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A highly specific and sensitive electroanalysis strategy for microRNAs based on the amplified silver deposition by the synergic TiO₂ photocatalysis and guanine photoreduction using charge-neutral probes

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TiO₂ photocatalysis and guanine photoreduction were synergically combined for amplifying silver deposition for the electroanalysis of short-chain microRNAs with guanine bases using charge-neutral probes. It could allow for the highly specific and sensitive detection of microRNAs in blood as well as the identification of their mutant levels.

MicroRNAs (miRNAs) are non-coding short-chain RNAs with approximately 18-25 nucleotides in length.^{1,2} They are useful diagnostic and prognostic markers for many basic biomedical researches and evaluations.³⁻⁵ In particular, the expressing levels of miRNAs in peripheral blood have been well established to be the sensitive biomarkers for the cancer diagnostics and metastasis.³⁻⁵ The quantification of free miRNAs in blood, however, can be challenged by many factors like the short chain length and low expression level of miRNAs.⁶ For example, the common sandwiched detection of short-chain miRNAs of low melting temperature might in a way risk the unwinding of hybridized sequences. Therefore, exploring highly sensitive analysis strategies and suitably efficient detection modes for the detection of short-chain miRNA targets is of great interest.

In recent decades, many modern analytical methods have been established to quantify or identify miRNA levels, most known as the northern blotting,⁷ electrochemical biosensing,⁸ reverse transcription polymerase chain reaction,⁹ and surface enhanced Raman spectroscopy.¹⁰ Among these classic detection methods, the electrochemical biosensors have been recognized as particularly attractive alternatives with high analysis sensitivity, low detection limit, fast response time, cost effectiveness, and ease of automatization.¹¹ For example, Dong and co-workers described a label-free electrochemical

biosensor for the detection of miRNAs with the limit detection down to 67 fM.¹² Gao's group have reported the electrochemical analysis of miRNAs using some special catalyst labels of metal oxides.^{8,13,14} A sandwiched electroanalysis protocol has also been developed for the electroanalysis of low-level miRNAs in blood based on the silver deposition catalyzed by gold nanoclusters-incorporated enzyme.¹ Noticeably, the catalytic silver deposition procedure has been widely applied as a powerful signal amplification tool,^{1,15,16} nevertheless, a common challenge can be mostly encountered regarding the non-specific adsorption of positively charged silver ions onto the oppositely charged phosphodiester backbones probes of nucleic acids. Alternatively, many efforts have been devoted to the use of some nonionic nucleic acid analogs, such as peptide nucleic acids (PNAs)¹⁷ and morpholinos,¹⁸ in the solid-phase assays for nucleic acids. Especially, PNAs with charge-neutral pseudopeptide backbones could serve as outstanding probe candidates for the specific molecular recognition of nucleic acid targets in the biosensor designs.¹⁹

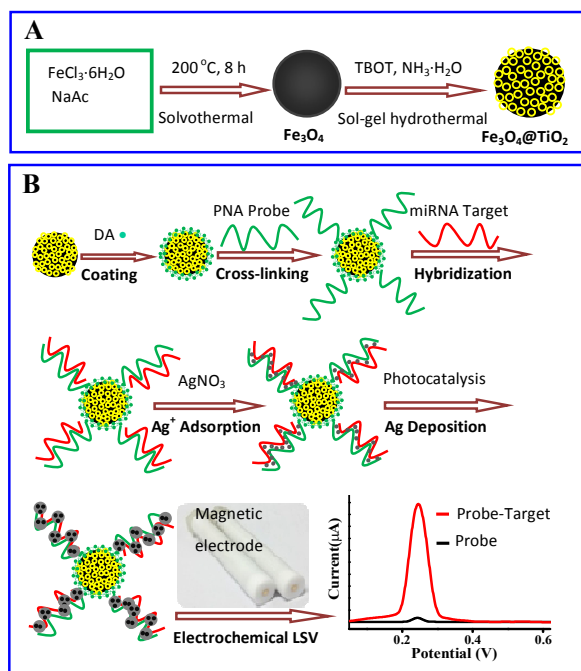
Moreover, titanium dioxide (TiO₂), a photocatalyst with excellent chemical stability, low toxicity, low cost, and redox properties, has been extensively applied for solar cells and photocatalytic cleanup of toxic organics in wastewater.²⁰⁻²² In 1997, Dunford and colleagues demonstrated that TiO₂ particles could catalyze the DNA damage in vitro or in cells.²³ Also, Warner et al. found that guanine, an easily oxidized DNA base, could play the vital role in the photo-oxidative damage of DNA oligonucleotides physisorbed on TiO₂ particles.²⁴ More importantly, Rajh and co-workers discovered an efficient light-induced crosstalk across the TiO₂ interface and DNA oligonucleotides with guanine bases for the photocatalytic deposition of metallic silver.^{25,26}

Inspired by the these pioneering works above, in the present work, a synergic combination of TiO₂ photocatalysis and guanine photoreduction has been proposed for the first time to amplify the silver deposition towards a highly sensitive and selective analysis method tailored for probing guanine-containing miRNAs of hsa-let-7a in blood using charge-neutral PNA probes. Here, the miRNA sequences from the hsa-let-7

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Scheme 1. Schematic illustration of (A) the synthesis procedure of magnetic $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites, of which Fe_3O_4 carriers were prepared via the solvothermal way and further shelled by TiO_2 layers through the hydrothermal method, and (B) the main detection procedure and principle for targeting miRNAs containing guanine bases using the $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$, including DA coating, PNA probe cross-linking, target miRNA hybridization, Ag^+ adsorption, photocatalytic silver deposition, and electrochemical signal output with magnetic electrodes.

family, of which the aberrant expression levels can be associated with the diagnostics and prognosis of cancers. The main detection principle and procedure are schematically illustrated in **Scheme 1**. As is shown in **Scheme 1A**, magnetic hollow Fe_3O_4 were first synthesized by a modified solvothermal method,²⁷ and then coated with TiO_2 shells by using a modified sol-gel method,²⁸ resulting in $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites of magnetic separation and photocatalysis functions. Scanning electron microscope (SEM) imaging was conducted to characterize the resulting Fe_3O_4 particles before and after TiO_2 coatings (**Fig. 1A**). It was found that Fe_3O_4 particles could display a uniformly defined spherical shape with the average size of about 500 nm. Furthermore, as shown in **Fig. 1B**, TiO_2 layers could be coated on Fe_3O_4 particles with the compact and lumpy structures, and the formed $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites showed an average size of about 570 nm. The crystalline structures of Fe_3O_4 spheres and $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites were further investigated using powder X-ray diffraction (XRD). One can note from **Fig. 1C** that the marked diffraction peaks of crystalline Fe_3O_4 can be obtained in good agreement with the well established data (JCPDS 75-1609). Importantly, the typical XRD pattern of crystallographic planes marked for anatase-phase TiO_2 could be additionally attained for $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites (**Fig. 1D**). Moreover, the as-prepared $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites were modified with dopamine (DA) to yield the $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$. Here, DA as an enediol ligand could be tightly anchored on

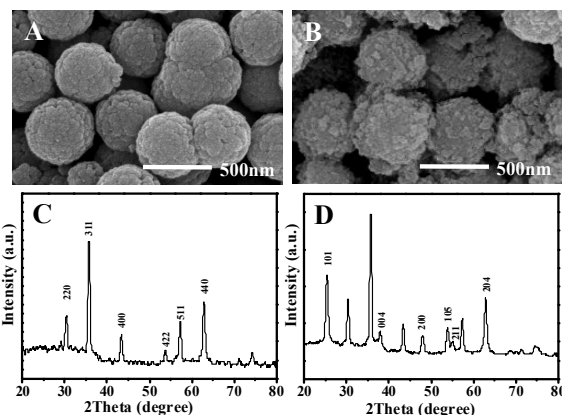


Figure 1. Representative SEM images of (A) Fe_3O_4 and (B) $\text{Fe}_3\text{O}_4@\text{TiO}_2$, corresponding to (C) and (D) XRD patterns with the labelled Fe_3O_4 and TiO_2 peaks, respectively.

the nanocrystalline TiO_2 nanoparticles to facilitate their tunable photocatalytic responses in the visible spectral region by adjusting the coordination geometry of Ti atoms on the TiO_2 surface.²⁹ And the amine groups-derivatized $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ could be further applied to covalently load the PNA probes for capturing guanine-containing miRNA targets in blood. The main detection procedure and principle are schematically illustrated in **Scheme 1B**. Herein, miRNA targets containing guanine bases were captured magnetically onto the PNA probe-modified $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$. After the introduction of Ag^+ ions, the silver deposition took place by the synergic photocatalysis of TiO_2 on $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ and photoreduction of guanine bases on the targeting miRNAs. The so amplified silver signals were electrochemically measured with the magnetic electrodes to expect the highly sensitive and selective electroanalysis of guanine-containing miRNAs in blood afterwards.

The analysis performances of silver deposition-based electroanalysis for miRNAs containing guanine bases were comparably investigated, of which the different detection conditions were schematically illustrated (**Fig. 2**). As shown in **Fig. 2A**, the electrochemical silver response is much larger in

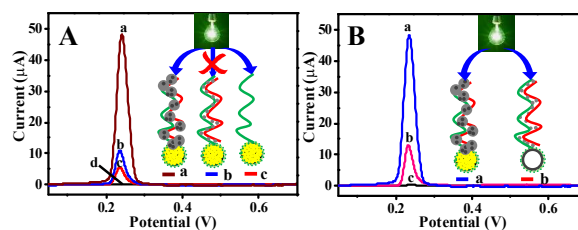


Figure 2. (A) Comparison of electrochemical responses to wild miRNAs (20 pM) captured by PNA probes-modified $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ after the catalytic silver depositions in the (a) presence and (b) absence of visible light, where the responses of the (c) PNA probes-modified $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ and (d) blank electrodes were provided as the controls, with the corresponding products schematically illustrated (inset). (B) Electrochemical responses to wild miRNAs (20 pM) captured by the magnetic probes (a) with and (b) without TiO_2 coatings after the photocatalytic silver depositions, taking (c) the blank electrode as the control, with the corresponding detection conditions schematically illustrated (inset).

the visible light (Curve a) than in the dark (Curve b), thus confirming that stronger photocatalytic silver deposition could be achieved under the visible light that would be chosen as the necessary condition for the detection of miRNA targets. Furthermore, the silver deposition signal could be neglected for the PNA probes alone, indicating that PNA probes as nonionic nucleic acid analogs could present no significant adsorption of Ag^+ ions (Curve c). Importantly, it demonstrates that the photocatalytic silver deposition might not occur in the absence of the targeting miRNAs with the photoreducing guanine bases. Moreover, **Fig. 2B** discloses that the $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ (Curve a) could exhibit much greater silver response than the $\text{Fe}_3\text{O}_4\text{-DA}$ without TiO_2 (Curve b), indicating that TiO_2 photocatalyst could play a vital role in the amplified silver deposition.

The $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ dosages were optimized for the electrochemical silver responses, showing that 1.0 mg mL^{-1} of $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ is enough (**Fig. S1A**, ESI†). Interestingly, too high concentrations of $\text{Fe}_3\text{O}_4@\text{TiO}_2\text{-DA}$ might lead to slightly decreased signals, presumably due to that the nonconductive nanocomposites could be overlaid on the electrode surfaces with too high density so as to conduct the negative effects on the electrochemical silver responses. Also, the influences of the Ag^+ amounts on the photocatalytic silver deposition were experimentally investigated, showing that the peak currents of silver responses could reach the constant at 1.0 mM AgNO_3 (**Fig. S1B**, ESI†). Furthermore, the time for the probe-target hybridization and photocatalytic silver deposition reactions was separately explored. As shown in **Fig. S1C**, (ESI†), the electrochemical silver responses could increasingly tend to be steady after 50 min, which was thus chosen as the hybridization reaction time. In addition, **Fig. S1D**, (ESI†) displays that the photocatalytic silver deposition reactions occurred very fast, with the saturate silver signals attained at about 5.0 min.

Under the optimized experimental conditions, the silver deposition-based electroanalysis method was employed to detect the wild and mutated miRNA samples (**Fig. 3**). **Fig. 3A** shows the electrochemical responses to wild miRNA targets of different concentrations in buffer. A linear current correlation to the logarithm of the miRNA concentrations was obtained across the concentration range from 2.0 fM to 2.0 nM ($R^2 = 0.9947$), with the detection limit of 1.3 fM , estimated according to the 3σ rule (**Fig. 3B**). Meantime, the developed electroanalysis method was applied to probe the levels of wild miRNAs spiked in blood samples with different concentrations (**Fig. 3C**). A linear detection relationship between the electrochemical responses and wild miRNA levels in blood was obtained over the miRNA concentrations ranging from 2.0 fM to 2.0 nM ($R^2 = 0.9833$), with the detection limit of about 1.6 fM . Moreover, the electrochemical responses of the silver deposition to the single-base and double-base mismatched miRNAs were examined by comparing to the wild miRNA targets (**Fig. 3D**). As expected, the single-base mismatched miRNAs (Curve b) showed much lower responses than the matched ones (Curve a), whereas the double-base mismatched

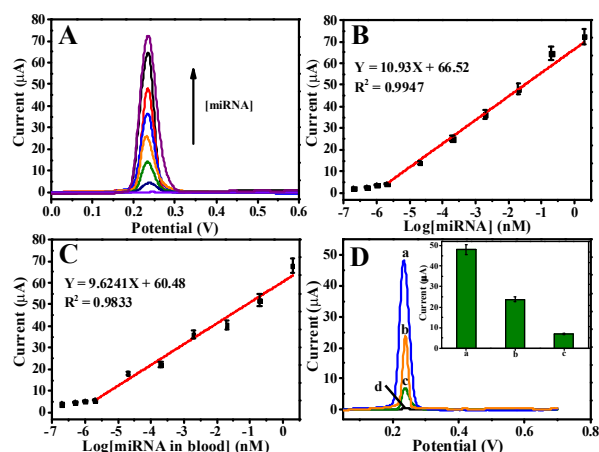


Figure 3. (A) Electrochemical responses to wild miRNA samples with different concentrations. The calibration curves describing the relationships between the current responses and different logarithmic concentrations of wild miRNA samples spiked in (B) buffer and (C) blood. (D) Comparable investigation of electrochemical responses of the developed electrodes to (a) matched, (b) single-base mismatched, and (c) double-base mismatched miRNAs (20 pM), corresponding to the current changes (inset), where (d) the response to the blank electrode is provided as the control.

targets could manifest the lowest responses (Curve c), as also illustrated in the inset of **Fig. 3D**. Accordingly, the as-developed electroanalysis method enables the discrimination of mutation levels of the single-base and double-base mismatched miRNAs. Herein, the mutations of single and double guanine bases of miRNAs might decrease the guanine-enabled photoreduction abilities for the silver deposition at different degrees. That is, the photoreduction of guanine bases of miRNAs is highly needed for the photocatalytic silver deposition aforementioned. In addition, the existence of mismatches in the miRNAs might additionally make an insulating barrier to prevent the hole migration to the guanine-guanine accepting sites, leading to a decrease in the TiO_2 photocatalysis for the silver deposition, as also demonstrated elsewhere.^{25, 26} Therefore, the developed silver deposition-based electroanalysis strategy could allow for the detection of wild miRNA targets in blood with high detection sensitivity and selectivity. Also, it can allow for the quantification of mutant levels of miRNA targets for profiling RNA expression pattern.

To summarize, in this work, a highly specific and sensitive electroanalysis methodology has been successfully developed for probing guanine-containing miRNA targets in blood based on the amplified silver deposition by the synergic combination of TiO_2 photocatalysis and guanine photoreduction using charge-neutral probes. This silver deposition-based electroanalysis method can possess some outstanding advantages over most of the traditional detection methods for short-chain miRNAs. First, the synergic combination of the photocatalysis of TiO_2 and the photoreduction of guanines of targeting miRNAs could achieve the dramatically amplified signal of silver deposition to promise the highly sensitive detection of miRNAs specifically containing guanine bases. Second, the coatings of DA on $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites could endow the shelled TiO_2 nanoparticles with tunable

photocatalytic responses in the visible light of great interest, in addition to the covalent loading of capture probes. Third, the use of charge-neutral PNAs might overcome any non-specific adsorption of silver ions to facilitate the highly selective detection of miRNAs. Forth, the introduction of magnetic carriers could allow for the convenient separation of miRNAs from the complicated blood samples without the tedious purification steps. Finally, the sensitive electrochemical output of silver deposition with magnetic electrodes could facilitate the sensitive detection of miRNAs. Therefore, the developed electroanalysis method could not only probe guanine-containing miRNAs in blood with high sensitivity and selectivity, but also identify their single-base and double-base mutation levels. Importantly, such an electroanalysis mode may be tailored for the short-chain nucleic acids containing reducing guanines. It holds great promise of wide applications in the clinical laboratory for the cancer diagnosis and the warning of cancer metastasis.

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