Highly Sensitive and Selective Strategy for MicroRNA Detection Based on WS₂ Nanosheet Mediated Fluorescence Quenching and Duplex-Specific Nuclease Signal Amplification

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ABSTRACT: MicroRNAs (miRNAs) play vital roles in physiologic and pathologic processes and are significant biomarkers for disease diagnostics and therapeutics. However, rapid, low-cost, sensitive, and selective detection of miRNAs remains a challenge because of their short length, sequence homology, and low abundance. Herein, we report for the first time that WS₂ nanosheet can exhibit differential affinity toward short oligonucleotide fragment versus ssDNA probe and act as an efficient quencher for adsorbed fluorescent probes. This finding is utilized to develop a new strategy for simple, sensitive, and selective detection of miRNA by combining WS₂ nanosheet based fluorescence quenching with duplex-specific nuclease signal amplification (DSNSA). This assay exhibits highly sensitive and selective with a detection limit of 300 fM and even discriminate single-base difference between the miRNA family members. The result indicates that this simple and cost-effective strategy holds great potential application in biomedical research and clinical diagnostics.

MicroRNAs (miRNAs) are a group of short, endogenous, noncoding RNAs that play vital regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression.1 Recent studies have found that the dysregulation of miRNA expression is closely associated with various diseases2,3 including human cancers.4 In addition, miRNAs have been regarded as biomarker candidates in clinical diagnosis and therapy.5 However, it is difficult to detect the miRNAs owing to their unique characteristics, including small size, sequence homology among family members, low abundance in total RNA samples, and susceptibility to degradation. Therefore, strategies for sensitive and selective detection of miRNAs are in urgent need, especially for early clinical diagnosis. Recent years have seen an increasing interest in the development of different isothermal amplification techniques for miRNA detection.6–9 Very recently a new duplex-specific nuclease signal amplification (DSNSA) strategy has been developed for highly sensitive detection of miRNA.10

Graphene, a two-dimensional nanomaterial, has been extensively used in biological applications.11–14 Recently, transition metal dichalcogenides (e.g., MoS₂, WS₂, etc.) as two-dimensional (2D) layered nanomaterials analogous to graphene attracted great attention due to their carrier mobility,15,16 optical,17,18 and catalytic properties.19,20 In contrast to graphene, transition metal dichalcogenides nanosheets can be facilely synthesized in large scale and directly dispersed in aqueous solution without the need of surfactants or oxidation treatment. Moreover, WS₂ nanosheets were reported to allow self-assembly of thiolated compounds on its surfaces, indicating another advantage of WS₂ nanosheets over graphene in facile surface modification.21 This implies that transition metal dichalcogenides nanosheets can hold great potential in biomedical applications because surfactants may cause denaturation of proteins and oxidation can alter the semiconductive property of the nanostructures. Recently, it has been demonstrated that MoS₂ nanosheet exhibits a capability for discriminating single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA), which may catalyze the applications of transition metal dichalcogenides nanosheets in biomedical areas.22

Herein, we report for the first time that another transition metal dichalcogenides nanosheets such as WS₂ nanosheets can also act as an efficient quencher for fluorescence dyes such as fluorescein, and more importantly, these nanosheets exhibit differential affinity toward short oligonucleotide fragment (<10 bases) versus ssDNA probe (>10 bases). On the basis of this finding, we develop a new strategy for simple, sensitive, and selective detection of miRNA by combining the super fluorescence quenching ability of the WS₂ nanosheet and duplex-specific nuclease signal amplification (DSNSA). As

Received: December 4, 2013
Accepted: January 22, 2014
Published: January 22, 2014
shown in Scheme 1, upon the addition of target miRNA, the ssDNA probe hybridizes to a target miRNA to form a DNA/RNA heteroduplex. The heteroduplex will become the substrate for duplex-specific nuclease (DSN) cleavage, since DSN only cleaves the ssDNA probe in the DNA/RNA duplex. The cleavage of the ssDNA probe allows the target miRNA to be released and hybridize with another ssDNA probe, which initiates a next round of cleavage, releasing, and hybridization. This cyclic reaction generates a great amplification of fluorescence signal. After the product from the DSNNSA reaction is incubated with WS2 nanosheets, the cleaved short FAM-linked oligonucleotide fragments will not be adsorbed on the nanosheets because of their weak affinity and thus retain a strong fluorescence signal. In contrast, in the absence of the DSNNSA reaction, the ssDNA probes remain intact and their fluorescence is almost entirely quenched due to their strong affinity to WS2 nanosheets.

The basis of the developed strategy is the differential affinity toward short oligonucleotide fragment versus ssDNA probe (22 bases). To demonstrate this finding, we first investigated the fluorescence quenching efficiency ($F_0 - F/F_0$) of WS2 nanosheet to ssDNA with different lengths. As shown in Figure S1 in the Supporting Information, a substantial increase in the fluorescence quenching efficiency of WS2 nanosheet to ssDNA was observed with the increasing base number of ssDNA. The fluorescence quenching efficiency of WS2 nanosheet to FAM-labeled ssDNA containing 5 and 9 bases were 42% and 72%, respectively. However, 97% fluorescence quenching efficiency was achieved with the ssDNA containing 22 bases. This phenomenon indicated that the affinity of the short ssDNA fragments (<10 bases) to WS2 nanosheet is much weaker than that of the long ssDNA (22 bases). Moreover, we have made a comparison of WS2 nanosheet with graphene oxide for fluorescence quenching performance, as shown in Figures S1 and S2 in the Supporting Information. It is found that graphene oxide also shows less quenching efficiency for shorter DNA. However, the quenching efficiency of graphene oxide (at the same concentration as the WS2 nanosheet) is lower than that of the WS2 nanosheet, and more importantly, the discrimination ratio (the ratio of quenching efficiencies for a long DNA versus a short DNA) for graphene oxide is lower than that of the WS2 nanosheet. This forms the basis for the design of the WS2 nanosheet based fluorescence biosensor for miRNA detection.

MircoRNA-21 (miR-21) was then selected as a model target, which was overexpressed in human breast cancers, to demonstrate the developed sensing platform. A FAM-labeled ssDNA probe (P-21) complementary to miR-21 was prepared (Table S1 in the Supporting Information). As shown in Figure 1A, in the absence of target miRNA, the fluorescence of P-21 was almost entirely quenched regardless of the presence or absence of DSN (curves a, b, c, d), which could be ascribed to the strong interaction between the long ssDNA probe and the WS2 nanosheets as well as the high fluorescence quenching efficiency of the nanosheets. However, when adding target miRNA, the fluorescence of P-21 was partially recovered in the presence of DSN.
presence of WS₂ nanosheets (curve e). This indicated a weaker interaction between DNA/RNA hybrid duplexes and WS₂ nanosheets than that between ssDNA probe and WS₂ nanosheets. In contrast, when incubating the ssDNA probe with target miRNA and DSN, the FAM-labeled probe was cyclically cleaved by DSN, producing short FAM-linked oligonucleotide fragments. Because of the weak affinity of the short FAM-linked oligonucleotide fragments to WS₂ nanosheets, a strong fluorescence signal was observed with a signal-to-background ratio of ∼14 (curve f). These results indicated that the proposed method can be used for sensitive detection of miRNA.

To verify the DSN-catalyzed cleavage mechanism, agarose gel electrophoresis analysis was performed. As shown in Figure 1B, the band of probe (lane 4) or target miRNA (lane 5), after separately incubating with DSN, appeared in the same position as those for the probe (lane 1) or target miRNA (lane 2). However, when incubating DNA/RNA hybrid duplex with DSN, the probe was cleaved to give a band of ∼10 bp length and the target miRNA still remained intact, a faint band of ∼25 bp was ascribed to the undigested DNA/RNA heteroduplex (lane 6). These results confirmed that the ssDNA probe and miRNA would not be cleaved by DSN, while the ssDNA probe in DNA/RNA hybrid duplex could be specifically cleaved by DSN.

In order to achieve the best assay performance, we optimized the amplification and sensing conditions including the working temperature and the amount of DSN in the DSNsA reaction as well as the concentration of WS₂ nanosheets. The reaction time of 30 min is chosen according to the previous report, the concentrations of P-21 and miR-21 were fixed at 100 nM and 20 nM, respectively. As shown in Figure S3 (Supporting Information), the highest fluorescence intensity ratio (F/Flₐ₉) was chosen for further experiments. Figure S5 (Supporting Information) illustrates the dependency of the F/Fl₀ on the concentration of WS₂ nanosheets. The F/Fl₀ gradually increased with an increasing concentration of WS₂ nanosheets. However, the F/Fl₀ decreased with a further increasing concentration of WS₂ nanosheets when the concentration of WS₂ nanosheets was over 6 μg/mL, because the short FAM-linked oligonucleotide fragment cleaved by DSN was adsorbed on the excessive amount of WS₂ nanosheets. Therefore, the WS₂ nanosheets concentration of 6 μg/mL was chosen for further experiments to reach the desired sensitivity and selectivity.

The performance of the developed strategy for quantitative analysis of miRNA was further investigated. As shown in Figure 2A, a dramatic increase in the fluorescence intensity was observed with the increasing concentration of miR-21. A quite wide dynamic range from 0 to 100 nM was achieved for this assay. Figure 2B shows the relationship between the fluorescence intensity and different miR-21 concentrations in logarithmic scale. The logarithm of fluorescence intensity possesses a linear correlation to the logarithm of miR-21 concentration in the range from 0.001 nM to 10 nM. The calibration equation was ln F = 2.6198 + 0.1696 ln C, with a correlation coefficient R² = 0.9917, where F is the fluorescence intensity and C is the concentration of miR-21. The detection limit was estimated to be 30 fM according to the 3σ rule, which was better or comparable to some previously reported fluorescence sensors for miRNA detection. It is noteworthy that such a high sensitivity was achieved in less than 40 min. The excellent sensitivity mainly relied on the high fluorescence quenching capability of WS₂ nanosheets and the strong cleavage activity of DSN.

Furthermore, we performed a series of contrast experiments using miR-143, miR-141, and single-base-mismatched miR-21 (SM miR-21) to evaluate the specificity of the proposed miRNA assay. As shown in Figure 3, there was weaker fluorescence intensity ratio (F/Fl₀) in the presence of SM miR-21 compared with the complementary target miR-21. Besides, nearly negligible fluorescence change was observed in the addition of miR-143 and miR-141 compared with the blank.
The high specificity due to the excellent capability of DSN for discriminating perfectly from nonperfectly matched DNA/RNA duplexes. This result indicates that this approach exhibits high selectivity and has potential application to discriminate a single-base difference between the miRNA family members.

To determine whether the developed method could be applied to real complex biological samples, we measured miR-21 from different cell extracts including the human breast cancer cell lines (MCF-7), cervical cancer cell lines (Hela), and mammary epithelial cell lines (MCF-10A). The relative expression levels of miR-21 in these three cell lysate samples were determined using the quantitative real-time polymerase chain reaction (qRT-PCR) and the proposed method, respectively. The results indicated that MCF-7 cell lines had higher miR-21 expression level than Hela and MCF-10A cell lines (Figure S8 in the Supporting Information), which is in good accordance with previous reports.27 We also determined the accuracy of the method by measuring the recovery of known amounts of miR-21 spiked into the MCF-10A cell lysates (Table S3 in the Supporting Information). The method reveals good recovery rates of standard addition from 97% to 104%. These results indicated that the proposed method was capable of detecting miRNA sensitively in real complex samples.

In summary, we report for the first time that transition metal dichalcogenides nanosheets such as WS₂ nanosheets can exhibit differential affinity toward short oligonucleotide fragments versus ssDNA probes and can act as an efficient quencher for the adsorbed fluorescence DNA probes. On the basis of this finding, we develop a new strategy for simple, sensitive, and selective detection of miRNA by combining super fluorescence quenching ability of WS₂ nanosheet and duplex-specific nuclease signal amplification. Compared with other miRNA detection methods, this method shows high sensitivity, improved single-base mismatch discrimination, lowered fluorescence background, and decreased cost due to high fluorescence quenching efficiency of WS₂ nanosheets and the use of a single-label probe. In virtue of these advantages, the proposed strategy implies the potential of combining the WS₂ nanosheets based fluorescence quenching with nucleic acid amplification techniques as a high-performance sensing platform for biomedical research and clinical diagnostics.

![Figure 3. Specificity of miRNA assay. Bars represent the fluorescence intensity ratio (F/F₀) upon the different miRNAs targets with the same concentration of 10 nM, where F₀ and F are the fluorescence signals in the absence and the presence of miRNAs, respectively. Error bars are the standard deviation of three repetitive experiments.](image)

**REFERENCES**


