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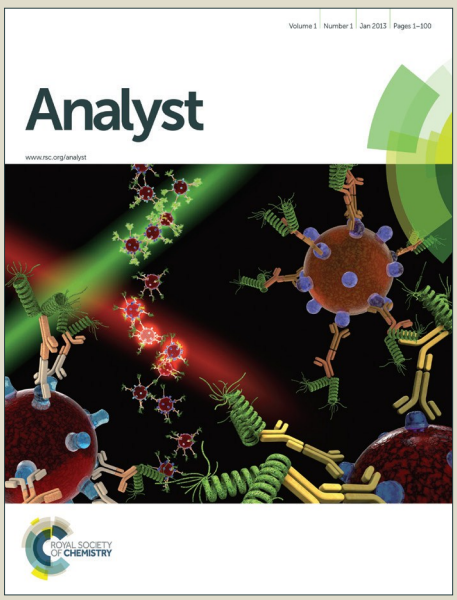

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Proximity hybridization-regulated electrochemical stripping of silver nanoparticles via nanogold induced deposition for immunoassay

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A simple and disposable electrochemical immunosensor was developed for sensitive and selective detection of protein biomarker via target-induced proximity hybridization and electrochemical stripping analysis of silver nanoparticles (AgNPs). The immunosensor was prepared by assembling single-stranded DNA modified gold nanoparticels (ssDNA@AuNPs) on graphene oxide modified disposable screen-printed carbon electrode (SPCE). In the presence of target protein and two DNA-labeled antibodies, the proximate complex formed in homogeneous solution could hybridize with the assembled DNA to take away AuNPs, which decreased AuNPs-catalyzed deposition of AgNPs on immunosensor surface, and thus the anodic stripping signal. The proposed method avoided the interference of dissolved oxygen. Using carcinoembryonic antigen (CEA) as model analyte, this method showed a linear range of four magnitude orders with detection limit down to 3.9 pg/mL. The electrochemical immunosensor possessed preparation convenience, good stability and high sensitivity, and could be extended to sensitive biosensing of other analytes, showing a potential application in point-of-care testing.

Introduction

Sensitive and selective detection of cancer biomarkers and cancer cells is of great importance in early clinical diagnosis and biomedical research because of the positive correlation between the levels of tumor biomarkers in serum/tissue and the stages of tumors.[1-3] Various immunoassay methods, such as enzyme-linked immunosorbent assays (ELISA),[4] electrochemical,[5,6] fluorescent,[7] luminescent[8] and colorimetric immunoassays,[9] have been designed for the detection of protein biomarkers. Due to the high sensitivity, inherent simplicity, portability and low cost, the electrochemical immunoassay methods have attracted considerable interest. This technology has been coupled with a proximity ligation of a pair of oligonucleotide-labeled antibodies in the presence of target protein for extending its application in point-of-care testing.[10-12]

The proximity ligation assay (PLA) is a recently developed strategy for protein analysis.[13] Through a DNA ligation reaction of oligonucleotides, the early detection methods used a pair of proximity probes to bind target protein and form an amplifiable DNA strand suitable for real-time PCR.[13-15] These methods showed the extremely sensitive and specific detection of proteins. By using electrochemical[10,16] and chemiluminescent[17] readout, this strategy has conveniently been used for sensitive quantitation of proteins. In these electrochemical PLAs, a DNA strand such as hairpin DNA or aptamer is covalently immobilized on electrode surface for capture of the proximity ligation product, which brings the electrochemical active label for signal-on detection. To achieve point-of-care testing, here, a screen-printed carbon electrode (SPCE)[18] was used for the immobilization of the DNA strand through the hydrophobic and/or π-stacking interaction between single-stranded DNA (ssDNA) and graphene oxide (GO), and the proximity-dependent surface hybridization led to the release of the immobilized DNA, which produced a signal-off detection strategy.

The readout of electrochemical signal can generally be performed with electroactive species,[12] enzyme, quantum dots[19,20] or metallic nanoparticles as the labels. The latter can be directly stripped by anodic oxidation to obtain the current signal.[21-24] In comparison with gold nanoparticles (AuNPs), silver nanoparticles (AgNPs) can be electrochemically oxidized at a relatively lower potential with a relatively sharp peak, thus, they are the more favorable signal tag than AuNPs. To improve the sensitivity, these metallic nanoparticles can be used as catalyst to further catalytically deposit AgNPs on sensor surface.[25-27] To introduce the catalytic silver deposition into the electrochemical signal readout, here ssDNA functionalized AuNPs was prepared and immobilized on the GO modified SPCE.

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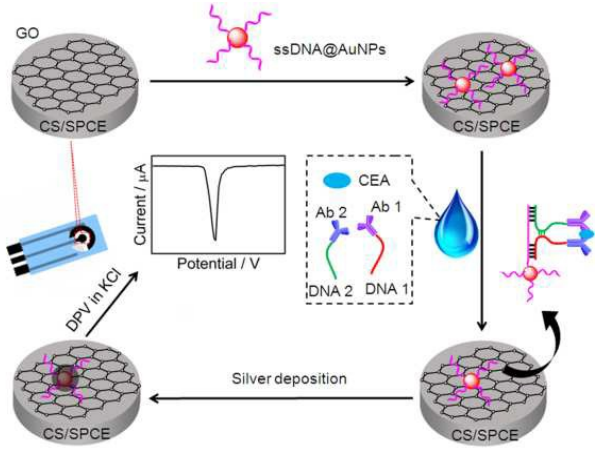
Using carcino-embryonic antigen (CEA) that plays a key role in the diagnosis and screening for colon cancer, colorectal cancer and other malignancies[28,29] as a model tumor marker, the ssDNA functionalized Au NPs could conveniently detach from the GO surface upon its hybridization with a complementary DNA strand.[30] Thus, the target-induced proximity hybridization was introduced to form a proximate complex for the design of a regulated DNA biogate, which provided the complementary DNA strand for the detachment of ssDNA functionalized Au NPs by using two DNA strand-labeled antibodies to recognize the target protein. The formed sequence could hybridize with the immobilized ssDNA to take away AuNPs from sensor surface, which decreased AuNPs-catalyzed deposition of AgNPs and thus the anodic stripping signal (Scheme 1).



Scheme 1. Schematic representation of preparation of immunosensor and detection strategy by stripping analysis of AgNPs catalytically deposited on the immunosensor surface by gold nanolabels.

The electrochemical immunoassay methods for CEA with enzyme or nanomaterials as label have extensively developed.[31-34] In comparison with these methods, the immunological recognition of the proposed method happened in homogeneous solution, and both the silver deposition enhancement and the well-defined silver stripping peak improved the detection sensitivity. Moreover, the positive potential range for anodic stripping excluded the interference of dissolved oxygen. These advantages greatly improved the analytical performance, thus the proposed method shows a potential application for point-of-care testing.

Experimental

Materials and reagents

CEA, anti-CEA antibody (anti-CEA, mouse monoclonal antibodies, clone nos. Z-2011 and Z-2012) and prostate-specific antigen (PSA) were purchased from Keybiotech Co. Ltd. (Beijing, China). Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-car-boxylate (SMCC) was supplied by Heowns Biochem LLC (China), and dithiothreitol (DTT) was from Sangon Biotechnology Co. Ltd. (Shanghai, China). Chitosan (CS, ≥95%) and silver enhancer solution were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate were obtained from Shanghai Reagent Co. (Shanghai, China). GO was obtained from XFNano Materials Tech Co., Ltd. (Nanjing, China). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore) was used in all assays. TE buffer (10 mM, containing1 mM EDTA and 0.3 M NaCl, pH 7.9) was used for preparation of oligonucleotide stock solutions. PBS1 (55 mM, containing 150mM NaCl and 20 mM EDTA, pH 7.2) and PBS2 (55 mM, containing 150 mM NaCl and 5 mM EDTA, pH 7.2) were used to prepare DNA-labeled antibodies. 10 mM PBS containing 150 mM NaCl (pH 7.4) was used for proximity ligation. The washing buffer was 10 mM pH 7.4 PBS containing 0.01% Tween-20.

Human serum samples were generously provided by Jiangsu Province Tumor Hospital. All DNA oligonucleotides were synthesized by Sangon Inc. (Shanghai, China), and their sequences are given below:

ssDNA for functionalization of AuNPs: 5'-SH−TTTTTTTTTTTTTT TAAGCGATCGATAGTC-3'

DNA 1: 5'-SH-TACGTCCAGAACTTTACCAAACCACACCCTTT TTTTGTCTTGGATCGCTT-3'

DNA 2: 5'-GACTATCATCAAGACTTTTTTTATCACATCAGGC TCTAGCGTATGCTATTG-SH-3'

Instrumentation

The scanning electron microscopic (SEM) images were obtained from an S-4800 scanning electron microscope (Hitachi, Japan). The UV-vis spectra were recorded on UV-3600 UV-vis-NIR spectrophotometer (Shimadzu Co., Kyoto, Japan). Dynamic light scattering (DLS) measurement was performed with a BI-200SM light scattering apparatus (Brookhaven, U.S.A). Electrochemical experiments, including differential pulse voltammetric (DPV) measurements, were conducted on CHI 660B electrochemical workstation (Shanghai CH Instruments, China).

Preparation of ssDNA@AuNPs

AuNPs with 13-nm diameter were prepared according to the previous protocol.[35] 15 μL of ssDNA (40 μM) was then mixed with 200 μL of 5.0 nM AuNPs solution and incubated for 0.5 h. Afterward, 25 μL100 mM PBS (pH 7.4) containing 0.1% Tween-20 was added to the mixture and incubated at room temperature for 20 min. Small aliquots of 2.0 M NaCl in 10 mM PBS (pH 7.4) containing 0.01% Tween-20 were added stepwise to raise the NaCl concentration to 1.0 M, during which 10-s sonication and 20-min incubation were required for each addition step of NaCl. The mixture was incubated over 16 h at room temperature. Subsequently, a centrifugation process was performed to remove the excess oligonucleotides and obtain the DNA functionalized AuNPs (ssDNA@AuNPs), which were resuspended in 200 μL of 10 mM PBS containing 150 mM NaCl, and stored at 4ºC prior to use.

Preparation of DNA-labeled antibodies

The DNA-labeled antibodies were prepared with a modified coupling procedure.[36] Anti-CEA (2 mg/mL) was first reacted with a 20-fold molar excess of SMCC in PBS1 for 2 h at room temperature. Meanwhile, 12 μL of 100 μM thiolated oligonucleotide (DNA 1 or DNA 2) was reduced with 16 μL of 100 mM DTT in PBS1 at 37 °C for 1 h. The obtained anti-CEA-SMCC and reduced oligonucleotide were purified by ultrafiltration using 100 KD and 10 KD millipore respectively (10000 rmp, 10 min). Then, the two reaction products were mixed in PBS2 to incubate overnight at 4 °C, and the unreacted anti-CEA and DNA were removed by uzltrafiltration using a 100 KD millipore (10000 r, 10 min) for several times, and the obtained DNA-labeled antibodies were collected in 50 μL of PBS2.

Preparation of electrochemical sensor

The screen-printed carbon electrode (SPCE) containing a graphite working electrode (2 mm in diameter), a graphite auxiliary electrode

and a silver pseudo-reference electrode was prepared according to our previous report.[37] The insulating layer printed around the working area constituted an electrochemical microcell.

The SPCE was pretreated electrochemically to generate the carboxylic acid groups on the working electrode by cyclic voltammetric scanning between -0.3 and +0.6 V for 10 cycles at 0.5 V/s in 0.01 M PBS (pH 7.4).[38] Immediately, 2 μL 2.5 mg/mL CS was dropped on the working electrode for improving adhesion force between electrode and GO. After drying for 15 min, 3 μL 1 mg/mL GO was coated on chitosan surface and dried in air at room temperature. Then, 5 μL of the prepared ssDNA@AuNPs was cast onto the GO modified SPCE for about 40 min. After washed with washing buffer and pH 7.4 PBS, the SPCE was stored at 4ºC prior to use.

Detection of CEA

The immunoassay was performed by dropping 5 μL mixture of 250 nM Ab1-DNA1 and Ab2-DNA2, various concentrations of CEA or serum sample on the prepared SPCE. After incubation at 37 °C for 40 min, the SPCE was rinsed with washing buffer and pH 7.4 PB. Meanwhile, silver enhancer solutions A and B were 20-fold diluted and mixed in equal volume. Then 3 μL of the mixture of silver enhancer solutions was delivered to the electrochemical microcell for 4 min (under dark), followed by rinsing with water. Subsequently, DPV measurement was performed form -0.15 to 0.25 V at 50 mV/s in 1.0 M KCl solution to record the stripping current.

Results and discussion

Characterization of ssDNA@AuNPs

The UV-vis spectrum of AuNPs solution showed an adsorption peak at 519 nm (Fig. 1A, curve a), from which the diameter of 13 nm was obtained. The DLS measurement of AuNPs showed an average hydrodynamic diameter of 17 nm (Fig. 1B). After the AuNPs was functionalized with ssDNA, the UV-vis spectrum of formed ssDNA@AuNPs showed a red shift of the absorption peak to 531 nm and a new absorption at approximately 260 nm (Fig.1A, curve b) due to the DNA strand on AuNPs surface (Fig. 1A, curve c), while the hydrodynamic diameter of increased to 29 nm (Fig. 1B), suggesting the successful formation of DNA functionalized AuNPs. The DLS results were in good agreement with previous work,[39] confirming the good dispersity of ssDNA@AuNPs in aqueous media.

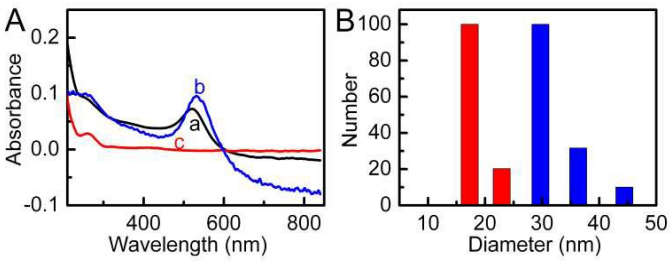


Fig. 1 (A) UV-vis absorption spectra (a) AuNPs, (b) ssDNA@AuNPs, and (c) ssDNA, and (B) hydrodynamic diameters of AuNPs (red) and ssDNA@AuNPs (blue).

Characterization of immunosensor

SEM was used to characterize the surface morphologies of immunosensor before and after proximity ligation. Before coating CS on the working electrode, the bare SPCE showed a rough surface (Fig. 2A). After the SPCE was coated with CS and then GO, the GO could be observed on the immunosenseor surface (Fig. 2B), implying that the CS and GO were successfully immobilized on the working electrode. Afterward, the coating of ssDNA@AuNPs on the electrode brought a lot of AuNPs on the surface (Fig. 2C), indicating the assembly of ssDNA@AuNPs on GO via the strong interaction. After the proximity ligation of two DNA labeled antibodies with target CEA and the hybridization of the formed complex with ssDNA, the number of ssDNA@AuNPs on the surface was obviously decreased (Fig. 2D). This appearance demonstrated the successful formation of the DNA2-Ab2/target CEA/DNA1-Ab1 complex and its hybridization with ssDNA@AuNPs to weaken the interaction between DNA and GO, which led to the release of AuNPs from immunosensor surface.

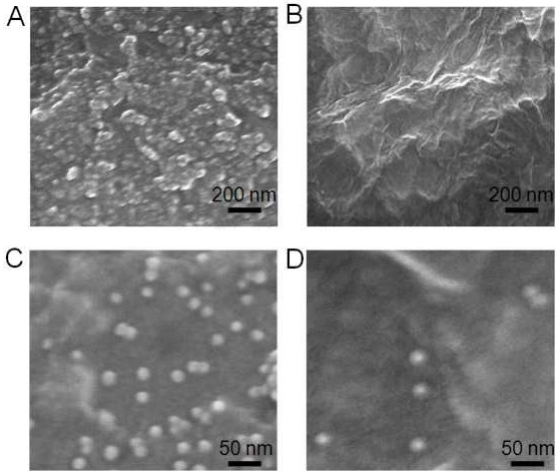


Fig. 2 SEM images of (A) bare SPCE, (B) GO modified SPCE, (C,D) ssDNA@AuNPs on GO modified SPCE as the immunosensor before (C) and after (D) incubation with the mixture of 250 nM Ab1-antibody, 250 nM Ab2-antiboday and 100 ng/mL CEA.

Optimization of immunosensor preparation and detection conditions

To obtain the excellent analytical performance, the conditions for immunosensor preparation were firstly optimized. Due to the low electric conduction of GO, high concentration of GO would increase the resistance of the immunosensor, thus the DPV stripping current of deposited AgNPs decreased (Fig. 3A). Contrarily, too low concentration of GO could not meet the saturated coverage of ssDNA@AuNPs, which also led to low stripping current. The optimized concentration of GO was 1.0 mg/mL. On this modified electrode, the stripping current increased with the increasing incubation time of ssDNA@AuNPs and trended to constant values after an incubation time of 40 min (Fig. 3B), which was used for preparation of the immunosensor.

The time for proximity ligation of the two DNA labeled antibodies with target protein and hybridization of the ligation product with the immobilized ssDNA@AuNPs played an important role in the immunoassay. The stripping current quickly decreased with the increasing reaction time, indicating the increasing release of AuNPs from electrode surface (Fig. 3C). The release reached a steady state at about 40 min. Thus, the whole reaction time for proximity ligation and hybridization was selected as 40 min.

In the proposed immunoassay, silver deposition was a key step. The deposition time was optimized with the ratio of signal to noise (inset in Fig. 3D). Here the noise was the stripping current of AgNPs

deposited on GO modified SPCE with the same silver deposition step. When the stripping current of the immunosensor increased with the increasing deposition time, the noise also increased (Fig. 3D), and the maximum ratio of signal to noise occurred at a deposition time of 4 min. Thus, 4 min was adopted as the optimal silver deposition time in this work.

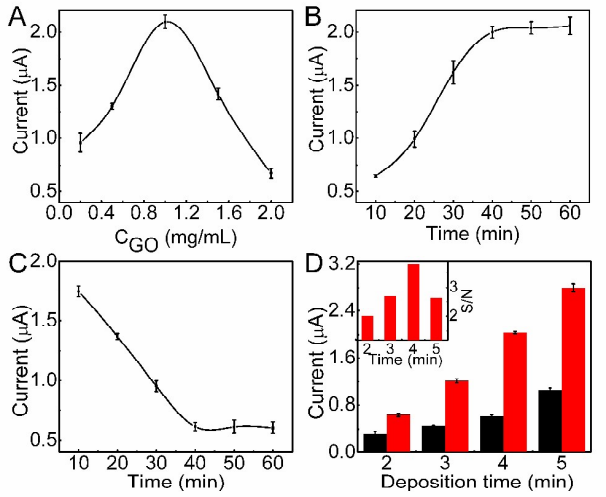


Fig. 3. Effects of (A) GO concentration and (B) incubation time of ssDNA@AuNPs for immunosensor preparation, (C) incubation time of 250 nM Ab1-antibody, 250 nM Ab2-antiboday and 100 ng/mL CEA, and (D) silver deposition time on stripping current of AgNPs in 1.0 M KCl. Inset in D: ratio of signal to noise at different times (n = 3 for error bars).

Analytical performance of immunosensing method

Under optimal conditions, the DPV response decreased proportionally with the increasing concentration of CEA (Fig. 4A). The calibration plot showed a good linear relationship between the DPV peak current and the logarithm value of CEA concentration in the range of 0.01 to 100 ng/mL with a correlation of 0.9965 (Fig. 4B). The linear regression equation was I (μA) = -0.3549 log [C] (ng/mL) + 1.3549. The detection limit corresponding to a signal-to-noise ratio of 3 was 3.9 pg/mL. Such a detection limit was lower than 10 pg/mL[40,41] and 5 pg/mL[31] with other signal amplification strategies.

The non-specific binding characteristic of the proposed immunoassay was evaluated by comparing the current responses toward solutions containing either CEA or other antigen only, for example prostate-specific antigen (PSA), or a mixture of CEA and PSA. The DPV responses in the absence of both target CEA and PSA and in the presence of only 100 ng/mL PSA were 2.28 μA and 2.05 μA, respectively, while the DPV response to 100 ng/mL CEA or the mixture of 100 ng/mL PSA and 100 ng/mL CEA was 0.641

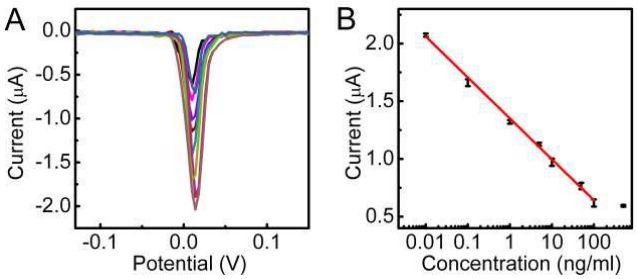


Fig. 4 (A) DPV responses to 0, 10^{-2}, 10^{-1}, 1, 5, 10, 50, 100 and 500 ng/mL CEA (from high to low) and (B) calibration curve of the proposed method for CEA detection.

and 0.638 μA. As expected, the immunoassay method showed obvious response to target CEA, and negligible response to PSA was observed, indicating little non-specific binding of non-specific antigen. Both the intra-assay and interassay precisions of the immunosensor were examined with 1 ng/mL CEA. The relative standard deviations (RSD) for five measurements were 4.3% and 5.8%, respectively, showing good precision and acceptable fabrication reproducibility.

Real sample analysis

To assess the application of the proposed method in complex biological systems, the analysis of CEA in clinical serum samples was carried out. The assay results in five clinical serum samples were in good agreement with the reference values from the commercial electrochemiluminescent single-analyte test as shown in Table 1. The relative errors less than 9.46% indicated this method possessed good reliability, promising a powerful protocol for point-of-care analysis.

Table 1. Assay results of CEA in serum samples using the proposed and reference methods.

Samples	Proposed method (ng/mL)	Reference method (ng/mL)	Relative error (%)
1	0.72	0.70	1.7
2	1.43	1.48	-3.4
3	4.56	4.88	-6.6
4	19.2	17.55	9.7
5	90.7	95.8	-5.3

Conclusions

A simple electrochemical immunoassay method is proposed by a designed proximity hybridization regulated deposition of AgNPs and followed electrochemical stripping on a disposable immunosensor. The immunosensor can conveniently be prepared by casting ssDNA@AuNPs on a graphene oxide modified SPCE. The homogeneous proximity ligation and the hybridization of the product with the immobilized ssDNA can be completed in single step. The AuNPs catalyzed silver deposition improves the analytical sensitivity. Using CEA as a model target, the proposed assay shows a wide detection, high sensitivity, convenient operability, and acceptable stability and accuracy. This methodology can conveniently be extended to detect a wide range of analytes with available affinity ligands to form the proximate complex and thus provides a great promise in clinical application.

Acknowledgements

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