

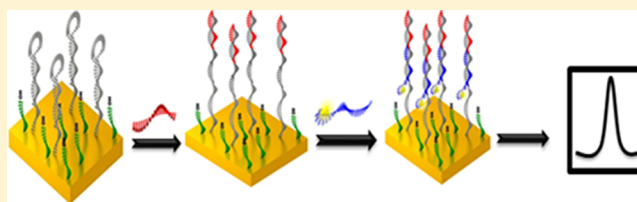
Trace and Label-Free MicroRNA Detection Using Oligonucleotide Encapsulated Silver Nanoclusters as Probes

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ABSTRACT: A simple, sensitive, and label-free method for microRNA (miRNA) biosensing was described using oligonucleotide encapsulated silver nanoclusters (Ag-NCs) as effective electrochemical probes. The functional oligonucleotide probe integrates both recognition sequence for hybridization and template sequence for in situ synthesis of Ag-NCs, which appears to possess exceptional metal mimic enzyme properties for catalyzing H_2O_2 reduction. The miRNA assay employs gold electrodes to immobilize the molecular beacon (MB) probe. After the MB probe subsequently hybridizes with the target and functional probe, the oligonucleotide encapsulated Ag-NCs are brought to the electrode surface and produce a detection signal, in response to H_2O_2 reduction. An electrochemical miRNA biosensor down to 67 fM with a linear range of 5 orders of magnitude was obtained. Meanwhile, the MB probe allows the biosensor to detect the target with high selectivity. The Ag-NCs-based approach provides a novel avenue to detect miRNA with high sensitivity and selectivity while avoiding laborious label and signal amplification. It is convinced that rational introduction of signal amplification strategy to the Ag-NCs-based bioanalysis can further improve the sensitivity. To our best knowledge, this is the first application of the electrocatalytic activity of Ag-NCs in bioanalysis, which would be attractive for genetic analysis and clinic biomedical application.



MicroRNA (miRNA) encoding in the genomes of different species is a class of small endogenous noncoding RNAs (~22 nucleotides).^{1–3} It plays a significant regulatory role at the post-transcriptional level in a diverse range of biological processes^{4–6} via an RNA-induced silencing protein complex, which can partially or completely complement the 3'-untranslated region of target mRNAs to mediate mRNA cleavage or prevent protein synthesis.^{7–9} Their expression is temporally regulated and specific to cells and tissues. Recently, it has been found that alternative expression patterns of miRNA is associated with a variety of cancers,^{10,11} and miRNA is serving as a class of clinically significant diagnostic and prognostic markers, and it can be used as a useful tool in basic biomedical research.^{12–14} Current standard methodologies (including real-time reverse transcription polymerase chain reaction, Northern blotting, and miRNA arrays) are laborious, semiquantitative, and require expensive equipment or radioactive/toxic labels.^{15–17} Therefore, the establishment of a novel, reliable, simple, and sensitive miRNA profile appears significant and is an urgent need in miRNA exploration.

Nanomaterials provide a promising sensing platform, because of the high surface areas for improved mass transport, the high loading of receptor molecules for synergistic amplification of the target response, and unique optical, electronic, and catalytic properties for translating of the biorecognition events to an electrochemical or spectroscopic response.^{18–20} The nanoparticle is emerging as an increasing role in biosensing. By coupling the biorecognition events with the metal nanoparticle

labels, the amplified detection of biorecognition events can be fulfilled through the conductivity measurements of the deposited metals,²¹ stripping voltammetric analysis of the dissolving metal ion,²² and direct detection of the redox signal due to the mimic enzyme property of the metal nanoparticles.²³ The use of metal nanoparticles as a catalyst is analogous to the way of enzymes and might overcome some of the problems related to the inherent thermal and environmental instability of the enzyme.

Molecular quantum clusters, possessing sizes below 2 nm and typically consisting of several to some tens of atoms, are a fascinating area of contemporary interest in nanomaterials.^{24,25} The size-dependent discrete energy levels of nanocluster lead to "molecular" properties such as strong fluorescence and catalytic activity.^{26,27} With regard to their luminescence, the clusters represent a new class of exceptional fluorophores for bioimaging labeling^{28,29} and chemical/biological detection.^{30,31} However, little effort has been dedicated to the application of catalytic activity of metal nanoclusters in bioanalysis.³²

Here, a simple, sensitive, selective, and label-free electrochemical biosensing strategy for detection of miRNA was designed by using oligonucleotide encapsulated silver nanoclusters (Ag-NCs) as effective electrochemical label. As shown

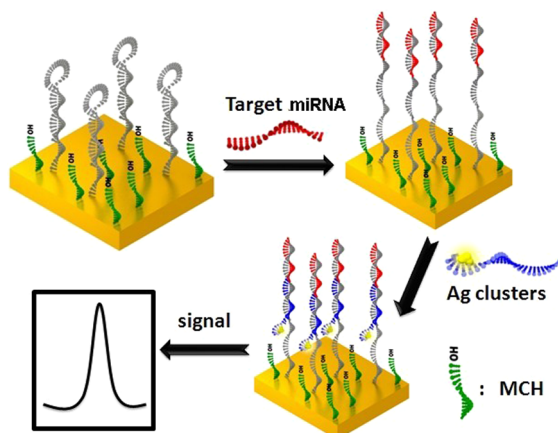
Received: July 4, 2012

Accepted: September 17, 2012

Published: September 17, 2012

in Scheme 1, the design of functional oligonucleotide probe combined the recognition sequence for hybridization with

Scheme 1. Illustration of Electrochemical Detection of MiRNA Using Oligonucleotide Encapsulated Ag-NCs



template sequence for in situ synthesis of Ag-NCs,³³ which displayed efficient catalytic property toward H_2O_2 reduction. The subsequent hybridization between molecular beacon (MB) probe immobilized on gold electrode (GE) with target and functional probe brought the oligonucleotide encapsulated Ag-NCs to the electrode surface. The high catalytic capability of Ag-NCs toward H_2O_2 reduction resulted in a sensitive and label-free electrochemical miRNA biosensor, while the inherent selectivity of the MB³⁴ probe endows the biosensor with high base discrimination ability. To the best of our knowledge, this work demonstrated for the first time the application of the electrocatalytic activity of Ag-NCs in bioanalysis, which provides a promising platform for genetic target analysis and clinic biomedical application.

EXPERIMENTAL SECTION

Materials and Reagents. 6-Mercapto-1-hexanol (MCH), tris (carboxy-ethyl)phosphine (TCEP), and citric acid were purchased from Sigma (St. Louis, MO). Sodium borohydride (NaBH_4) and silver nitrate (AgNO_3) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Hybridization buffer (HB) was 100 mM pH 7.0 phosphate buffered saline (PBS), which was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 (0.2 M) and adjusting the pH with NaOH (0.1 M) and H_3PO_4 (0.1M). 0.05% Tween-20 was spiked into (PBS) as wash buffer (PBST) to minimize unspecific adsorption. Store buffer (SB) was tris-(hydroxymethyl)aminomethane (Tris)-HCl (10 mM, pH 8.0) containing ethylenediaminetetraacetic acid (EDTA) (1 mM). Ultrapure water obtained from a Millipore water purification system ($\geq 18 \text{ M}\Omega$, Milli-Q, Millipore) was used in all runs. All other reagents were of analytical grade and used as received. The oligonucleotides with the following sequences were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China) and purified using high-performance liquid chromatography. Their sequences were as follows:

primer MB probe:

5'SH TTT TTT TTT TTT ACT GTC TTA GCA CGC
CAA TAT TTA CGT GCT GCT AA-3'

functional oligonucleotide probe:

5'TGC TAA GAC AGT CCC TAA CTC CCC 3'

All the RNA sequences were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, PRC) and purified using high-performance liquid chromatography, which were listed as follows:

target:

5'UAGCAGCACGUA~~AA~~AUAUUGGCG 3'

1-base mismatch:

5'UAGCAGCACGUA~~AA~~AUAUUG~~C~~CG3'

3-base mismatch:

5'UAA~~CAG~~CACGUA~~AA~~AUAUUG~~C~~CG3'

Instruments. The morphologies of Ag-NCs were examined with multimode three-dimensional (3D) atomic force microscopy (AFM) (Bruker, USA) and transmission electron microscopy (TEM) (using a JEM 2100 TEM microscope). AFM measurement was carried out under tapping mode. A droplet of Ag-NCs dispersion was dropped onto a freshly cleaved mica surface, and the sample was kept at room temperature to allow water evaporation for obtaining the sample. The image was obtained at room temperature (25 °C) with a humidity of 30%. The UV–visible (UV-vis) absorption and X-ray-photoelectron spectroscopy (XPS) analyses were recorded with an UV-1800 spectrophotometer (Shimadzu, Japan) and an ESCALAB 250 spectrometer (Thermo-VG Scientific, USA), respectively. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed with a CHI 660B electrochemical analyzer (Co. CHI, TX). All measurements were conducted on a standard cell with a Ag/AgCl reference electrode, a platinum wire as counter electrode and a bare or Ag-NCs modified Au electrode (diameter = 2.5 mm) as working electrode.

Synthesis of Ag-NCs. Ag-NCs were synthesized in accordance with the previous literature.³⁵ Briefly, 50 μL of template strand (100 μM) and 0.8 μL of AgNO_3 solution (50 mM) were added to 50 μL of citrate buffer (20 mM, pH 7.0), with a Ag^+ /oligonucleotide relative concentration ratio of 8:1. Then, 0.8 μL of fresh NaBH_4 solution (25 mM) was added to the solution under ice temperature, and the resulting solution was vigorously shaken for 1 min. The oligonucleotide encapsulated Ag-NCs were obtained by keeping the solution overnight in darkness at 4 °C.

Preparation of MiRNA Biosensor. The Au electrode was first polished with 0.3 μm alumina, sonicated in water, and then electrochemically cleaned with a series of oxidation and reduction cycle in the solution (0.01 M KCl, 0.05 M H_2SO_4) before modification. The clean electrode then was dried with nitrogen, 5 μL of 50 nM primer MB probe solution containing 2 μM TCEP in the PBS (100 mM, pH 7.0) was deposited dropwise onto the clean gold surface, and the electrode was kept at room temperature for 16 h, to immobilize the MB probe. The electrode then was washed thoroughly with PBST to remove nonspecifically bound oligonucleotides. The resulted Au electrode was immersed in a 1 mM MCH for 1 h to block

the surface. The surface was then rinsed with PBST, and 5 μL of different concentrations of target MIRNA was dropped to the surface, hybridized at 37 $^{\circ}\text{C}$ for 30 min. Afterward, the electrode was rinsed with PBST again and incubated with 5 μL solution of oligonucleotide encapsulated Ag-NCs (50 nM) at 25 $^{\circ}\text{C}$ in darkness for 30 min. The obtained electrode was rinsed with PBST thoroughly and used to measure the electrochemical response in PBS (100 mM, pH 7.0) containing H_2O_2 (3 mM).

RESULTS AND DISCUSSION

Characterization of Ag-NCs. To obtain the desired size and size distribution, the growth of the nanocluster is often encapsulated using a capping agent. Silver nanoclusters are typically synthesized using single sequence DNA (ssDNA) as a template and NaBH_4 as the reducing agent for reduction of Ag cation.³³ The ssDNA provides preformed templates and stops the nanoclusters from growing once a desired size is reached.³⁶ The properties of Ag-NCs strongly depend on the particles size, stabilizer, surrounding medium, and aggregation state.^{37–40} Therefore, the obtained Ag-NCs were first characterized. The morphology of the prepared Ag-NCs was investigated by AFM and TEM, which provided the direct evidence of the formation of Ag-NCs (Figure 1). As shown in Figure 1A, the typical AFM

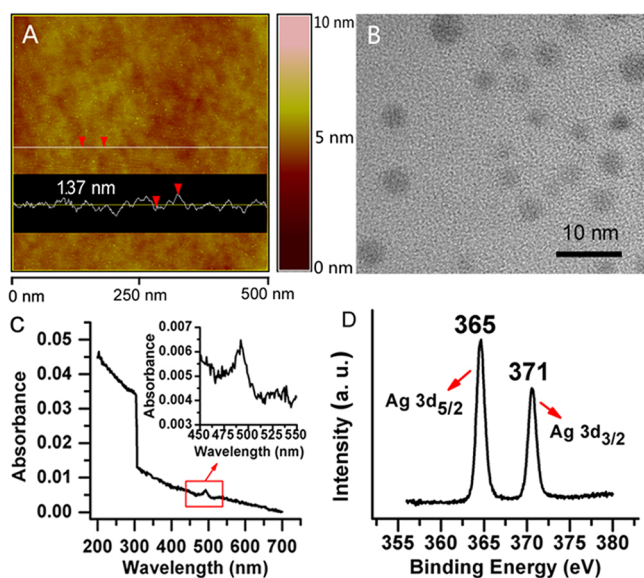


Figure 1. Characterization of Ag-NCs: (A) AFM image, (B) TEM image, (C) UV-vis spectrum, and (D) XPS spectrum of Ag-NCs.

image displays both the height and cross-section analysis of Ag-NCs. The average undulate height is 1.37 nm with a narrow size-distributed, agreeing with some previous reports.^{33,36} The TEM image clearly exhibits that the Ag-NCs are spherical and the average diameter was approximate 2 nm (Figure 1B). It can be also easily found that a narrow size distribution of Ag-NCs was obtained, which is useful for bioanalysis. The UV-vis spectrum and X-ray photoelectron spectroscopy (XPS) of Ag-NCs were further employed to prove the successful synthesis of the Ag-NCs. The UV-vis spectrum of the Ag clusters shows a strong absorption band at 492 nm (Figure 1C), which is diverse from the absorption of the Ag nanoparticle with strong absorption band at 400 nm^{41,42} and indicates the formation of the Ag-NCs. As shown in Figure 1D, the XPS in the Ag 3d region exhibited two strong absorption peaks at 365 and 371

eV associated with Ag 3d_{5/2} and Ag 3d_{3/2}, respectively, which indicated the formation of metallic silver.^{35,43}

Catalytic Property of Ag-NCs. It is well-known that some noble nanoparticles, such as Pt and Ag nanoparticles, possess mimic enzyme properties to catalyze H_2O_2 reduction, which have been widely used in bioanalysis.^{23,44} In principle, the Ag-NCs with the more-active surface, compared to the silver nanoparticles, also have the capability to catalyze the reduction of oxidation of H_2O_2 , which inspires us to explore the catalytic property of the Ag-NCs for bioanalysis. As a proof of concept, the catalytic capability to reduce H_2O_2 by Ag-NCs was first investigated. As shown in Figure 2, compared to the bare Au

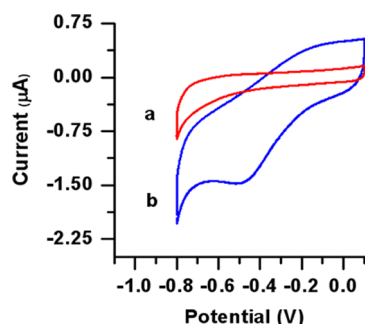


Figure 2. Cyclic voltammetry (CV) responses of Au electrode (curve a) and Ag-NCs modified Au electrode (curve b) to H_2O_2 (3 mM) in N_2 -saturated PBS (100 mM, pH 7.0).

electrode exhibiting negligible catalytic response to 3 mM H_2O_2 in N_2 -saturated PBS (curve a), the Ag-NCs modified Au electrode displayed obvious catalytic current toward the reduction of H_2O_2 at -0.49 V under the same condition (curve b). The reduction of H_2O_2 occurred in the potential of -0.8 – 0.1 V, which indicated that the Ag-NCs had high fidelity in catalyzing the reduction of H_2O_2 and had promising potential in bioanalysis.

Optimization of Biosensing Conditions. The concentrations of the primary MB probe anchored on the electrode surface and the Ag-NCs used for catalyzing H_2O_2 reduction intensely influence the response. The performance of the biosensors prepared with 5 μL of primary MB probe at different concentrations after hybridization with 1 nM target miRNA and 50 nM functional oligonucleotide probe is shown in Figure 3A. The current increased along with the increase of the concentration of primary MB probe from 1 to 50 nM, and leveled off at higher concentrations. Thus, 5 μL of 50 nM MB probe was selected for all subsequent work. Similarly, the effect

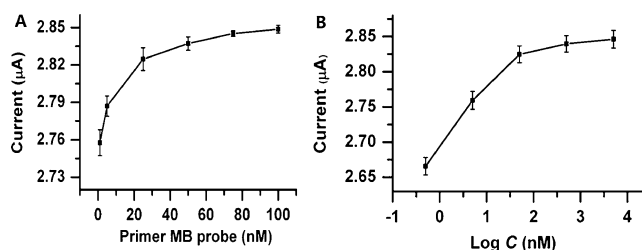


Figure 3. Influences of the concentrations of (A) MB probe used for preparation of modified Au electrode at 1 nM target miRNA and 50 nM Ag-NCs and (B) Ag-NCs used for detection at 50 nM MB probe and 1 nM target miRNA on the DPV response of H_2O_2 (3 mM) in N_2 -saturated PBS (100 mM, pH 7.0).

of the concentration of Ag-NCs on the response was investigated at 50 nM MB probe and 1 nM target miRNA concentration. As seen in Figure 3B, the current increased rapidly with the concentration of Ag-NCs increasing from 0.5 nM to 50 nM. At higher concentrations, the signal increased slowly due to the steric effect of oligonucleotide encapsulated Ag-NCs, which hindered the effective hybridization. Therefore, 50 nM of Ag-NCs was used in all subsequent work.

Electrochemical miRNA Assay. Under optimal conditions, the electrochemical response of the biosensor to H_2O_2 reduction was measured after subsequent hybridization of the MB probe with the target and functional oligonucleotide probe. As shown in Figure 4, the amplified electrochemical signal

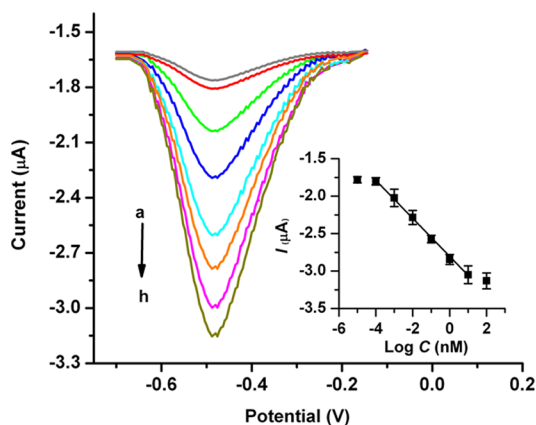


Figure 4. DPV curves of Ag-NCs modified Au electrodes in N_2 -saturated PBS (100 mM, pH 7.0) containing H_2O_2 (3 mM) at 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM target miRNA (from a to h). Inset: plot of the peak current versus the logarithm of target concentration.

increased with the increasing concentration of target used for hybridization. The plot of the current intensity versus the logarithm value of target concentration displayed a linear relationship in the range from 10 nM to 100 fM (Figure 4, inset), the limit of detection (LOD) was 67 fM at four times the standard deviation of the control (free of target miRNA). This LOD of 67 fM corresponds to 0.335 zmol of target in 5 μL of sample solution. The result was much lower than other metal mimic enzyme-based DNA detection system with a LOD of 10 pM,²³ a LOD of 6 pM,⁴⁵ and was also lower than Ag nanoparticle-based DNA detection system with a LOD of 14 nM.⁴⁶ This method also showed superior sensitivity than electrocatalytic nanoparticle tag-based miRNA detection.⁴⁴ The result indicated the excellent catalytic capability of Ag-NCs toward reduction of H_2O_2 . The rational introduction of signal amplification strategy to the Ag-NCs-based bioanalysis could further improve the LOD for ultrasensitive electrochemical detection of miRNA hybridization.

Specificity. The selectivity of the Ag-NCs-based miRNA biosensor was investigated by measuring the DPV response of the proposed biosensor to three types of miRNA sequences at the same concentration (100 pM), including complementary target, single-base mismatched strand, and three-base mismatched strand in the same condition. As shown in Figure 5, the proposed system displayed high fidelity in discriminating perfectly complementary target and the mismatched stands. The current of perfectly complementary target was 3.95 times of that for single-base mismatch sequence, and the response to

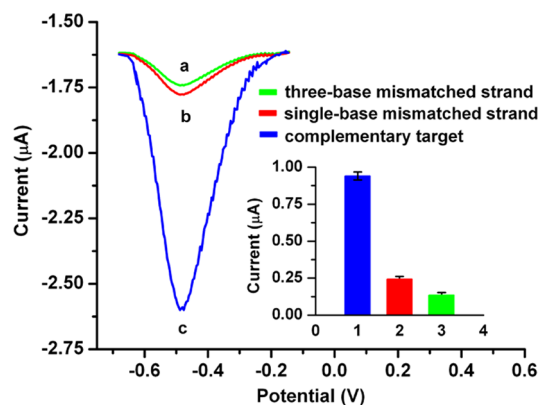


Figure 5. DPV curves of Ag-NCs modified Au electrodes in N_2 -saturated PBS (100 mM, pH 7.0) containing H_2O_2 (3 mM) for detection of 100 pM complementary target, single-base mismatched strand, and three-base mismatched strand. Inset shows the corresponding peak currents.

the three-base mismatch strand was only 14% of that for the perfectly complementary target (see Figure 5, inset). The high sequence specificity may be attributed to unique specificity of the MB in comparison with linear DNA probe.³⁴ These results suggested that the sensor had high sequence specificity, and this detection approach had potential application in single nucleotide polymorphism analysis.

CONCLUSIONS

In this work, a simple, sensitive, selective, and label-free miRNA biosensing strategy based on the efficient catalytic property of Ag-NCs toward H_2O_2 reduction was proposed. A functional oligonucleotide probe consisting of recognition sequence for hybridization and template sequence for in situ synthesis of Ag-NCs was designed. The resulting Ag-NCs with well-proportioned size were characterized by atomic force microscopy (AFM), transmission electron microscopy (TEM), ultraviolet–visible (UV-vis) analysis, and X-ray photoelectron spectroscopy (XPS). The high catalytic capability of Ag-NCs in response to H_2O_2 reduction resulted in a sensitive electrochemical miRNA biosensor without any laborious label and signal amplification, while the inherent selectivity of the MB probe endows the biosensor with high base discrimination ability. It is convinced that the sensitivity can be further sharply improved by simple rational introduction of signal amplification strategy to the Ag-NCs-based bioanalysis. The proposed approach provides a promising detection platform for genetic analysis and clinic biomedical applications.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded by China Postdoctoral Science Foundation (No. 11175012), the Chinese Central Universities Funds (NO. 06199017, NO. 06108037, 06199019, 06108101), the National Natural Science Foundation of China (Grant Nos.

21127007 and 21103009), Beijing Natural Science Foundation (Grant No. 2122038)

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