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RESEARCH ARTICLE

In situ biosensor for detection miRNA in living cells based on carbon nitride nanosheets with catalytic hairpin assembly amplification

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Abstract

In this study, an ultrasensitive fluorescence turn-on assay for *in situ* sensing of intracellular microRNA (miRNA) was developed utilizing a carbon nitride nanosheet (CNNS) and a catalytic hairpin assembly (CHA). The CHA showed favourable signal amplification for low-level biomarkers, and CNNS was an excellent candidate as a fluorescence quencher and gene vector. Moreover, the hairpin DNA of CHA could be adsorbed onto the surface of CNNS. An enzyme-free fluorescence biosensor for ultrasensitive sensing of intracellular miRNA in cells based on CHA and CNNS was designed. When faced with target miRNA, the fluorescence was recovered due to the miRNA, which could trigger cycling of CHA circuits, leading to the production of a marked enhanced fluorescence signal. Compared with traditional methods, the proposed method is convenient, with low cytotoxicity, and high specificity and ultrasensitivity. It has promising potential for detection low-level biomarkers.

KEYWORDS

carbon nitride nanosheet, catalytic hairpin assembly, intracellular signal amplification, microRNA, nanoprobe

1 | INTRODUCTION

MicroRNAs (miRNAs) can regulate gene expression and play crucial roles in a variety of biological processes.[1–3] The abnormal expression of miRNAs is related to various diseases.[4–7] miRNA expression is at low levels in cells, thus ultrasensitive detection of miRNAs is vital for miRNA-related biological studies. Some techniques including microarrays, northern blotting analysis, real-time polymerase chain reaction (RT-PCR) and other new methods[8–12] have been employed to heighten the sensitivity of low-level miRNA detection. However, pre-treatment could cause cell death and miRNA expression in living cells could not be detected by these methods. Recently, some techniques for the *in situ* detection of intracellular miRNA have been developed,[13–16] but these techniques lacked signal amplification, therefore an *in situ* amplification strategy for ultrasensitive detection of intracellular miRNAs is still a urgent need.

Graphene oxide (GO) has been extensively used in the biosensing fields. More recently, bulk graphitic-phase carbon nitride, a two-dimensional layered graphene analogue, has been applied successfully in biological fields.[17,18] CNNS, which could be stripped from the bulk graphitic-phase carbon nitride, has also drawn increasing attention due to its unique properties[19,20] that include low

Abbreviations used: $A\beta_{1-42}$, amyloid beta 42 peptide; CHA, catalytic hairpin assembly; CNNS, carbon nitride nanosheet; DMEM, Dulbecco's modified Eagle's medium; FAM, carboxyfluorescein; FAM-P1, FAM-labeled hairpin DNA probe 1 of CHA; FAM-P2, FAM-labeled hairpin DNA probe 2 of CHA; FL, fluorescence; GO, Graphene oxide; P1/P2–CNNS, CNNS simultaneously assembled with FAM-P1 and FAM-P2; P1–CNNS, CNNS assembled with FAM-P1; P2–CNNS, CNNS assembled with FAM-P2; PBS, phosphate-buffered saline; PC12 cells, pheochromocytoma cells; TEM, transmission electron micrograph.

cytotoxicity, good biocompatibility and an efficient fluorescence quenching ability.

Due to the progress of oligonucleotide nanotechnology, oligonucleotide-based sensing has achieved excellent signal amplification.[21,22] Recently, catalytic hairpin assembly (CHA) has been widely used in sensitive analyses[23–27] but CHA-based strategies in living cells have been rarely reported, however. Additionally a CNNS-based CHA enzyme-free biosensor has not been used previously to detect intracellular miRNAs in cells.

Here, we propose an ultrasensitive fluorescence biosensor for monitoring intracellular miRNA by coupling CHA with CNNS. Enzyme-free CHA can generate large signal amplification, and CNNS is a excellent candidate as a fluorescence quencher and gene vector. As shown in Scheme 1a, FAM-labeled hairpin DNA probe 1 and probe 2 of CHA (P1 and P2) were have been designed based on the miRNA-106a (miR-106a) sequence. Parts 1, 2 and 3 sequences of P1 were complementary to the miR-106a sequence, and the P2 parts 3, 4*, 3*, and 2* sequences were complementary to the P1 sequence (Scheme 1a). P1 and P2 were labeled with dye. P1/P2–CNNS was prepared by assembling P1 and P2 onto the CNNS surface through strong π–π interactions (Scheme 1a).[28,29] P1/P2–CNNS was transfected into cells, therefore intracellular miR-106a could hybridize specifically with P2 parts 1, 2 and 3 to open up the P1 hairpin to form miRNA–P1complexes and release other P1 parts. P1 Part 3* was then hybridized with P2 part 3 to initiate CHA, which displaced miR-106a, opened up P2 to form P1–P2 double-stranded DNA (dsDNA), and weakened the π–π interaction between CNNS and the bases. The interactivity between the CNNS and dsDNA was weak, leading to clear fluorescence recovery, and recycled miR-106a initiated the next CHA (Scheme 1b). This protocol established an ultrasensitive biosensor for miRNA monitoring in living cells. The proposed method offers a new, convenient, ultrasensitive and high specificity tool for detection of intracellular miRNA.

2 | EXPERIMENTAL

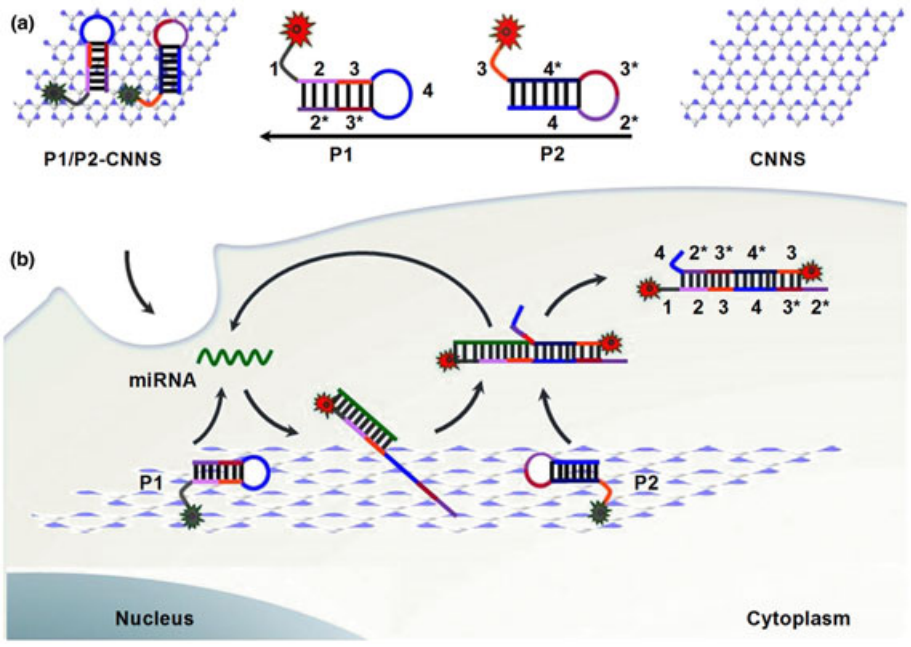
2.1 | Reagents

PC12 cells were obtained from the Shanghai Institute of Cellular Biology of Chinese Academy of Sciences, MTT was purchased from KeyGen Biotech. Co. Ltd (Nanjing, China); $A\beta_{1-42}$ was purchased from Sigma-Aldrich Inc. (USA); 1 × SPSC buffer (0.75 M NaCl and 50 mM Na_2HPO_4, pH 7.4), Opti-MEM and Lipofectamine 2000 were obtained from Invitrogen (USA); HPLC-purified RNA and diethylpyrocarbonate (DEPC)-treated water were purchased from GenePharma Co., Ltd (Shanghai, China); HPLC-purified DNA was synthesized by the Sangon Biological Biotechnology Co., Ltd (Shanghai, China). All oligonucleotides were dissolved in RNA-free water. Oligonucleotides sequences are listed in Table 1.

The mismatched bases are highlighted in lowercase.

2.2 | Apparatus

Absorbance was measured using a UV–visible spectrophotometer (UV-2401PC, Shimadzu, Japan). Fluorescence measurements were performed using a spectrofluorometer (RF-5301PC, Shimadzu, Japan). The cytotoxicity assay was conducted using a microplate reader (VICTOR™ 5, PerkinElmer, USA). TEM measurements were made using a JEOL JEM-2100 transmission electron microscope (Japan). Flow cytometric analyses were conducted using a flow cytometer (Beckman Coulter FC500, USA).



SCHEME 1 Scheme for (a) P1/P2–CNNS preparation and (b) sensing of miRNA in living cells

TABLE 1 Oligonucleotides sequences

Name	Sequences (5′ → 3′)
miR-106a	5′-AAAAGUGCUUACAGUGCAGGUAG-3′
Inhibitor-106a	5′-CUACCUGCACUGUAAGCACUUUU-3′
FAM-P1	5′-FAM-CTACCTG (1) CACTGTAA (2) GCACTTTT (3) CCATGTGTAGT (4) AAAAGTGC (3*) TTACAGTG (2*)-3′
FAM-P2	5′-FAM-GCACTTTT (3) ACTACACATGG (4*) AAAAGTGC (3*) TTACAGTG (2*) CCATGTGTAGT (4)-3′
Non-complementary RNA	5′-AGCAGCAUUGUACAGGGCUAUCA-3′
Three-base mismatched RNA	5′- AAAAGUGC*g*UAC*g*GUG*a*AGGUAG-3′
Single-base mismatched RNA	5′-AAAAGUGCUU*g*CAGUGCAGGUAG-3′

2.3 | Synthesis of CNNS

CNNS was synthetized based on previous methods.[19,30] Bulk graphitic-phase carbon nitride was synthetized by the polymerization of melamine molecules. CNNS was synthetized by sonicating bulk graphitic-phase carbon nitride in water, then the sonicated mixture was centrifuged at 5000 rpm to remove aggregated graphitic-phase carbon nitride particles to give a CNNS suspension, which was centrifuged at 15 000 rpm to acquire CNNS.

2.4 | Preparation of the nanoprobe

FAM-P1 and FAM-P2 were denatured to fold into a hairpin structure for later use. The concentration of CNNS for FAM-P1(50 nM) and FAM-P2 (50 nM) to prepare P1/P2–CNNS was 200 μg/ml. To obtain P1/P2–CNNS, CNNS was dispersed in SPSC buffer, then sonicated for 5.0 min. FAM-P1 and FAM-P2 were next mixed with the CNNS solution and gently stirred for 30 min, and then centrifuged to acquire P1/P2–CNNS. After redispersion, the P1/P2–CNNS concentration was expressed in terms of the CNNS concentration. P1–CNNS and P2–CNNS were acquired by a similar process, the concentration of CNNS for FAM-P1(50 nM) or FAM-P2(50 nM) to prepare P1–CNNS or P2–CNNS was 100 μg/ml.

2.5 | Cell culture

The pheochromocytoma cells (PC12 cells) were cultured in DMEM plus 10% fetal calf serum, with a humidified atmosphere of 5.0% carbon dioxide and 95% air at 37°C. Opti-MEM, a serum-reduced medium that could increase transfection efficiency, was used for cell transfection.

2.6 | MTT assay

PC12 cells were seeded in replicate 96-well microtitre plates at 10 000 cells/well with 100 μl Opti-MEM for 12 h. After Opti-MEM had been removed, PC12 cells were treated with CNNS 0 ~ 800 (0, 50,100, 200, 300, 400, 600, 800) μg/ml for 8.0 h; cells that were cultured with Opti-MEM medium only served as controls. These cells were washed thoroughly with PBS, and 50 μl MTT solution (1.0 mg/ml) was added to the wells. After 4.0 h, the remaining medium was removed, and 150 μl dimethylsulfoxide was added. Absorbance measurements were performed using a VICTOR™ 5 microplate reader at 490 nm.

2.7 | *In vitro* fluorescence quantitative detection of miRNA

The maximum excitation wavelengths of FAM-P1 and FAM-P2 were 490 nm, the fluorescence emission maximum occurred at 516 nm, and the fluorescence spectrum were recorded between 497 nm and 650 nm. P1/P2–CNNS (200 μg/ml) was incubated with different concentrations (0, 0.5, 25, 100, 200, 300, 400, or 600 pM) of miR-106a in SPSC buffer for 4.0 h at room temperature, the fluorescence measurements were performed using a RF-5301PC spectrofluorometer.

2.8 | Monitoring of miRNA in living cells

PC12 cells were seeded into Petri dishes at 100 000 cells per dish with a volume of 5.0 ml/dish for 12 h. Cells were divided into several groups:

a. Control group, cultured with Opti-MEM.
b. Model group, incubated with 5.0 μM $A\beta_{1-42}$ for 24 h. $A\beta_{1-42}$-induced PC12 cells were regarded as an Alzheimer's disease model, and miR-106a is closely related to Alzheimer's disease.
c. Inhibitor-treated group, transfected with 50 nM inhibitor-106a for 24 h, and the inhibitor was transfected using Lipofectamine 2000.
d. Co-action group (model group incubated with inhibitor-106a), incubated with 50 nM inhibitor-106a for 24 h after pre-treatment with 5 μM $A\beta_{1-42}$ for 24 h.

These dishes were cultured in a humidified atmosphere of 5.0% carbon dioxide and 95% air at 37°C. After washing with PBS for three times, these cells were incubated with 200 μg/ml P1/P2–CNNS for 8.0 h. Because the fluorescence of the H2–CNNS transfected cells was derived from the baseline of the flow cytometer and interference signals, PC12 cells incubated with 200 μg/ml P2–CNNS served as a blank to eliminate background. Then flow cytometric analysis were performed using a Coulter FC-500 flow cytometer.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of CNNS and nanoprobe

TEM images of CNNS displayed a two-dimensional ultra-thin structure (Figure 1A) and possessed an electronegative zeta potential of

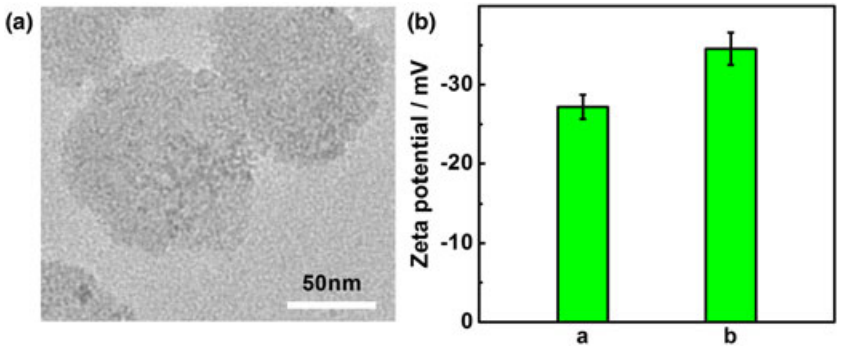


FIGURE 1 (a) TEM image of CNNS. (B) zeta potential analysis for (a) CNNS and (b) P1/P2–CNNS

−28.8 mV (Figure 1B, column a), which was favourable for biosensing application.

Successful preparation of P1/P2–CNNS was evaluated. After FAM-P1 and FAM-P2 were assembled on CNNS, the zeta potential of P1/P2–CNNS became more negative than that of CNNS (Figure 1B, column b), due to FAM-P1 and FAM-P2 negative-charged phosphates. The P1/P2–CNNS absorbance spectrum displayed the characteristic FAM peak and significant hypochromicity with respect to free FAM-P1 and FAM-P2 (Figure S1),[29,31] which indicated that FAM-P1 and FAM-P2 were assembled on CNNS, thus a successful preparation of the nanoprobe was confirmed.

3.2 | Quenching effect of CNNS

Effective quenching of FAM-P1 and FAM-P2 fluorescence by CNNS was a vital component for the biosensor, so the quenching ability of CNNS was investigated. As shown in Figure 2A, when FAM-P1 and FAM-P2 concentrations were fixed at 50 nM, the fluorescence signal was gradually decreased when increasing amounts of CNNS were added; quenching was effective when CNNS concentration was 200 μg/ml (Figure S2). CNNS at high concentrations can reduce the sensitivity of the sensing platform because amplification products could be adsorbed onto the CNNS surface, so concentration given above was used to prepare P1/P2–CNNS. The concentrations to prepare P1–CNNS or P2–CNNS were also optimized at 100 μg/ml CNNS to 50 nM FAM-P1 or FAM-P2 (Figure S3).

3.3 | Signal amplification of the biosensor

To verify the amplify strategy, P2–CNNS and P1–CNNS were hybridized with miR-106a and the results compared. As shown in Figure 2A, in the absence of miR-106a, the fluorescence signal of nanoprobe was weak (Figure 2B, curve d). Upon addition of miR-106a, there was no obvious fluorescence enhancement compared with P2–CNNS (Figure 2B, curve c), but some enhancement of fluorescence intensity compared with P1–CNNS could be observed, due to one-step hybridization between miR-106a and FAM-P1 to form the P1–RNA complex (Figure 2B, curve b), far away from CNNS. The fluorescence signal was clearly enhanced after the miR-106a reaction with P1/P2–CNNS (Figure 2B, curve a), suggesting that the P1/P2–CNNS fluorescence signal arose from miRNA-triggered CHA. This result confirmed successful signal amplification.

3.4 | Specificity and stability of the biosensor

To evaluate the specificity of the miRNA sensing platform, the control alone or with non-complementary RNA, three-base mismatched RNA, or single-base mismatched RNA was incubated with the P1/P2–CNNS at the same concentration of miR-106a, respectively. As shown in Figure 3A, only miR-106a could induce significant fluorescence enhancement. The fluorescence enhancement produced by miR-106a was at least 4.5-fold that produced by other RNAs, suggesting the miRNA biosensor has excellent specificity, due to the P1/P2 stem–loop structure. Furthermore, no obvious fluorescence enhancement from P1/P2–CNNS could be observed with the pH change from 8.0 to 4.7, which is the acidic

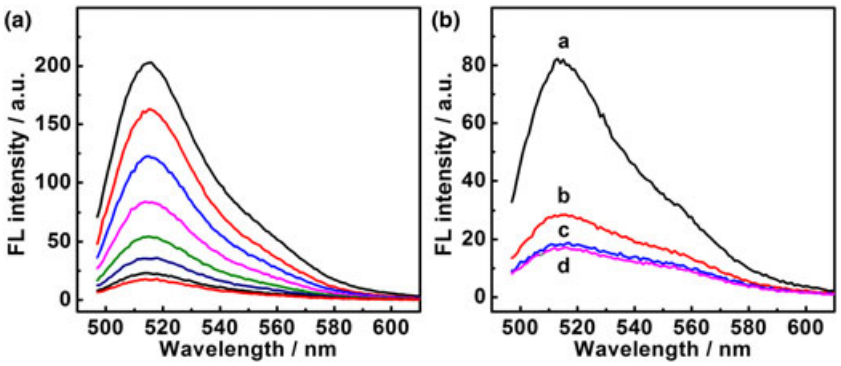


FIGURE 2 (a) fluorescence spectra of 50 nM FAM-P1 and FAM-P2 incubated with various amounts of CNNS at 37°C for 0.5 h (from top to bottom: 0, 30, 60, 90, 120, 150, 180, and 200 μg/ml). (B) fluorescence spectra of 200 μg/ml P1/P2–CNNS (a), P1–CNNS (b) and P2–CNNS (c) incubated with 300 pM miR-106a at 37°C for 4 h, respectively. P1/P2–CNNS (d) as control

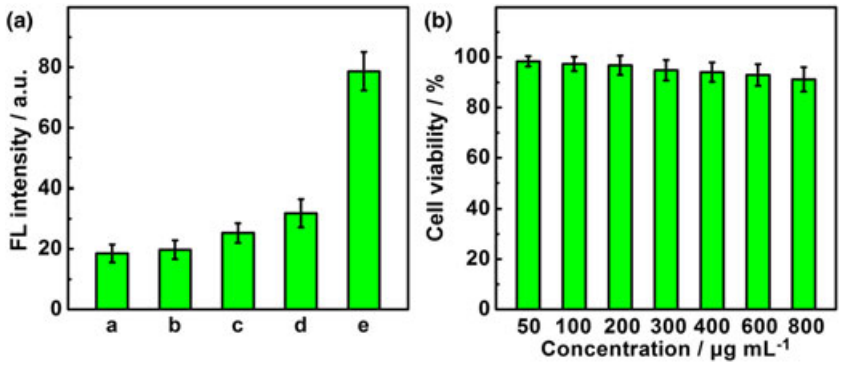


FIGURE 3 (a) fluorescence intensities obtained by incubating 200 μg/ml P1/P2–CNNS (a, control) with 300 pM non-complementary RNA (b), single-base mismatched RNA (c), three-base mismatched RNA (d) and miR-106a (e) at 37°C for 4 h. (B) cytotoxicity of materials in PC12 cells. PC12 cells were incubated with various amounts of CNNS at 37°C for 8 h

environment of the endocytic pathway in living cells (Figure S4). Therefore, the biosensor has high sequence specificity and excellent stability for application in living cells.

3.5 | MTT assay of the materials

To detect intracellular miR-106a in cells, CNNS is expected to possess low cytotoxicity and good biocompatibility, therefore an MTT assay was performed to investigate cytotoxicity. PC12 cells were incubated with the increasing concentrations of CNNS, which displayed limited effects on PC12 cells viability. Even up to a concentration of 800 μg/ml, CNNS induced only a small decrease in PC12 cell viability (Figure 3B), indicating that CNNS has only low cytotoxicity. CNNS was found to possess better biocompatibility than GO (Figure S5). Therefore 200 μg/ml CNNS was used for miRNA sensing, which ensured high viability of the cells.

3.6 | Detection of miRNA with the biosensor *in vitro*

The capability of the CNNS-based enzyme-free biosensor to detect miRNA *in vitro* was evaluated. As shown in Figure 4, after P1/P2–CNNS was incubated with different concentrations of miR-106a, fluorescence intensity enhancement (ΔI) increased dramatically with increase in miR-106a concentration (c). This correlated linearly with equation $\Delta I = 0.1761C + 3.1$, with a correlation coefficient R^2 of 0.9962, and a concentration range of 0.5–600 pM. The detection limit was 0.10 pM. The proposed CNNS-based enzyme-free biosensor displayed high sensitivity, which was derived from the excellent fluorescence quenching capacity of CNNS and the signal amplification of CHA.

3.7 | *In vivo* amplify strategy of the biosensor

To investigate signal amplification of the developed strategy in living cells, processing time and P1/P2–CNNS concentration were evaluated to be 8.0 h and 200 μg/ml (Figure S6). P2–CNNS and P1–CNNS served as controls. PC12 cells that expressed miR-106a were cultured with P1/P2–CNNS, P1–CNNS or P2–CNNS for 8.0 h, respectively. As shown in Figure 5A, the fluorescence signal from P1/P2–CNNS was distinctly observed (Figure 5A, column a), and the fluorescence signal from the P1–CNNS was markedly weaker than that of P1/P2–CNNS (Figure 5A, column b), indicating that the P1/P2–CNNS fluorescence signal derived from miR-106a-triggered CHA. In contrast, a faint fluorescence signal was observed from the P2–CNNS cultured cells (Figure 5A, column c), due to baseline and interference signals. P2–CNNS cultured cells therefore were used as the blank to compensate for background signals for flow cytometric analysis.

3.8 | Monitoring of miRNA in living cells

PC12 cells were incubated with $A\beta_{1-42}$ and miRNA inhibitor to acquire different miRNA expression levels. PC12 cells treated with P1/P2–CNNS served as the control group. The miR-106a expression level of the $A\beta_{1-42}$-induced group (model group) was about 70.7% of the control group (Figures 5B, column b, and S7B). The miR-106a expression level of the inhibitor-106a-treated group was 51.7% of the control group (Figures 5B, column c, and S7c), whereas the miR-106a expression level of the co-action group (model group treated with inhibitor-106a) was 34.8% of the control group (Figures 5B, column d, and S7d). Thus, the proposed method was able to achieve *in situ* detection of relative intracellular miRNA expression levels.

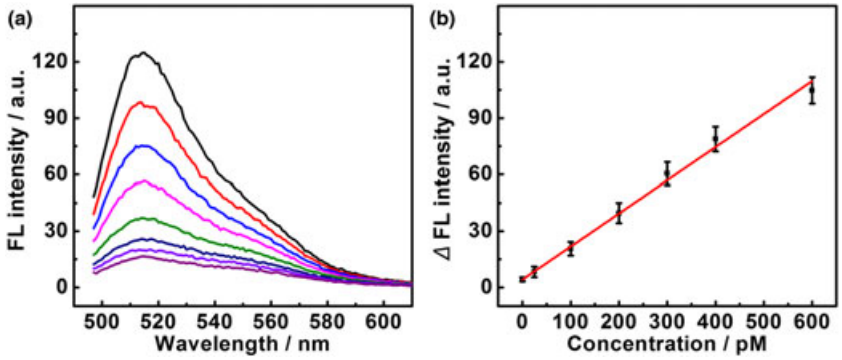


FIGURE 4 Fluorescence spectra of (a) 200 μg/ml P1/P2–CNNS in the presence of various miR-106a concentrations at 37°C for 4 h (from bottom to top: 0, 0.5, 25, 100, 200, 300, 400, and 600 pM). (B) calibration curve for the fluorescence intensity enhancement versus miR-106a concentrations

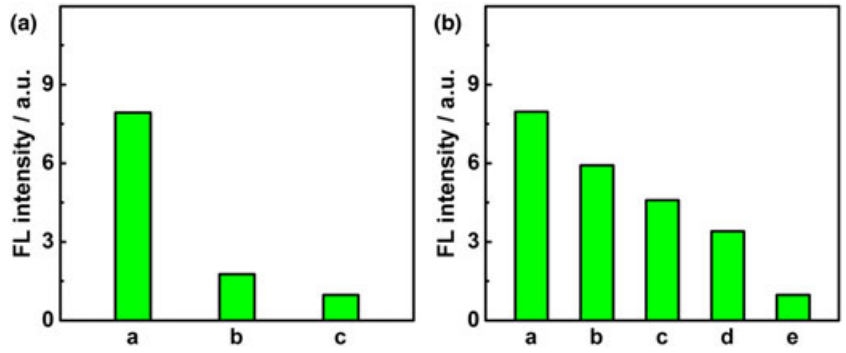


FIGURE 5 (a) fluorescence intensities obtained by flow cytometric analysis of PC12 cells transfected with 200 μg/ml P1/P2–CNNS (a), P1–CNNS (b), and P2–CNNS (c). (B) fluorescence intensities obtained by flow cytometric analysis of PC12 cells (a), $A\beta_{1-42}$-induced PC12 cells (b), PC12 cells treated with 50 nM inhibitor-106a for 24 h (c), $A\beta_{1-42}$-induced PC12 cells treated with 50 nM inhibitor-106a for 24 h (d) at 37°C for 8 h, respectively, and PC12 cells transfected with P2–CNNS as blank (e)

4 | CONCLUSIONS

In conclusion, a novel signal-on biosensor for ultrasensitive and convenient monitoring of miRNA in living cells was designed based on target-triggered signal amplification and the strong fluorescence quenching ability of CNNS. The proposed strategy is convenient as no thermal cycling or enzyme is needed. Excellent amplification of CHA led to good sensitivity and specificity for monitoring intracellular miRNA in cells. Due to its strong fluorescence quenching ability and low cytotoxicity, CNNS can serve as a biocompatible gene vector. Therefore, the proposed method provides a convenient, sensitive and specific biosensor for monitoring miRNA in living cells.

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SUPPORTING INFORMATION

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