An Impedimetric-Fluorescence Double-Checking Biosensor with Enhanced Reliability Based on Graphene Oxide

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MicroRNA (miRNA) is a class of clinically significant diagnostic and prognostic markers for some diseases, especially for cancer. Graphene oxide (GO) has been reported to bind and quench dye-labeled single-stranded DNA (ssDNA) probes while it has less affinity toward double-stranded DNA (dsDNA) or hybrids of DNA/RNA. This property makes it very suitable to construct a double-checking system for miRNA detection, thus increasing the reliability of detection. This kind of double-checking sensor will have great potential in clinical diagnosis. Based on this concept, in this simple and sensitive electrochemical impedance spectroscopy biosensor for miRNA-21 is developed using a GO-coated electrode. Simultaneously, the fluorescence of the remaining hybridization solution which contains dissociated duplex of ssDNA/miRNA is also investigated to double check the signal and increase its reliability. Therefore, a GO-based double-checking biosensor is developed for miRNA-21 detection, which shows simplicity, high sensitivity and selectivity, and outstanding reliability. This is the first time to integrate the electrochemical impedimetric method and fluorescence method in one sensing system for detecting miRNA based on GO. It is believed that this work will have a profound impact on the design of graphene-based biosensors for miRNA detection.

1. Introduction

MicroRNAs (miRNAs) are a class of small-sized (18–22 nucleotides), noncoding, single-stranded RNA molecules that play crucial roles in cell proliferation, differentiation, and apoptosis.[1–3] Accumulating evidence also showed that aberrant miRNA expression is associated with cancer initiation, tumor stage, and tumor response to treatments,[4–7] which indicates miRNAs can be serving as a class of clinically significant diagnostic and prognostic markers. In order to practically utilize miRNAs as useful biomarkers in clinical processes, it is very important to develop sensing technologies for quantitative measurement of the expression levels of specific miRNAs.[8] However, some current methodologies (including qRT-PCR, Northern blotting, and miRNA arrays, etc.) are laborious, semiquantitative, and require expensive equipment or radioactive/toxic labels.[9–11] Therefore, developing simple, sensitive, and reliable methods for miRNA analysis is still a great challenge.

Due to their special chemical, topological, optical, and electronic properties, nanomaterials have become increasingly important in electronics, catalysis, optics, sensors, and other fields.[12–16] The use of graphene oxide (GO), an exfoliated and oxidized form of graphite, for the construction of biosensor, has been the object of attention due to its high water dispersibility, its affinity toward hydrophobic and/or π-conjugated biomolecules and its fluorescence quenching capability.[17–22] It has been reported that GO can bind and quench dye-labeled single-stranded DNA (ssDNA) probes, while it has less affinity toward double-stranded DNA (dsDNA) or hybrids of DNA/RNA, so the fluorescence of a DNA probe will be recovered when it forms a duplex with its target and dissociates from GO.[23–25] This unique ssDNA–GO interaction has been used in developing biosensors for DNA, proteins, and small molecules by fluorescence method.[26–28] Recently, based on GO and some enzymes, several miRNA biosensors have also been developed by fluorescence method.[29–32] In fact, the property of the unique GO–ssDNA interaction includes the adsorption–desorption of ssDNA on GO surface and quenching recovering of fluorescence of the ssDNA. This unique property of GO is very suitable to be utilized to construct a double-checking system for miRNA detection, thus increasing the reliability of detection. This kind of double-checking sensor will have great potential in clinical diagnosis. Up to now, there is no report about the construction of this kind of double-checking sensor for miRNA detection based on GO.

In the electrochemical field, it is facile to convert adsorption–desorption events into detectable electrochemical signals.[33–38] Among many electrochemical methods, electrochemical
impedance spectroscopy (EIS) has been proven as one of the most powerful tools for probing the features of surface-modified electrodes without any other electrochemical labels (such as horse radish peroxidase). To date, the EIS method has been used to construct miRNA biosensors based on DNA enzymes, polyaniline, p19 protein, etc. \cite{39-41} However, the EIS method has not been used to develop miRNA sensors based on the unique ssDNA–GO interaction.

In this study, based on the unique ssDNA–GO interaction, we propose a simple and sensitive EIS miRNA sensor by measuring the impedance change of the GO coated electrode. Unlike other EIS-based biosensors, \cite{39-41} where the charge transfer resistance (\(R_{ct}\)) always positively reflects the concentrations of miRNA based on the hybridization of miRNA with its complement ssDNA or PNA immobilized on the electrode surface, in this work, the change of \(R_{ct}\) are inversely proportional to the concentrations of miRNA because the hybrids of ssDNA/miRNA can be easily dissociated from GO surface. Simultaneously, the fluorescence of the remaining hybridization solution which contains the hybrids of ssDNA/miRNA released from GO is also measured to double check the quantity of miRNA and increase the reliability of the detection. Therefore, a GO-based double-checking biosensor was developed for miRNA assay. It is not a simple combination but an ingenious integration for the two detection methods in one sensing system because the two detection processes are not separate. Instead, the signal in the fluorescence method is just come from the fluorescence amidite (FAM)-labeled DNA probes dissociated from the electrode surface, thus the increasing fluorescence signal corresponding to the decrease of the charge transfer resistance (\(R_{ct}\)) of the electrode surface, which can be used to reflecting the same concentrations of miRNA. So it is very suitable for these two methods to be used to check out each other. This integration is not suitable for any two detection methods and it is not suitable for any other nanomaterial except for GO and its analogous. To our knowledge, this is the first time to utilize the unique ssDNA–GO interaction in miRNA analysis by EIS method. This is also the first time to integrate the EIS method and fluorescence method in one sensing system for detecting miRNA based on GO.

In this setting, we used the miRNA-21 as a model case, which has been identified as the only miRNA overexpressed in 11 types of solid tumors. \cite{42,43} The developed double-checking miRNA-21 sensor shows high sensitivity, selectivity, and outstanding reliability. We believe that this work will have profound impact on the design of graphene-based biosensor for miRNA detection.

2. Results and Discussion
2.1. Characterization and Feasibility

Scheme 1 depicts the working principle of the GO-based impedimetric-fluorescence double-checking biosensor for miRNA. GO is immobilized onto the surface of electrode by physical adsorption. Then FAM dye-labeled ssDNA probes are immobilized on the GO modified glassy carbon (GC) electrode using \(\pi\)-stacking interactions between the ring of nucleobases and the hexagonal cells of GO. The fluorescence signal of the FAM-DNA probes is quenched by GO. When the ssDNA modified electrode is immersed in a hybridization solution which contains target miRNA, the ssDNA probes form duplexes with target miRNA during hybridization. Then, partial release of the DNA probes from GO surface occurs because of the formation of stable hydrogen bonds among nucleobases.
and their shielding inside the phosphate backbone after hybridization, which will disturb the interaction between the labeled DNA probe and GO. Concurrently, the fluorescence signal of the FAM-DNA probes in the DNA/RNA hybrids will be recovered. The adsorption and desorption of DNA probes from the electrode surface can be monitored by EIS method, and the resulting hybridization solution can be investigated by fluorescence assays. Thus a GO-based double-checking biosensor was developed for miRNA assay.

GO nanoflakes synthesized in this work is single layered with a \( \approx 1.1 \) nm thickness, which is consistent with previous reports.\(^{44,45}\) The chemically synthesized GO was readily water dispersible due to the presence of oxygenic groups at the surface (Figure S1, Supporting Information). To check the adsorption of ssDNA on GO, X-ray photoelectron spectroscopic (XPS) measurement on GO and GO-ssDNA were performed using indium-tin oxide (ITO) conductive glass as substrate. As shown in Figure 1A, a small peak of N 1s (399.5 eV) can be detected in the GO on ITO sample, because NaNO\(_3\) was involved in the preparation process of GO. After the adsorption of FAM-DNA probes on the GO surface, the peak of N 1s increases sharply due to the existence of nitrogen in DNA. In addition, a peak assigned as P 2p at 131.4 eV can be detected in the ssDNA-GO sample (Figure 1B), while no peak appears for GO nanoflakes without DNA adsorption because phosphorus is not present in GO but in DNA. The XPS results indicate that ssDNA probe has been successfully adsorbed on the surface of GO.

The EIS method was used to investigate the impedance of the modified electrode at each modification step. The charge transfer process associated to the redox reaction of the couple K\(_3\)[Fe(CN)\(_6\)]/K\(_4\)[Fe(CN)\(_6\)] at the applied potential is strongly influenced by any electrode surface modification. Figure 2 illustrates the impedance spectroscopy results presented as Nyquist plots (\( Z_{\text{im}} \) vs \( Z_{\text{re}} \)). In order to give more detailed information, a Randle’s equivalent circuit was chosen to fit the impedance spectroscopy. In this circuit, \( R_c \) corresponds to the resistance of the solution, \( R_t \) represents the resistance for charge transfer between the solution and the electrode surface, \( W \) is the Warburg impedance and CPE (constant phase element) is associated with the capacitance of the double layer. After the deposition of GO layer (curve b), a considerable increase of 0.11 \( \pm \) 0.03 k\( \Omega \) in the \( R_t \) value was observed compared with bare GC electrode (curve a) because the formation of the GO layer insulated the electrode and blocked the interfacial electron transfer between the redox probe and the electrode surface considerably. When the ssDNA probes were attached to the GO-modified electrode (curve c), the charge repulsion between the negatively charged phosphate backbone of ssDNA and the [Fe(CN)\(_6\)]\(^{3-/4-}\) probe resulted in a reduced ability for electron transfer on the electrode surfaces and led to a further increase of 4.63 \( \pm \) 0.29 k\( \Omega \) in the \( R_t \) value. This result indicates the successful modification of the electrode surface.

Then the modified electrode was used to hybridize with miRNA. The EIS and fluorescence result was showed in Figure 3. When the ssDNA coated GC electrode was incubated in the blank solution for 1 h (Figure 3A, curve a), no significant difference appeared in the \( R_t \) value. However, after hybridization with 1 \( \times \) 10\(^{-11}\) M target miRNA, a significant decrease of 2.63 \( \pm \) 0.45 k\( \Omega \) in the \( R_t \) value was observed (Figure 3B, curve b). As shown in the scheme, the reason for impedance decrease after hybridization with the target miRNA is the partial release of the ssDNA.
probes from the electrode surface, which decreases the total charge present onto the electrode surface, thus reducing the resistance to charge transfer. So the decrease of the $R_\text{ct}$ reflects the number of ssDNA/miRNA duplexes formed or the ssDNA probes being cleaved off the GO surface during hybridization and hence the concentration of the target miRNA. The EIS results confirmed that GO has less affinity toward hybrids of DNA/RNA than the ssDNA and illustrate the feasibility of the proposed GO-based EIS method for detecting miRNAs.

As mentioned above, after hybridization, the hybrids of ssDNA/miRNA will be dissociated from GO, so the fluorescence of dye-labeled ssDNA will be recovered in the remained hybridization solution, which can be detected by fluorescence method. Figure 3B shows the fluorescence spectra of the remained hybridization solution after the ssDNA modified electrode hybridized with blank solution and 10$^{-11}$ M miRNA. As expected, no obvious fluorescence was observed for the remained solution which the electrode was incubated in the blank solution (Figure 3B, curve a). However, after hybridizing with 10$^{-11}$ M miRNA, a significant fluorescence enhancement was observed for its remained hybridization solution (Figure 3B, curve b), suggesting DNA/RNA duplex has been dissociated from GO surface, so the enhancement of the fluorescence can also be used to reflect the same concentration of miRNA as the decrease in $R_\text{ct}$ value in the electrochemical method (Figure 3A, curve b). Therefore, these two methods can be used to check out each other in one system.

2.2. EIS Measurements

The ssDNA probes adsorbed on GC electrode was used to hybridize with target miRNA. To obtain a reproducible sensing platform, the concentration of ssDNA probes to be immobilized onto the electrode surface was optimized at 50 × 10$^{-6}$ M. This concentration can ensure full coverage of the electrode surface and it is high enough to underrate nonspecific adsorption of the target miRNA (Figure S2, Supporting Information). The sensitivity of the GO-based electrochemical impedance miRNA biosensor was investigated by varying the concentration of the complementary target miRNA (Figure 4). As mentioned above, the addition of the target miRNAs causes the decrease of the $R_\text{ct}$ value due to the formation of the DNA/RNA hybrids and the partial release of the DNA probes from the GO surface. The analytical signal, $\Delta R_\text{ct}$ ($\Delta R_\text{ct} = |R_\text{ct, hybrids} | - |R_\text{ct, DNA probe}|$), had a linear relationship with the logarithmic value of the target miRNA concentration ranging from 5 × 10$^{-13}$ to 1 × 10$^{-10}$ M. Further increasing the concentration of target miRNAs did not cause an additional change in the signal as a plateau was reached. The detection limit of this electrochemical impedance biosensor is about 3.1 × 10$^{-14}$ M (at S/N of 3.0).

The capability in discriminating different miRNAs is crucial in miRNA expression profiling. The selectivity of the GO-based electrochemical impedance miRNA sensor was investigated using three kinds of different target miRNA (fully complementary, one-base mismatched sequences, and noncomplementary sequences, see the Experimental Section) to hybridize with the probe ssDNA immobilized on the electrode. As shown in Figure 5, a pronounced increase in the $\Delta R_\text{ct}$ value corresponding to the hybridization with 10$^{-11}$ M fully complementary miRNA sequence was observed and a lower or negligible increase in the $\Delta R_\text{ct}$ value was obtained when incubating with one-base mismatched sequences or noncomplementary sequences. This result suggests that the GO-based impedimetric miRNA sensor has high selectivity.

2.3. Fluorescent Assays

The remaining hybridization solution was used for fluorescence assays. As expected, fluorescence signal in the hybridization solution was observed due to the release of the DNA miRNA (fully complementary, one-base mismatched sequences, and noncomplementary sequences, see the Experimental Section) to hybridize with the probe ssDNA immobilized on the electrode. Therefore, these two methods can be used to check out each other in one system.

Figure 3. A) Complex impedance plot of a) DNA-coated GO-GC electrode hybridizes with blank solution; b) a typical complex impedance result of DNA probes modified electrode hybridizes with 1 × 10$^{-11}$ M miRNA-21. B) Fluorescence spectra of the remaining hybridization solution for a) and b) as in (A).

Figure 4. Representative Nyquist plots of DNA-GO-GC electrode hybridized with blank solution and target miRNA at different concentrations: a) 5 × 10$^{-13}$, b) 1 × 10$^{-12}$, c) 5 × 10$^{-12}$, d) 1 × 10$^{-11}$, e) 5 × 10$^{-11}$, and f) 1 × 10$^{-10}$. Inset was the plot of $\Delta R_\text{ct}$ versus logarithm of the concentration of target miRNA. Frequency range: from 100 mHz to 100 kHz; electrolyte: 5 × 10$^{-3}$ M [Fe(CN)$_6$]$_3$/-4/ (1:1) in 0.01 M phosphate buffer solution (pH 7.4) containing 0.1 M KCl; amplitude: 5 mV.
probes from the GO surface (Figure 6). Contrary to the result of EIS, the fluorescence intensities increased with the increase in target miRNA concentration. There was a good linear correlation between the fluorescence ratio and the amount of miRNA-21 in the concentration range of $5 \times 10^{-13}$ to $5 \times 10^{-11}$ M with a detection limit of $\approx 8.3 \times 10^{-14}$ M (at S/N of 3.0), which suggested that the proposed method is also suitable for quantitation of miRNA. Importantly, it provides an adoptable method to double check the signal for the above EIS sensor in the same sensing system, thus increasing the reliability for each concentrations of miRNA detection.

The selectivity of this fluorescence sensor was also examined and the result is shown in Figure 7, the fluorescence obtained with the wild-type sequence is more significant than that measured with one-base mismatched sequences or noncomplementary sequences, suggesting the fluorescence sensor for miRNA is highly selective. This result is also consistent with the EIS results and indicated that the selectivity of the double-checking sensor can also be verified by the integration of the two detection methods in one sensing system.

In our work, only one nanomaterial (i.e., GO) was used and one step is needed for the fabrication of electrode. The preparation of the sensing interface is easy. When the EIS experiment is ended, the remaining hybridization solution can be detected by fluorescence method directly without adding any other steps. So this double-checking sensor is very simple. When two sensors are integrated together, the sensitivity of the whole sensor should accord to the relatively insensitive method. So the detection limit of the fluorescence sensor ($\approx 8.3 \times 10^{-14}$ M) should be as the detection limit of the double-checking sensor. This value is better than or comparable to some other type of miRNA biosensors.[29–32,46,47] So this double-checking sensor is sensitive. As for the reliability, each concentration of miRNA will be double checked by two methods, thus increasing the reliability of the detection. This is the main aspect of the enhanced reliability we want to express in this work. On the other hand, the interference of false positive signals and false negative signals can also be reduced because different results may be obtained by these two methods due to their different sensing ability. This is another aspect of the enhanced reliability. The stability of the double-checking sensor will depend on the stability of the modified electrode, we found that after storage at 4 °C for two weeks, there was still 85% miRNA-sensing ability remained for the modified electrodes, indicating that this sensor was highly stable.

2.4. Detection of Saliva Sample

In order to evaluate the applicability of the proposed double-checking miRNA biosensor, the saliva samples spiked with different concentrations of target miRNA were detected with our biosensor. Table S1 (Supporting Information) clearly showed that the proposed miRNA sensor has a strong resistance to the complex matrix of saliva and can be used to detect ultratrace target miRNA in real saliva samples with a recovery of 93%–107% and a RSD < 7% (n = 3). Moreover, the results
obtained by the EIS method were observed to agree fairly well with those obtained from fluorescence method, illustrating that our double-checking miRNA sensing system is powerful in increasing the reliability of the results. These results indicated that the GO-based double-checking sensing system could be preliminarily used for the detection of target miRNA in the complex sample system.

3. Conclusion
In summary, based on the unique ssDNA–GO interaction and two sensitive techniques, i.e., electrochemical impedimetric technique and fluorescence techniques, we developed a double-checking biosensor for miRNA detection. The three sensing elements integrated in one system link simplicity, high sensitivity and selectivity, and great reliability of the miRNA detection. This double-checking sensing method can be used to detect miRNA, RNA, DNA, and other biomolecules. This double-checking sensing method can also be expand to other analogous of GO, such as MoS_2, WS_2, which have been reported to possess the similar interactions with ssDNA as that of GO. The proposed method would be of great scientific value for the design and engineering for sensor applications.

4. Experimental Section
Materials: All DNA or miRNA sequences were synthesized and purified by Invitrogen Inc. The oligonucleotides have the following sequences:

Probe oligonucleotides: 5′-FAM-TCAA CAT CAG TCT GAT AAG CTA-3′

miRNA-21: 5′-UAG CUU AUC AGA CUG AUG UUG A-3′

Single-based mismatch miRNA: 5′-UAG CUU AUC AGA CUG AAG UUG A-3′

Noncomplementary miRNA: 5′-GUA AGG CAU CUG ACC GCA GCC A′-3′

All other reagents and solvents were of analytical reagent grade. To prepare GO, graphite powder (4 g) was oxidized in a hot solution (80 °C) of concentrated H_2SO_4 (24 mL) containing K_2S_2O_5 (8 g), and P_2O_5 (8 g). The resulting mixture was isolated and slowly cooled to room temperature. The mixture was then diluted, filtered, and dried overnight. The obtained GO was further dispersed in a water slurry using a sonicator. The dispersion was then filtered to obtain a clear GO solution.

Preparation of GO: GO was prepared from graphite through modified Hummers method. Briefly, graphite powder (4 g) was oxidized in a hot solution (80 °C) of concentrated H_2SO_4 (24 mL) containing K_3[Fe(CN)]_6 (8 g) and K_4[Fe(CN)]_6 (8 g). The resulting mixture was isolated and slowly cooled to room temperature. The mixture was then diluted, filtered, and dried overnight. The obtained GO was dispersed in a water slurry using a sonicator. The dispersion was then filtered to obtain a clear GO solution.

Analysis for Complex Samples: To investigate the analytical applicability of the proposed double-checking miRNA-21 biosensor, artificial saliva samples were prepared. Under the direction of previously established protocols, unstimulated saliva samples were collected between 9 a.m. and 10 a.m. Subjects were required to refrain from eating, drinking, smoking, and oral hygiene procedures for at least 1 h before the collection. Saliva samples were centrifuged at 610 g for 15 min at 4 °C. Then, the supernatant was taken and diluted with hybridization buffer (1:100). The different concentrations of target miRNA were spiked into the diluted saliva sample and the concentrations of target miRNA in the as-prepared samples were assayed using the developed sensing protocol.

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

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