by Fujirebio Diagnostics AB (Göteborg, Sweden). Human IgG was purchased from Wuhan Boster Biological Technology Ltd. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays. The clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of analytical grade and used as received.

Tris-HNO_3 buffer (0.1 M, pH 7.2) was prepared and used as the working solution. The washing buffer was 0.1 M Tris-HNO_3 containing 0.05% (w/v) Tween-20. 0.1 M Tris-HNO_3 containing 5% (w/v) BSA was used as blocking solution. The mixture solution of 0.50 mM $AgNO_3$ and 0.25 mM AA was prepared daily for silver-deposition enhancement.

Apparatus: The morphology of the CNT/Ag NPs was examined using a JEM 2100 high-resolution TEM(Japan). Scanning electron micrographs were obtained with a Hitachi S-4800 SEM(Japan) at an acceleration voltage of 10 kV. XPS measurements were performed using a PHI5000 Versa Probe spectrometer (ULVAC-PHI, Japan) with an ultrahigh vacuum generator. The IR spectra were recorded using a NEXUS870 Fourier-transform infrared (FTIR) spectrometer (NICOLET, USA). All electrochemical immunoassays were performed on a CHI 660B electrochemical workstation (Chenhua, Shanghai, China). The reference levels of the tumor markers in the human serum samples were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche).

Preparation of Trace Tag: Firstly, the carboxylated CNTs were prepared by 4 h sonication treatment with 3:1 H_2SO_4/HNO_3 and repeated washing with water until pH 7.0 was reached.[15] Next, the CNT/Ag NPs nanohybrids were synthesized according to the previous report with minor modification.[33] Briefly, 0.5 mg of carboxylated CNTs was dispersed in 0.5 mL of water with an ultrasonic bath for about 10 min, and then placed in a flask with a magnetic stirrer. To improve the deposition of Ag NPs on CNTs, 10 mg of citrate was dissolved in the dispersion. While stirring at

room temperature, 0.6 mL of 10 mM $AgNO_3$ solution was added dropwise into the flask and reacted for 36 h. The resulting CNT/Ag NPs nanohybrids were collected by centrifuging at 12000 rpm for 15 min and washing three times with water, and then dispersed in 1.0 mL of water.

50 μL of 1.0 mg mL^{-1} streptavidin was added to 200 μL of CNT/Ag NP suspension, which was previously adjusted to pH 6.7 with 50 mM phosphate buffer,[39] and stirred at room temperature for 120 min. The mixture was then centrifuged at 3800 rpm for 10 min. Afterwards the sediment was washed three times with pH 7.2 Tris-HNO_3 to remove the excessive streptavidin and dispersed in 200 μL of pH 7.2 Tris-HNO_3 to obtain the suspension of CNT/Ag NPs/streptavidin trace tag.

1.0 mL of 3 μg mL^{-1} biotin-anti-CEA or biotin-anti-AFP signal antibody was added into the obtained CNT/Ag NPs/streptavidin suspension and gently mixed at room temperature for 50 min. The final product was acquired by centrifugation at 3500 rpm for 10 min. After twice washing with pH 7.2 Tris-HNO_3, the tagged antibodies were obtained and resuspended in 400 μL of pH 7.2 Tris-HNO_3 containing 0.1% BSA as the assay solution.

Preparation of Immunosensor Array: The SPCEs containing two graphite working electrodes (W1 and W2, diameter: 2 mm), a Ag/AgCl reference, and a graphite auxiliary electrode were prepared with screen-printing technology according to our previous report.[15] The insulating layer printed around the working area constituted an electrochemical microcell.

The immunosensor array was constructed by immobilizing corresponding capture antibodies at the working electrodes of SPCEs through chitosan coating and glutaraldehyde cross-linking. Firstly, 1.0 μL of 0.25 mg mL^{-1} chitosan was coated onto the working electrodes and dried at room temperature. After activating with 2.5% glutaraldehyde (in 50 mM pH 7.4 phosphate buffer) for 2 h and washing with water, 1.0 μL of 0.5 mg mL^{-1} anti-CEA and anti-AFP capture antibodies were dropped onto the corresponding working electrodes W1 and W2, respectively, and reacted at room temperature for 60 min and then 4 °C overnight in a 100% moisture-saturated environment. Subsequently, excess antibodies were washed out with washing buffer and pH 7.2 Tris-HNO_3. A drop of 20 μL of blocking solution was dropped on the array and incubated for 60 min at room temperature to block possible remaining active sites against nonspecific adsorption. After another washing with washing buffer and pH 7.2 Tris-HNO_3 the resulting immunosensor array was obtained.

Measurement Procedure: To carry out the immunoreaction and electrochemical measurements, the immunosensor array was firstly incubated with a 15-μL drop of the mixture of CEA and AFP standard solutions or serum samples for 40 min at room temperature, followed by washing with washing buffer and pH 7.2 Tris-HNO_3. It was then incubated with 15 μL mixture of tagged antibodies for 40 min at room temperature. After washing with washing buffer and Tris-HNO_3 again, 15 μL of silver-deposition solution containing 0.50 mM $AgNO_3$ and 0.25 mM AA was dropped onto the electrochemical microcell for 4 min, which was protected from light. After silver deposition, the immunosensor array was rinsed with water, and linear sweep voltammetry from –0.15 to 0.25 V at 50 mV s^{-1} was performed in 1.0 M KCl solution to record the stripping currents at W1 and W2 for simultaneous detection of CEA and AFP.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was financially supported by National Basic Research Program (2010CB732400), the National Science Fund for Creative Research Groups (20821063) and the projects (20875044, 21075055) from National Natural Science Foundation of China, and Natural Science Foundation of Jiangsu (BK2008014).

Received: February 21, 2011
Published online: May 26, 2011

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