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RESEARCH ARTICLE

Ultra-sensitive fluorescent sensor for intracellular miRNA based on enzyme-free signal amplification with carbon nitride nanosheet as a carrier

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Abstract

A novel ultra-sensitive fluorescent sensor for monitoring microRNA (miRNA) in living cells was constructed by utilizing a hybridization chain reaction (HCR) as the signal amplification with a carbon nitride nanosheet (CNNS) as a carrier. The Cy5-labeled hairpin DNA could be adsorbed onto the surface of CNNS, resulting in fluorescence quenching of Cy5. When treated with complementary miRNA, the fluorescence was recovered because miRNA could efficiently trigger an HCR, which led to the release of the HCR products from the CNNS. This intracellular HCR strategy can be used for ultra-sensitive monitoring of intracellular miRNA. The main advantages of the proposed method are its simplicity, high sensitivity, high specificity and low toxicity for monitoring low-level biomarkers.

KEYWORDS

carbon nitride nanosheet, fluorescence biosensor, intracellular signal amplification, microRNA

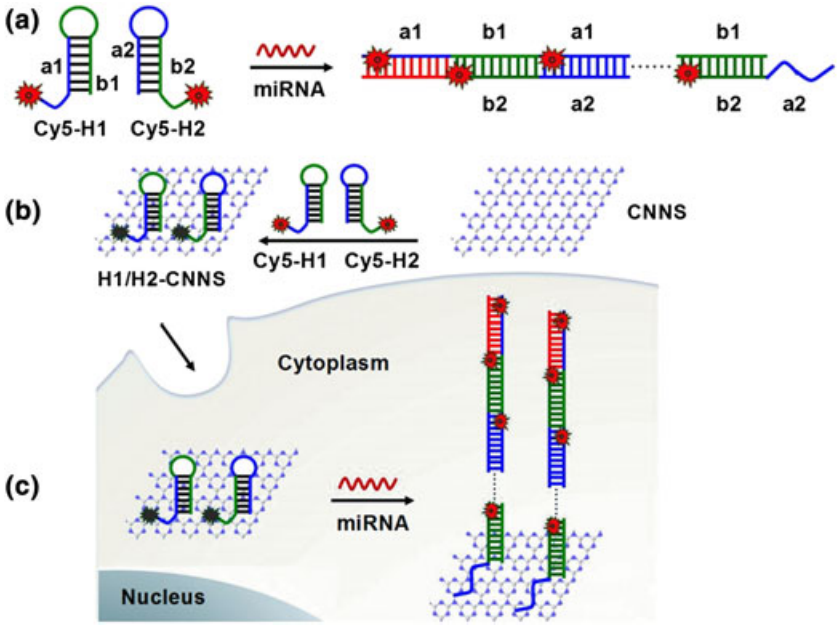
1 | INTRODUCTION

MicroRNAs (miRNAs) play significant regulatory roles in biological processes,[1–4] and the abnormal expression of miRNAs can contribute to various diseases.[5–9] Notably, miRNAs are expressed at low levels in cells. Thus, ultra-sensitive sensing of miRNAs is crucial to detection of miRNA-related diseases. Some methods have been developed to improve the sensitivity of miRNA analysis, including microarrays, RT-PCR, northern blot analysis and various new strategies.[10–14] However, none of these procedures allowed monitoring of miRNA in living cells. Recently, a few methods for *in situ* monitoring of intracellular miRNA have been established,[15–19] but these methods were short of signal amplification strategies, therefore ultra-sensitive monitoring of miRNAs in living cells is still a challenge.

Due to its simple operation and signal amplification, HCR has aroused much attention for sensitive analyses,[20–22] but the use of HCR-based intracellular signal amplification has been rarely reported. Recently, bulk graphitic-phase carbon nitride has been widely utilized in the biological fields.[23–25] CNNS, which could be extracted from bulk graphitic-phase carbon nitride to directly disperse in aqueous solution without the need for surfactants or oxidation treatment,[26] has several unique emerging properties, including strong fluorescence quenching ability, low cytotoxicity, and good biocompatibility. Our previous studies have proposed use of confocal imaging using CNNS for detection of intracellular miRNA,[16,27] but CNNS has not been used for HCR-based detection of intracellular miRNAs to date, so we propose here the use of an ultra-sensitive fluorescent sensor for monitoring intracellular miRNA by utilizing HCR with CNNS.

Cy5-labeled hairpin DNA 1 and hairpin DNA 2 of HCR (Cy5–H1 and Cy5–H2) were designed according to the miRNA-107 method (Scheme 1A). When miRNA-107 was presence, it could specifically hybridize with one part of H1 (a1) to open the hairpin and release the other part of H1 (b1), then b1 hybridized with one part of H2 (b2) and opened the hairpin to expose the other part of H2 (a2), which was identical in sequence to the miRNA-107 and could trigger another cycle. The H1/H2–CNNS complex was prepared by loading Cy5–H1

Abbreviations: $A\beta_{1-42}$, amyloid beta 42 peptide; CNNS, carbon nitride nanosheet; Cy5–H1, Cy5-labeled hairpin DNA 1 of HCR; Cy5–H2, Cy5-labeled hairpin DNA 2 of HCR; H1/H2–CNNS, CNNS simultaneously assembled with Cy5–H1 and Cy5–H2; H1–CNNS, CNNS assembled with Cy5–H1; H2–CNNS, CNNS assembled with Cy5–H2; FL, fluorescence; HCR, hybridization chain reaction; PBS, phosphate-buffered saline; PC12 cells, pheochromocytoma cells; TEM, transmission electron micrograph.



SCHEME 1 SCHEME for (a) HCR, (b) H1/H2-carbon nitride nanosheet (CNNS) preparation and (c) sensing of miRNA in living cells

and Cy5–H2 onto the CNNS surface by strong π–π interactions (Scheme 1B) with fluorescence quenching,[28,29] the quenching could be attributed to static electronic communication between dyes and CNNS.[29] After H1/H2–CNNS was transfected into cells, intracellular miRNA-107 could specifically trigger HCR to autonomously assemble a DNA polymer, which weakened the π–π interaction between bases and CNNS, leading to the release of the HCR products from the CNNS and fluorescence recovery (Scheme 1C). By detecting the change in fluorescence, a non-destructive and specific fluorescence strategy for miRNA sensing in living cells could be realized. The proposed method offers a new method with simplicity, high sensitivity and specificity for monitoring miRNA in living cells.

2 | EXPERIMENTAL

2.1 | Chemicals and materials

PC12 cells were from the Shanghai Institute of Cellular Biology of Chinese Academy of Sciences; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from KeyGen Biotech. Co. Ltd (Nanjing, China); $A\beta_{1-42}$ was from Sigma-Aldrich Inc. (USA); 1× sodiumphos-phate -sodium chloride buffer solution (SPSC buffer) (50 mM Na_2HPO_4 and 0.75 M NaCl, pH 7.4), Opti-MEM and Lipofectamine 2000 were from Invitrogen Corporation (USA); RNA were synthesized by GenePharma Co., Ltd. (Shanghai, China); DNA was synthesized by Sangon Biological Biotechnology Co., Ltd. (Shanghai, China). The sequences are listed in Table 1. The T_m of miRNA-107 was 58.4°C, which was calculated with computation of mfold.[30]

2.2 | Instrumentation

UV–vis absorption measurements were performed on a UV-2401PC UV–vis spectrophotometer (Shimadzu, Japan). Fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu, Japan). A microplate reader (VICTOR™ 5, PerkinElmer, USA) was used for the MTT assay. A JEM-2100 TEM instrument (JEOL, Japan) was used for transmission electron micrograph (TEM) imaging. A Coulter FC-500 flow cytometer (Beckman-Coulter, USA) was used for flow cytometric analysis.

2.3 | Preparation of the carbon nitride nanosheet

The carbon nitride nanosheet (CNNS) was prepared from bulk graphitic-phase carbon nitride (g-C_3N_4) according to previously published methodology.[16,29] Firstly, bulk g-C_3N_4 was prepared by

TABLE 1 Oligonucleotides sequences

Name	Sequences (5′-3′)
miRNA-107	5′-AGCAGCAUUGUACAGGGCUAUCA-3′
Inhibitor-107	5′-UGAUAGCCCUGUACAAUGCUGCU -3′
Cy5–H1	5′-ATTGTACAGGGCTATCACAAAGTTGATAGCCCTGTACAATGCTGCT- Cy5–3′
Cy5–H2	5′-Cy5-ACTTTGTGATAGCCCTGTACAATAGCAGCATTGTACAGGGCTATCA -3′
Non-complementary RNA	5′-UGGAGUGUGACAAUGGUGUUUG-3′
Single-base mismatched RNA	5′-AGCAGCAUUGU*g*CAGGGCUAUCA-3′
Three-base mismatched RNA	5′-AGCAGCA*g*UGU*g*CAG*a*GCUAUCA-3′

Mismatched bases are in lowercase letters.

polymerization of melamine molecules at high temperature. Then CNNS was prepared by sonicating the bulk g-C_3N_4 in water. The sonicated mixture was first centrifuged at 5000 rpm to remove aggregated bulk g-C_3N_4 particles and obtain the CNNS suspension, which was centrifuged at 15 000 rpm to obtain CNNS.

2.4 | Preparation of nanoprobe

To prepare H1/H2–CNNS, CNNS was diluted with the SPSC buffer and sonicated for 5 min, Cy5–H1 and Cy5–H2 were added and gently stirred for 0.5 h, then centrifuged to obtain the H1/H2–CNNS product. After redispersion, the concentration of H1/H2–CNNS was expressed as the concentration of CNNS. The concentration of CNNS for 50 nM Cy5–H1 and 50 nM Cy5–H2 to prepare H1/H2–CNNS was 200 μg/mL. The H1–CNNS and H2–CNNS materials were prepared by a similar process. Cy5–H1 or Cy5–H2 was incubated at 95°C for 5 min, then slowly cooled to room temperature for 1 h to fold into hairpin structures before use.

2.5 | Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 80 U/mL penicillin, and 80 μg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.6 | Cytotoxicity assay

The PC12 cells were seeded at 10 000 cells per well in a 96-well microplate with 100 μL Opti-MEM for 12 h. After the medium was removed, these PC12 cells were incubated with 100–800 μg/mL CNNS for 8 h, and the PC12 cells incubated with Opti-MEM served as the control. The PC12 cells were washed with phosphate-buffered saline (PBS) three times, and 50 μL 1 mg/mL MTT was added to each well for 4 h. After the medium was removed, and 150 μL dimethylsulfoxide was added to each well to dissolve the formed formazan dye for 15 min, then the absorbance measurements for each well were obtained at 490 nm.

2.7 | *In vitro* fluorescence detection of miRNA

Next, 200 μg/mL H1/H2–CNNS were incubated with different concentrations of miRNA-107 in SPSC buffer for 4 h at room temperature, the fluorescence signals were recorded. Fluorescence of Cy5 were excited at 643 nm, the fluorescence emission maximum occurred at 667 nm.

2.8 | Monitoring of miRNA in living cells

The PC12 cells were seeded at 100 000 cells per dish in culture dish with a volume of 5 mL/well for 12 h. Cells were separated into four groups: (a) Control group, treated with Opti-MEM at 37°C; (b) $A\beta_{1-42}$-induced group, treated with 5 μM $A\beta_{1-42}$ oligomers for 24 h at 37°C; (c) inhibitor-107 group, treated with 50 nM inhibitor-107 for 24 h at 37°C; and (d) $A\beta_{1-42}$-induced + inhibitor-107 group, treated with 50 nM inhibitor-107 for 24 h after pre-treatment with 5 μM $A\beta_{1-42}$ oligomers for 24 h at 37°C. After washing with PBS, these cells were treated with 200 μg/mL H1/H2–CNNS for 8 h to perform the flow cytometric analysis. The normal PC12 cells transfected with 200 μg/mL H2–CNNS were used as the blank, and inhibitor-107 was transfected by Lipofectamine 2000 according to the instruction manual.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of nanoprobe

The TEM image of CNNS clearly showed a dimension of around 135 nm with a planar structure (Figure 1a). The successful preparation of H1/H2–CNNS was examined. When Cy5–H1 and Cy5–H2 were adsorbed onto CNNS, the H1/H2–CNNS zeta potential became more negative than the CNNS zeta potential (Figure 1b), which was due to the negatively charged phosphate of Cy5–H1 and Cy5–H2. And the absorption spectrum of H1/H2–CNNS showed the characteristic peak of Cy5 and remarkable hypochromicity relative to the free Cy5–H1 and Cy5–H2 (Figure S1),[29,31] which confirmed that Cy5–H1 and Cy5–H2 were adsorbed onto CNNS, thus further verified the successful preparation of the nanoprobe.

3.2 | The fluorescence quenching capability of CNNS

CNNS served as a fluorescence quencher and a novel carrier to construct the nanoprobe, whether or not the fluorescence of Cy5–H1 and Cy5–H2 could be effectively quenched by CNNS was crucial for effectiveness of the sensor, so the fluorescence quenching capability of CNNS was evaluated. As shown in Figure 2(a), when the concentrations of Cy5–H1 and Cy5–H2 were fixed, the fluorescence intensity

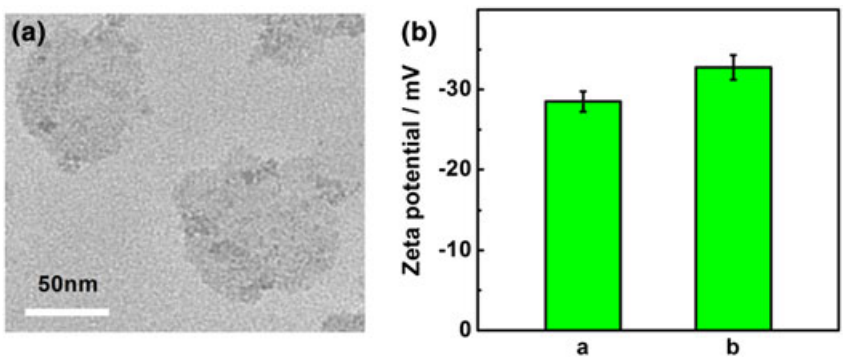


FIGURE 1 (a) TEM image of CNNS, (b) zeta potential analysis for (a) CNNS and (b) H1/H2-CNNS

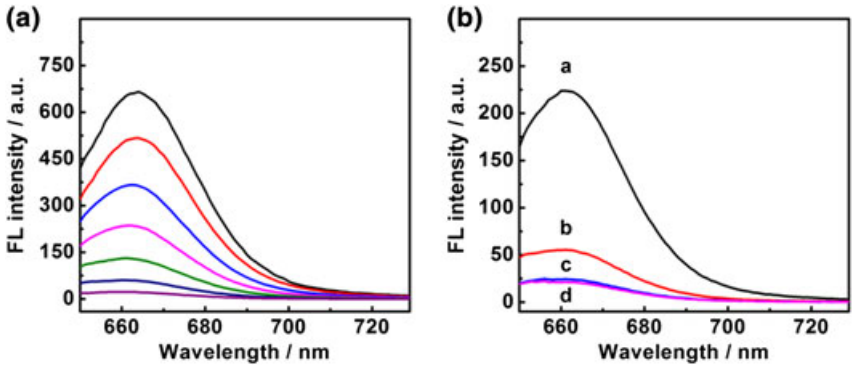


FIGURE 2 Fluorescence spectra of (a) 50 nM Cy5-H1 and Cy5-H2 incubated with various amounts of CNNS at 37°C for 0.5 h (from top to bottom: 0, 40, 80, 120, 160, 180, and 200 μg/mL), and (B) 200 μg/mL H1/H2-CNNS (a), H1-CNNS (b) and H2-CNNS (c) incubated with 400 pM miRNA-107 at 37°C for 4 h, respectively, H1/H2-CNNS (d) as a contrast

was gradually decreased with increasing CNNS concentration, and tended to be efficiently quenched when the CNNS was at the 200 μg/mL concentration (Figure S2), so this ratio was used to prepare H1/H2–CNNS. The ratio to prepare H1–CNNS or H2–CNNS was also evaluated to be 100 μg/mL CNNS for 50 nM Cy5–H1 or Cy5–H2 (Figure S3).

3.3 | *In vitro* response of the sensing platform

The signal amplification of the sensing platform was evaluated. H2–CNNS and H1–CNNS served as the control to verify successful realization of amplification. Upon addition of miRNA-107, no obvious fluorescence enhancement from H2–CNNS could be observed (Figure 2b, curve c), and the fluorescence enhancement from H1–CNNS could be observed because of one-step hybridization between miRNA-107 and Cy5–H1 (Figure 2B, curve b), but it was significantly weaker than the fluorescence intensity derived from H1/H2–CNNS (Figure 2b, curve a), indicating the fluorescence intensity of H1/H2–CNNS indeed resulted from miRNA-triggered HCR, which verified the successful signal amplification of the proposed strategy.

3.4 | Specificity and stability of the proposed strategy

The specificity of the proposed strategy was evaluated. Non-complementary RNA, three-base mismatched RNA, single-base mismatched RNA and miRNA-107 at the same concentration were incubated with H1/H2–CNNS, respectively. Non-complementary RNA was used to evaluated the possibility of fluorescence enhancement from the replacement of the adsorbed Cy5-labeled hairpin DNA on CNNS with the off-target RNA. Only miRNA-107 could induce a great fluorescence enhancement, while non-complementary RNA, three-base and single-base mismatched RNA failed to generate significant fluorescence signals, indicating that the proposed approach has high sequence specificity (Figure 3a), which due to the stem–loop structure of H1/H2. Notably, no obvious fluorescence enhancement could be observed with the pH change from 4.7 to 8.0, which is the acidic environment of the endocytic pathway (Figure 3b). Thus, H1/H2–CNNS has excellent specificity and stability potentials for monitoring miRNA in living cells.

3.5 | Cytotoxicity assay of the materials

To realize *in situ* monitoring of miRNA-107 in living cells, CNNS is expected to have good biocompatibility and low cytotoxicity. PC12 cells were treated with increasing amounts of CNNS, and showed negligible effects on cell viability. Even up to 800 μg/mL, CNNS induced little reduction in the viability of PC12 cells (Figure 4a), suggesting that CNNS possessed low cytotoxicity. And the CNNS has better security than GO (Figure 4b). Throughout the present study, we used CNNS with a concentration of 200 μg/mL, thereby ensuring high cell viability.

3.6 | Detection of miRNA with the proposed strategy *in vitro*

The ability of H1/H2–CNNS to detect miRNA *in vitro* was investigated. As shown in Figure 5, after H1/H2–CNNS was incubated with different concentrations of miRNA-107, the fluorescence intensity increased with the increasing concentration of miRNA-107, with a

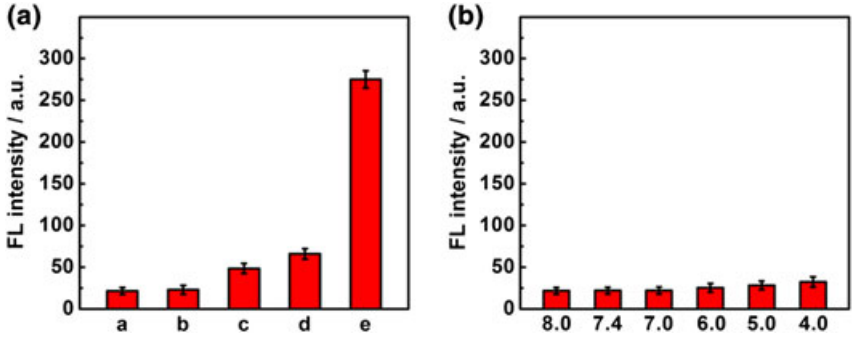


FIGURE 3 (a) fluorescence intensities obtained by incubating 200 μg/mL H1/H2-CNNS (a, contrast) with 500 pM non-complementary RNA (b), single-base mismatched RNA (c), three-base mismatched RNA (d) and miRNA-107 (e) at 37°C for 4 h. (B) fluorescence intensities obtained by incubating 200 μg/mL H1/H2-CNNS in buffer of varying pH values

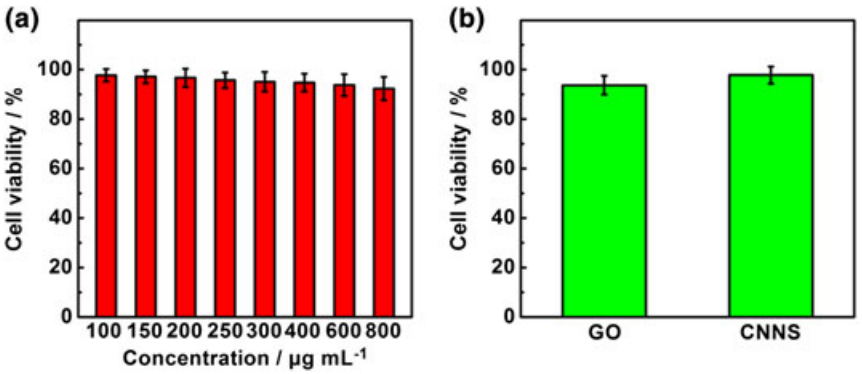


FIGURE 4 Cytotoxicity of materials in PC12 cells. PC12 cells incubated with (a) various amounts of carbon nitride nanosheet (CNNS), and (b) 200 μg/mL graphene oxide (GO) or CNNS at 37°C for 8 h

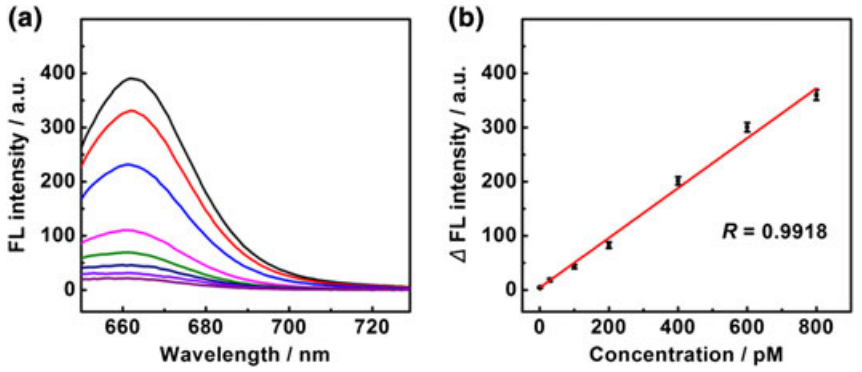


FIGURE 5 Fluorescence spectra of (a) 200 μg/mL H1/H2-CNNS in the presence of various miRNA-107 concentrations at 37°C for 4 h (from bottom to top: 0, 1, 30, 100, 200, 400, 600, and 800 pM). (b) calibration curve for the fluorescence change versus miRNA-107 concentrations

linear range from 1 pM to 800 pM and a detection limit of 0.20 pM on the basis of 3σ per slope, suggesting that the proposed strategy has excellent sensitivity.

3.7 | *In vivo* response of the sensing platform

To confirm the amplification of the proposed strategy, the operation time and concentration of H1/H2–CNNS were optimized to be 8 h and 200 μg/mL with flow cytometric analysis (Figure S4). $A\beta_{1-42}$-induced PC12 cells were commonly used as an Alzheimer's disease cell model, and miRNA-107 was closely associated with Alzheimer's disease. After $A\beta_{1-42}$-induced PC12 cells were incubated with H1/H2–CNNS, H1–CNNS or H2–CNNS for 8 h, the fluorescence intensity derived from H1/H2–CNNS was clearly observed (Figure 6A, a), and the fluorescence intensity from the H1–CNNS was significantly weaker than that for H1/H2–CNNS (Figure 6A, b), suggesting that the fluorescence intensity of H1/H2–CNNS indeed resulted from miRNA-107 triggered HCR. Whereas faint fluorescence was observed from the H2–CNNS transfected cells (Figure 6A, c), which derived from the baseline of the flow cytometer and interference signals. So H2–CNNS transfected cells were used as the blank to eliminate background values for flow cytometric analysis.

3.8 | Monitoring of miRNA in living cells

The PC12 cells were treated with $A\beta_{1-42}$ oligomers and miRNA inhibitor to obtain different expression levels of miRNA. The PC12 cells incubated with H1/H2–CNNS served as the control. The miRNA-107 level of the $A\beta_{1-42}$-induced group was 57.7% of the control group (Figures 6b,b and 7b), and the miRNA-107 levels of the inhibitor-107 treated group showed values that were 50.4% of the control group (Figures 6b,c and 7c), while the miRNA-107 levels of the $A\beta_{1-42}$ and inhibitor-107 simultaneously treated group showed values that were about 27.3% of the control group (Figures 6b,d and 7d). So, the

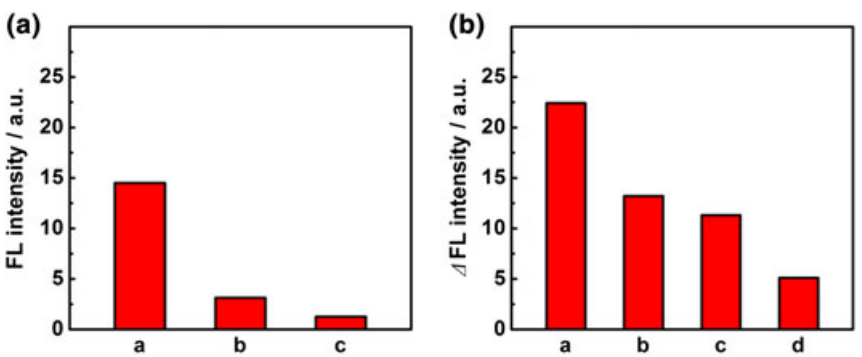


FIGURE 6 (a) fluorescence intensities obtained by flow cytometric analysis of Aβ1–42-induced PC12 cells transfected with 200 μg/mL H1/H2-CNNS (a), H1-CNNS (b) and H2-CNNS (c). (b) fluorescence change obtained by flow cytometric analysis of PC12 cells (a), Aβ1–42-induced PC12 cells (b), PC12 cells treated with 5 nM inhibitor-107 for 24 h (c), Aβ1–42-induced PC12 cells treated with 5 nM inhibitor-107 for 24 h (d) at 37°C for 8 h, respectively

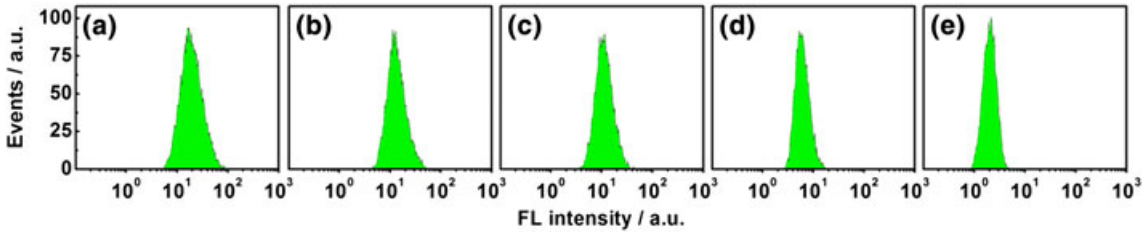


FIGURE 7 Histograms obtained by flow cytometric analysis of PC12 cells (a), Aβ1–42-induced PC12 cells (b), PC12 cells treated with 5 nM inhibitor-107 for 24 h (c), Aβ1–42-induced PC12 cells treated with 5 nM inhibitor-107 for 24 h (d), and the normal PC12 cells transfected with H2-CNNS as blank (e)

proposed strategy could realize detection of relative expression levels of miRNA in living cells.

4 | CONCLUSION

In summary, we have utilized the strong quenching ability of CNNS on Cy5 fluorescence to design a novel fluorescent switch-on sensor for ultra-sensitive and specific monitoring of intracellular miRNA. The PCR-free nature of the proposed strategy, which can avoid false-positive results, and the enormous intensity amplification of HCR, led to high sensitivity and specificity conditions for monitoring of miRNA in living cells. Furthermore, due to the strong fluorescence quenching ability and low cytotoxicity of CNNS, the proposed strategy was more biocompatible. Therefore, the proposed strategy provides a simple, sensitive and specific platform for detection of miRNA in living cells.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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