Rapid electrochemical detection of MiRNA-21 facilitated by the excellent catalytic ability of Pt@CeO₂ nanospheres†

Peiwu Chen,‡a Lan Jiang,‡a Xianjin Xie,‡a Dong Sun,‡b Jinyao Liu,‡a Yufeng Zhao,‡a Yuhao Li,§a Abel Ibrahim Balbín Tamayo,†c Baolin Liu,*d Yuqing Miao,*a and Ruizhuo Ouyang†d,*a

Pt@CeO₂ nanospheres (NSs) were first synthesized by simply mixing Ce(NO₃)₃ and K₂PtCl₄ under the protection of pure argon at 70 °C for 1 h, which exhibited excellent catalytic ability toward hydrogen peroxide (H₂O₂). An electrochemical biosensor was successfully developed using Pt@CeO₂ NSs as a capture probe for the ultra-sensitive and fast detection of miRNA-21, a new type of biomarker for disease diagnostics, especially for cancer. During the step-by-step construction process of the RNA sensor, Pt@CeO₂ NSs were functionalized with streptavidin (SA) to obtain SA-Pt@CeO₂ NSs through amide bonds. Gold nanoparticles (Au NPs) were electrodeposited on the surface of the glassy carbon electrode to improve the transmission capacity of electrons and provided Au atoms for fixing the thiolated capture probe (SH-CP) with a hairpin structure on the electrode via forming Au–S bonds. The target miRNA-21 specifically hybridized with SH-CP and opened the hairpin structure to form a rigid duplex so as to activate the biotin at the end of the capture probe. SA-Pt@CeO₂ NSs were thus specially attached to the electrode surface through the biotin-streptavidin affinity interaction, finally leading to the significant signal amplification. The ultra-sensitive and rapid detection of miRNA-21 was finally realized as expected benefiting from the excellent catalytic ability of Pt@CeO₂ NSs toward H₂O₂ in a wide linear concentration range from 10 fM to 1 nM with the detection limit as low as 1.41 fM. The results achieved with this new RNA sensor were quite satisfactory during the blood sample analysis.

1. Introduction

MicroRNAs (miRNAs), a type of endogenous non-coding short RNA molecules containing approximately 19–23 nucleotides, hinder protein translation or degrade messenger RNA to post-transcriptionally regulate gene expression in a variety of animals, plants, and viruses.¹ ² The abnormal expression of miRNAs has been proved to be particularly related to a variety of cancers, genetic diseases and immune inefficiencies.³ Therefore, miRNAs have gradually become a new type of clinical diagnosis and prognostic biomarker, and potential therapeutic target for new drug discovery.⁴ However, due to the unique characteristics of miRNAs including small size, low abundance, and similar sequences in homogenous families, the detection of miRNAs is still quite challenging.⁵ Conventional miRNA analysis methods like Northern blotting, microarray and RT-qPCR usually suffer from different inherent shortcomings, such as low sensitivity, false positive signals and expensive equipment.⁶–⁹ As a result, there is an urgent need for a variety of rapid, sensitive and reliable miRNA detection strategies to analyze miRNAs in vitro or in vivo.¹⁰ ¹¹

The inherent advantages of electrochemical methods like high sensitivity, simplicity, cost-effectiveness and good compatibility with micro-fabrication techniques have attracted widespread attention in nucleic acid analysis.¹² ¹³ In order to amplify the hybridization signal, various electrocatalytic enzyme labels and electroactive nanoparticles (NPs) have been utilized to develop electrochemical nucleic acid biosensors.¹⁴ Among these methods, the nanomaterial-based electrochemical signal amplification platform displays a broad application prospect owing to the large surface area, excellent electrical conductivity, good biocompatibility, high stability and advanced catalytic performance.¹⁵ For example, by using metal
ion-functionalized titanium phosphate NPs as a kind of tracer, a highly sensitive miRNA electrochemical biosensor was developed.\textsuperscript{16} The noble metal Pt NPs are the most effective mimic enzyme candidates for a variety of reactions\textsuperscript{17} and have been widely used to catalyze CO oxidation, oxygen reduction reactions (ORR) and hydrogen evolution reactions (HER) without causing damage to normal tissues during the development of nanomedicines.\textsuperscript{18–20} Moreover, compared with other electroactive metal NPs like Cu, Fe and Ni,\textsuperscript{21} Pt NPs often display significantly higher catalytic activity and more excellent electrical conductivity.

On the other hand, metal oxides have received a wide use in biosensors because of their low cost, good stability and particularly the distinctive electrocatalytic capabilities, which makes metal oxides excellent to produce redox electron pairs for creating electrochemical sensing platforms.\textsuperscript{22} For instance, cerium dioxide (CeO\textsubscript{2}) plays a vital role in fabricating biosensors due to its excellent catalytic performance, high surface oxygen mobility, good anti-inflammatory effect, favorable biocompatibility as well as high adsorption capacity. More importantly, the biological characteristics and unique catalytic performance of CeO\textsubscript{2} NPs result from their variable oxidation state and reversible conversion between Ce\textsuperscript{4+} and Ce\textsuperscript{3+}.\textsuperscript{23,24} However, the redox signal of CeO\textsubscript{2} NPs detected in the neutral and mild buffer solution is too weak to serve as a biosensing probe. The integration of Pt catalyst with CeO\textsubscript{2} NPs is regarded as a promising strategy and can reduce the formation energy of oxygen vacancies, thereby generating additional oxygen vacancies.\textsuperscript{25–27}

Therefore, Pt@CeO\textsubscript{2} NSs are proposed to exhibit enhanced catalytic performance and catalytic efficiency toward H\textsubscript{2}O\textsubscript{2} reduction.

Herein, a new electrochemical biosensor was developed for the highly sensitive detection of miRNA-21 benefiting from the powerful electrocatalysis of Pt@CeO\textsubscript{2} NSs toward H\textsubscript{2}O\textsubscript{2} reduction.\textsuperscript{28} Pt@CeO\textsubscript{2} NS were prepared as capture probes through mixing Ce(NO\textsubscript{3})\textsubscript{3} with K\textsubscript{2}PtCl\textsubscript{4} protected by pure Ar gas at 70 °C for 1 h, followed by immobilizing streptavidin (SA) through amide bond to obtain SA-Pt@CeO\textsubscript{2} NSs. The step-by-step construction process of the proposed RNA sensor was illustrated in Scheme 1. Briefly, the biotinylated probe SH-CP with a hairpin structure was first attached to the surface of glassy carbon electrode (GCE) covered by Au NPs through Au–S bonds. Biotin readily binds to biological macromolecules such as proteins and nucleic acids as marker, forming biotin derivatives that maintain the original biological activity of the macromolecules and bind the corresponding affinity molecules with four biotin binding sites.\textsuperscript{29} The subsequent addition of miRNA-21 would specifically hybridize the capture probe marked with biotin to open the hairpin structure and thus guarantee the specificity of the sensor. Finally, the SA-Pt@CeO\textsubscript{2} NSs bound to SH-CP through biotin-avidin affinity interaction. As predicted, Pt@CeO\textsubscript{2} NSs displayed extraordinary catalytic activity due their synergistic effect\textsuperscript{30} and generated significantly amplified electrochemical response to H\textsubscript{2}O\textsubscript{2}, which dramatically improved the sensitivity of the designed biosensor. The cumbersome modification process of the electrode could be simplified to a large

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{The schematic diagram of (A) the preparation of Pt@CeO\textsubscript{2} NSs and (B) the detection principle of the proposed Pt@CeO\textsubscript{2}-based electrochemical miRNA sensor.}
\end{figure}
extent and endow the biosensor with super high sensitivity owing to the excellent catalytic activity of Pt@CeO₂ NSs. Moreover, the hairpin capture probe gave the biosensor good ability to recognize base mismatches, increased the specificity of the biosensor, and thus provided a promising detection platform for clinical applications.

2. Experimental sections

2.1 Reagents and materials

Potassium tetrachloroplatinate (K₂PtCl₄) powder was purchased from Haoxi Nanotechnology Co., Ltd. (Shanghai, China). 6-Mercaptohexanol (MCH), tris(carboxyethyl)phosphine (TCEP), potassium chloride (KCl), hydrogen tetrachloroaurate trihydrate (HAuCl₄·3H₂O), diethyl pyrocarbonate (DEPC) water, potassium hexacyanoferrate-III (K₃[Fe(CN)₆]), potassium hexacyanoferrate-II (K₂[Fe(CN)₆]), hydrogen peroxide (H₂O₂), sodium sulfate (Na₂SO₄), ammonia solution (NH₄OH), cerium(III) nitrate hexa-, streptavidin (SA), N-puri- were of analytical grade and used as received without any further mide (EDC) and mercapto acetic acid (TGA) were all purchased (HAuCl₄).

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The detailed oligonucleotide sequences used in this work

Table 1

| Oligonucleotides | Sequence (from 5’ to 3’) |
|------------------|-------------------------|
| miRNA-21         | UAG CUU AUC AGA CUG AUG UUG A |
| SH-CP            | SH-AAAAAAAAATACACATCGAAGCTAGC | TAGG-biotin |
| S-RNA            | UAG CUU AUC GGA CUG AUG UUG A |
| T-RNA            | UUG CUU AUC GGA CUG AUG UUG A |
| N-RNA            | GUA AGG CAU CUG ACC GAA GGC A |
| miRNA-155        | UUUAGCGUAUUGGUGAUAGGGGGU |
3. Results and discussion

3.1 Preparation of the proposed Pt@CeO2-based RNA biosensor

As shown in Scheme 1, a layer of Au NPs was first electrodeposited on the surface of the bare GCE for the purpose to improve the transmission capacity of electrons and provide Au elements for forming Au-S bonds. Subsequently, the hairpin capture probe (SH-CP) labeled with biotin were immobilized on the surface of Au/GCE via the Au-S bonds. The unreacted Au sites on the electrode surface were sealed with MCH to avoid the non-specific adsorption. The hairpin structure capture was in a closed state without the target miRNA-21 which blocked the electrochemical tracer from interacting with the biotinylated end because of the large steric effect, as a result of SA-Pt@CeO2 not being immobilized on the electrode surface to catalyze H2O2. However, with the target present, the hairpin structure of the capture probe would be hydrolyzed and opened, forming a rigid duplex to activate the biotin at the end of the probe. Pt@CeO2 NSs was thus attached to the electrode surface through the affinity interaction between biotin and avidin. Under the strong catalytic ability of Pt@CeO2 NSs to reduce H2O2 to H2O, obvious redox electrochemical signal could be produced as predicted and the signal intensity increased with more miRNA-21 present. A preferred linear relationship was finally achieved within a certain concentration range to realize the qualitative and quantitative analysis of miRNA-21. The designed electrochemical miRNA sensor displayed preferred detection behaviour toward the target miRNA-21 due to the excellent catalytic performance of Pt@CeO2 as the electrochemical signal probe.

3.2 Characterizations of Pt@CeO2 NSs

A nanosphere shape of the prepared Pt@CeO2 NPs with uniform size and rough surface was clearly observed in the SEM image (Fig. 1a), which was confirmed by TEM images as well (Fig. 1b). The size of the Pt@CeO2 NSs was about 67 nm. The EDS map scanning of the Pt@CeO2 NSs shown in Fig. 1c–e revealed the uniform distribution of the three elements of O, Ce and Pt and the successful encapsulation of a large number of Pt NPs inside each nanosphere. Moreover, the presence of elemental peaks of O, Ce and Pt indicated the formation of Pt@CeO2 NSs (Fig. 2a).

The formation of Pt@CeO2 NSs was also verified by both XRD and XPS. The XRD results in Fig. 2b shows that the crystal planes (111), (220) and (222) of the Pt@CeO2 NSs appearing at 2θ of 28.55°, 47.48° and 59.09°, respectively, matched well the peaks in the standard diffraction card for CeO2 (JCPDS card no. 34-0394), and the (111) crystal planes at 38.76° perfectly matched the standard card for Pt (JCPDS card no. 04-0802). The above results verified the high purity and crystallinity of the synthesized Pt@CeO2 NSs. The presence of O 1s, Ce 3d and Pt 4f in the XPS full spectrum further confirmed the elemental chemical states and electronic structure of Pt@CeO2 NSs (Fig. 2c). In the fitted XPS spectrum (Fig. 2d), the four sets of peaks at 528.57, 529.86, 530.94 and 532.32 eV could be assigned to CeIV–O, CeIII–O, Pt–O and H–O bonds. In Fig. 2e, the fitted XPS spectrum of Ce 3d, the two peaks at 897.3 and 915.63 eV corresponded to CeIV 3d5/2 and CeIV 3d3/2, respectively, which were ascribed to CeO2. Meanwhile, the other two peaks at 881.47 and 900 eV could be assigned CeIII 3d5/2 and CeIII 3d3/2, respectively. The two peaks at 74.55 and 77.89 eV could be assigned to Pt 4f7/2 and Pt 4f5/2, respectively (Fig. 2f), which were generally attributed to PtIV. Moreover, the sub-bands at about 75.79 and 72.45 eV belonged to PtII 4f7/2 and PtII 4f5/2, respectively. These results proved the high crystal structure of Pt@CeO2 NSs with uniform morphology.

3.3 Electrochemical behavior of the prepared biosensing platform

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques could be used to qualitatively prove the successful construction of biosensors. In the solution of
5.0 mM $[\text{Fe(CN)}_6]^{3-}/4-$ containing 0.1 M KCl, CV was selected to investigate the surface properties of electrodes in different modification processes at a scan rate of 0.1 V s$^{-1}$ from 0.7 V to −0.2 V. Fig. 3a shows that a good reversible redox peak was clearly observed with the bare GCE (curve A). After Au NPs were electrodeposited on the electrode surface (curve B), the redox peak current increased significantly, indicating the good electrical conductivity and a larger specific surface area of Au/GCE. After the hairpin capture probe was subsequently fixed on the surface of Au/GCE, the peak current was found to greatly decrease (curve C) because of the mutual repulsion between the negatively charged phosphate group on the capture probe and $[\text{Fe(CN)}_6]^{3-}/4-$ which prevented $[\text{Fe(CN)}_6]^{3-}/4-$ from reaching the electrode surface. As a consequence, the peak current was reduced. Similarly, the sequential modification of MCH and miRNA-21 on the electrode surface further increased the steric hindrance of the electrode surface and accordingly hindered $[\text{Fe(CN)}_6]^{3-}/4-$ from reaching the electrode surface. The peak current was, therefore, continuously reduced (curves D and E). Clearly, the bare GCE showed a lower value of $R_p$ reflecting on a small semicircle (curve A). However, the layer of Au NPs on GCE decreased the semicircle diameter (curve B), indicating that the electron transfer over the electrode surface was promoted to a certain extent due to the good conductivity of Au NPs. While the diameter of the semicircle greatly increased after the SH-CP was fixed on the Au/GCE surface (curve C). The reason might be that the SH-CP hindered the electron exchange of the redox probe $[\text{Fe(CN)}_6]^{3-}/4-$ on the electrode surface, leading to the increase of $R_p$ value. Similarly, the presence of MCH and miRNA-21 on the electrode surface in sequence further enlarged the semicircle diameter of EIS (curves D and E). These results were indicative of the effective construction of the proposed sensor, which was in good consistence with the CV analysis.

3.4 The catalytic behavior of Pt@CeO$_2$ NSs

Signal amplification has been regarded as a very important strategy to improve the sensitivity of the electrochemical biosensors. In order of both Pt and CeO$_2$ were examined as well in 0.01 M pH 7.4 PBS containing 1 mM H$_2$O$_2$ for comparison. Fig. 3c shows that Pt exhibited lowest DPV peak current among the three signal probes, slightly lower than that of the signal probe CeO$_2$, while the highest peak current was generated using the Pt@CeO$_2$ NSs as signal probe. The greatly enhanced catalytic ability of Pt@CeO$_2$ NSs toward H$_2$O$_2$ reduction was thus verified by the much higher current response than that of Pt and CeO$_2$. Moreover, a sharp and strong paramagnetic signal was obviously seen at $g = 2.003$ from the EPR spectrum of...
Pt@CeO$_2$ NS in Fig. 3d, indicative of the appearance of oxygen vacancies as a result of doping Pt inside the lattice of CeO$_2$.

Therefore, the increasing oxygen vacancies might majorly contribute to the excellent catalytic ability of Pt@CeO$_2$ NSs.

3.5 Optimization of experimental conditions

Some experimental parameters used to construct a sensor will significantly affect the detection performance of the designed biosensor, which here included the concentration of the capture probe SH-CP, the incubation time of miRNA-21, the pH of PBS and the concentration of Pt@CeO$_2$ NSs. For the purpose to achieve the best detection performance, the above influencing factors need to be well optimized.

The concentrations of SH-CP influenced the DPV signal of the proposed sensor. As shown in Fig. 4a, the current response increased with more capture probe used in the concentration range from 0.1 to 1.0 $\mu$M, and reached the highest value at 1.0 $\mu$M. The higher the concentration of SH-CP was, the more the target chains could be captured until the concentration increased to 1.0 $\mu$M. Afterwards, the current decreased slightly owing to the steric hindrance on the electrode surface produced by the lower efficiency of DNA hybridization. Therefore, 1.0 $\mu$M SH-CP was selected for the subsequent experiments. The amount of the signal probe immobilized majorly depended on the reaction time between the target miRNA-21 and the capture probe. As seen in Fig. 4b, longer incubation time of miRNA-21 led to higher current signal. When the reaction lasted 120 min, the current tended to be saturated without changing significantly afterwards. Therefore, miRNA-21 was incubated with the capture probe for 120 min during the fabrication of the sensor. At the same time, the increasing pH value was found to be able to strengthen the current signal which reached the top at pH 7.4 and sharply decreased at higher pH values (Fig. 4c). All subsequent experiments would be carried out in pH 7.4 PBS.

Furthermore, the current response gradually rose up with more Pt@CeO$_2$ NSs applied and kept constant above 2.0 mM (Fig. 4d) which was used as the optimal concentration to construct the miRNA-21 biosensor.

3.6 Detection of miRNA-21

The DPV measurements of miRNA-21 at various concentrations were carried out under the optimal experimental conditions in the presence of 1 mM H$_2$O$_2$. As expected, the response current was found to proportionally go higher with the increasing miRNA-21 concentration from 10 fM to 1 nM (Fig. 5a), where a preferable linear relationship was achieved between response current $I$ and the logarithm of miRNA-21 concentration (lg $c$) with a linear equation fitted as $I = 8.765 \text{ lg } c + 152.314$ ($R^2 = 0.997$) shown in Fig. 5b. The limit of detection (LOD) was calculated to be 1.41 fM for miRNA-21 analysis at a signal-to-noise ratio of 3 (S/N) (see the calculation details in the ESI†). Most importantly, the detection limit was much lower than that of some previously reported biosensors listed in Table 2. A wider detection range and ultra-low detection limit were thus achieved with this developed miRNA-21 biosensor.
provided with the constructed biosensor for miRNA-21 detection, further confirming the advanced catalytic ability of the Pt@CeO₂ NSs toward H₂O₂ reduction and the ultra-high sensitivity of the proposed Pt@CeO₂-biosensor.

3.7 Selectivity, reproducibility and stability

Selectivity, reproducibility and stability are usually selected to evaluate the practicability of a sensor. Here, four small nucleic acid molecules with different sequences from miRNA-21 (S-RNA, T-RNA, N-RNA and miRNA-155) were chosen to assess the specificity of the proposed sensor through detecting the individual responses with and without miRNA-21 under the same condition (Fig. 6a). The proposed sensor was found to respond to each interferent much less in the absence of the target miRNA-21, however, the current values produced were almost the same when each of S-RNA, T-RNA, N-RNA and miRNA-155 coexisted with miRNA-21 and in good agreement with that for the detection of miRNA-21 only, suggesting that the proposed sensing strategy was highly selective for the detection of miRNA-21.

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Fig. 4  Effects of the experimental conditions on DPV response of the proposed sensor to miRNA-21 in 0.01 M PBS containing 1 mM H₂O₂ (pH 7.4): (a) SH-CP concentration, (b) incubation time of miRNA-21, (c) pH of PBS and (d) concentration of Pt@CeO₂ NSs. Error bar = RSD (n = 3).

Fig. 5 (a) DPV responses of the Pt@CeO₂-based electrochemical biosensor to different concentrations of miRNA-21 (0 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM) in 0.01 M PBS containing 1 mM H₂O₂ (pH 7.4); (b) the fitted linear curve of I versus lg c. Error bar = RSD (n = 3).
Both reproducibility and stability of the sensor are very important for the actual sample analysis. The results shown in Fig. 6b and c exhibited the excellent reproducibility and stability of this new sensor. No obvious current differences were observed among the five electrodes prepared individually and the response signal only decreased by 6.7% of the initial signal after the 8 days continuous measurement, which means that the excellent catalytic activity of noble metals was well preserved during the measurements. These results might lay a solid foundation for the practical application of the newly developed Pt@CeO2-based electrochemical sensor to detect miRNA-21 in real samples.

### 3.8 Analysis of miRNA-21 in human serum

In order to investigate the practical application of the Pt@CeO2-based miRNA-21 biosensor, the actual human blood samples collected from Changhai Hospital, Naval Medical University (Shanghai, China) were detected by the standard addition method. Three different concentrations of the target miRNA-21 were successively added to the 10 times diluted human serum. Table 3 shows that the recoveries of miRNA-21 in the blood samples with three different concentrations were between 98.2% and 102.6% with the RSD less than 4.56%, revealing the high accuracy, strong reliability and great potential of the sensor for analyzing the miRNA-21 level in real serum samples.

### 4. Conclusion

Taking full advantage of the excellent catalytic ability of Pt@CeO2 NSs toward H2O2, this work successfully developed a simple, ultra-sensitive and highly selective electrochemical biosensor to detect miRNA-21 in real blood samples. In the presence of the target miRNA-21, the capture probe with a hairpin structure was hybridized and opened to activate the biotin at the end of the probe, guaranteeing the specificity of the RNA sensor. Through the biotin-avidin affinity interaction, the hybridization event of the biomolecules immobilized on the
Electrode surface was successfully converted into the significantly amplified DPV signal in the PBS containing H$_2$O$_2$ resulting from the remarkable catalytic activity of Pt@CeO$_2$ NSs toward H$_2$O$_2$ reduction, realizing the ultra-sensitive detection of miRNA-21. Compared with other existing biosensors, this newly developed biosensor displayed better specificity, higher reproducibility, stronger stability and lower detection limit, providing a promising and potential detection platform for the real-time testing in clinical applications.

Conflicts of interest

There are no conflicts to declare.

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