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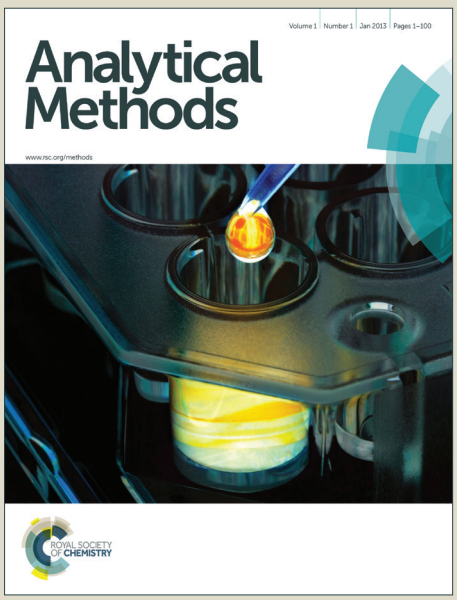

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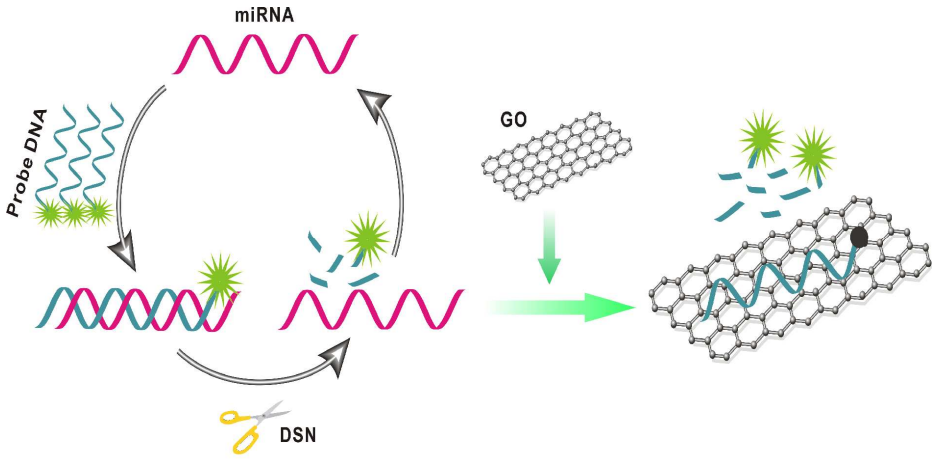

Graphene Oxide-based fluorescent sensing system for sensitive detection of miRNA with duplex-specific nuclease-aided signal amplification strategy

Amplified fluorescence sensing of miRNA by combination of graphene oxide with duplex-specific nuclease

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Abstract

A novel fluorescent sensing platform for miRNA detection is developed in this work, which simply combines the fluorescence quenching efficiency of graphene oxide (GO) and the duplex-specific nuclease (DSN)-induced target recycling. Fluorophore-labeled DNA strands acting as probes were physically adsorbed onto the GO surface, leading to fluorescence quenching. In the presence of target miRNA, the DSN cleaved the probe DNA in DNA-RNA hybrid duplex into small fragments and the miRNA was released from the duplex. Thus the recycling of the target miRNA was realized, producing numerous small DNA fragments. After the introduction of GO into the sensing solution, a strong fluorescence emission was observed due to the weak interaction between the short DNA fragments and GO. With this approach, a sub-picomolar detection limit of miRNA could be achieved within 40 min. What is more, this biosensor exhibited good sequence selectivity due to the great sequence discrimination ability of DSN. The proposed sensor is sensitive, specific, simple and rapid, paving a way to the miRNA analysis.

1 Introduction

MicroRNAs (miRNAs) are a group of short endogenous noncoding ribonucleic acids (RNAs) molecules found in eukaryotic cells with approximately 18-25 nucleotides (nt) in length and play critical regulatory roles in a variety of biological processes such as cell proliferation, differentiation, apoptosis, and immunological response by suppressing gene expression through incorporation into an active RNA-induced silencing protein complex.[1,2] Specifically, aberrant expression of miRNA is commonly observed in cancer initiation, oncogenesis, and tumor response to treatment,[3] and their expression patterns in cancers appear to be tissue-specific.[4] The detection of miRNAs has thus become a rapidly emerging field for further understanding the biochemical function of miRNAs and exploring useful diagnostic and prognostic markers of diseases. Currently, some technologies have been developed for the detection of miRNAs. The most commonly used ones are the northern blotting,[5] microarrays,[6,7] and real-time quantitative polymerase chain reaction (qRT-PCR).[8,9] However, these methods have some limitations, such as tedious procedure, poor reproducibility, contamination or low sensitivity. Furthermore, reverse transcription is always an essential step for the above approaches and it undoubtedly increases the experimental cost and design complexity. To avoid these disadvantages, a number of advanced methods have been established to identify and quantify miRNAs, such as rolling-circle amplification (RCA),[10-12] exponential amplification reaction (EXPAR),[13] as well as a variety of nanotechnology-based methods.[14,15] However, the miRNA analysis is still challenging, owing to its unique

characteristics, including short size, sequence homology among family members, quite low abundance expression levels (ca. 0.001% of the mass in total RNA sample or a few of molecules per cell), and susceptibility to degradation. From the above, the further improved profile strategy for rapid and sensitive analysis of miRNA is an insistent demand.

Graphene oxide (GO), a single-atom-thick, two-dimensional carbon material that prepared by acid exfoliation of graphite,[16] has attracted a growing interest in biological and biomedical applications due to its unique characteristics such as good water-solubility,[17] flexible modification and super fluorescence quenching ability.[18] Particularly, recent interest lies in the interaction of nucleic acids with GO. It has been proven that GO can adsorb single-stranded nucleic acids strongly *via* non-covalent π-stacking interactions between the rings in the nuclebases and the hexagonal cells of the GO but hardly interact with rigid double-stranded nucleic acids.[18] Given these remarkable properties, GO has been used as a platform for the detection of nucleic acids,[19-22] proteins,[23] enzyme activity,[24-26] and mental ions.[27] Several GO-based fluorescent sensors for the detection of miRNA have also been designed by combining with various enzymatic amplifications.[28,29] Recently, Zhao and coworkers made a detailed investigation on the difference of affinity of GO for ssDNA with different numbers of bases in length and proved that short ssDNA had weaker affinity to GO than long ssDNA.[29] Based on this discovery, the GO-base fluorescent biosensors had been successfully applied to detect bleomycin,[31] Cu^{2+} ,[32] mammalian Argonaute2 (Ago2) ,[33] *etc.*

Herein, we report on a new GO-based fluorescent platform for simple, quick, specific and sensitive detection of miRNA. This method relies on duplex-specific nuclease (DSN)-aided signal amplification and the GO's particular quenching capability. DSN is a nuclease purified from hepatopancreas of the Kamchatka crab (*Paralithodes camtschaticus*).[34,35] It displays a strong preference for cleaving dsDNA or DNA in DNA-RNA hybrid duplexes, and is practically inactive towards ssDNA or RNA substrates. Moreover, this enzyme shows a good capability to discriminate between perfectly and nonperfectly matched (up to one mismatch) short duplexes. Very recently, DSN has been employed to detect miRNA by duplex-specific nuclease signal amplification (DSNSA),[36] backbone-modified molecular beacons (MBs),[37] and electrochemical methods,[38] and shows great sensitivity. Compared with those reported approaches, our proposed design is much simpler and more efficient, and it does not need complex chemical modification or quencher labeling which would be time-saving and cost-effective.

2 Experimental section

2.1 Materials and reagents

The FAM-labeled DNA probe and the miRNAs were all purchased from Takara Biotechnology Co. Ltd. (Dalian, China), which were purified by HPLC. The sequences of these oligonucleotides are listed in Table 1. Duplex-specific nuclease (DSN) was obtained from Evrogen Joint Stock Company (Russia). Tris, DTT, NaCl and all the other chemicals were purchased from Generay Biotech Co. Ltd. (Shanghai,

China). Ultrapure water obtained from a Millipore water purification system (18.2 MΩ resistivity, Milli-Q Direct 8) was used in all runs. Graphite powder (99.9995%, 325 mesh) was purchased from Alfa Aesar (Ward Hill, MA, USA), and graphene oxide (GO) was synthesized from it by a modified Hummers' method.[39] The good characterization of GO had been described in our previously reported work.[22] The GO was then sonicated in Milli-Q purified water for 2 h to get a homogeneous brown solution (500 μg mL^{-1}) and stored at 4 ℃ for use. All the experiments involving miRNA were carried out in an RNase-free environment. All buffer solutions and water were treated with 0.1% DEPC and autoclaved. The tips and tubes are RNase-free and don't require pretreatment to inactive RNases.

(Table 1)

2.2 Apparatus

Fluorescent emission spectra were carried on a Hitachi F-4600 spectrophotometer (Hitachi Co.Ltd., Japan) equipped with a Xenon lamp excitation source. The excitation was set at 495 nm and the emission was monitored at 520 nm. The sample cell was a 0.35 mL quartz cuvette. The fitting of the experimental data was accomplished using the software Origin 8.0.

2.3 Procedure for the fluorescence detection

The miRNA detection procedure included two sequential steps. First, a volume of 20 μL reaction mixture containing 1×DSN master buffer (50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$, 1 mM DTT), 0.075 U DSN (disolved in 25 mM Tris-HCl, pH 8.0; 50% glycerol), probe DNA (4 μL, 1 μM) and target miRNA at different concentrations, was

incubated at 45 ℃ for 25 min, then cooled to room temperature. Subsequently the mixture was diluted with 178.8 μL of Tris-HCl buffer solution (25 mM, pH 7.4, 50 mM NaCl), after which the GO (1.2 μL, 500 μg mL^{-1}) was added. After incubation for 5 min, the fluorescence of the mixture was measured.

3 Results and discussion

3.1 Principle of amplified miRNA detection

The detailed principle of the GO-DSN based fluorescent sensing system is outlined in Scheme 1. We designed the probe DNA in which the 5′ end is labeled with a fluorophore carboxyfluorescein (FAM). In the absence of specific target miRNA, DSN shows no cleavage activity on ssDNA probe. So upon the addition of GO, the FAM-labeled probe DNA is physically absorbed onto the GO sheet, which brings the fluorescent dye close to the GO, and the fluorescence of the dye is quenched by GO through fluorescence resonance energy transfer (FRET). While in the presence of specific miRNA, the probe-target forms a DNA-RNA hybrid duplex, which becomes a substrate for DSN cleavage. It is found that DSN cuts the signal probes predominantly between fourth and fifth, and fifth and sixth nucleotides from the 5′ end when the signal probes are 10-mer. If the longer DNA duplexes are used as substrates, cleavage reaction yields products of 6 bp and shorter.[40] Thus in our sensing system, the probe DNA is cleavaged into short oligonuleotide fragments, releasing the miRNA from the duplex into the solution. In this manner, the released target miRNA can hybridize with another probe DNA to initiate a next round of cleavage. This

cyclic reaction will repeat again and again and the amount of the produced short FAM-linked oligonuleotide fragments is positively related to the concentration of the target miRNA at a fixed time. Finally, after the introduction of GO into the sensing solution, the FAM fluorescence partly remains ascribed to the weak interactions between GO and the short oligonuleotide fragments. Hence, miRNA can be quantitatively analyzed by measuring the fluorescence intensity after GO addition to the DSN reaction mixture.

(Scheme 1)

3.2 Verification of the amplified detection system

Subsequently, experiments were performed to verify the feasibility of the proposed amplified fluorescence sensing system. Fig. 1 shows the fluorescence emission spectra of FAM-labeled ssDNA (P_0) at different conditions. The fluorescence intensity of P_0 decreased rapidly to 13% of the original intensity in the presence of GO, suggesting efficient adsorption and fluorescence quenching of P_0 by the GO is achieved. While in the presence of 1 nM T_1, a remarkable signal enhancement was observed due to the DSN-induced target recycling. Meanwhile, the fluorescence intensity of the reaction solution without DSN was also measured for comparison. Evidently, it showed a small signal difference from the background signal in the absence of DSN, implying that the low target concentration was hard to be detected by a normal 1:1 ratio hybridization assay. These results confirm that the signal amplification is indeed realized by our design.

(Fig. 1)

3.3 Optimization of the assay conditions

DSN has a broad working temperature with optimum located at ~60℃.[35] In our experiment, although higher temperature could facilitate faster hybridization and subsequent dissociation miRNA, the higher temperature which was over T_m (the T_m of DNA-miRNA duplexes with 22 bp is ~50℃ according to the product's instructions[41]) may lead to unwinding. To obtain the best sensing performance, the working temperature of the DSN was fixed at 45℃. In addition, the incubation time of reaction mixture before adding GO influences the final result of this assay. If the incubation time is sufficiently long, this cyclic reaction will repeat again and again until all the probes are consumed and all fluorophores light up, resulting in significant fluorescent signal amplification. In order to realize quantification detection, we chose excess probes and fixed incubation time of 25 min in the work, as mentioned in the previously reported paper[36].

Thereafter, the other two main experimental variables need to be investigated, which are the amount of DSN and the concentration of GO. As shown in Fig. 2A, the signal-to-noise (S/N) ratio, which was expressed as F/F_0 value (F and F_0 are the fluorescence signals in the presence and the absence of 1 nM T_1, respectively), increased significantly as the concentration of GO increased over the range of 1.5-3.0 μg mL^{-1}. However, when the concentration of GO is up to 3.0 μg mL^{-1} or larger, the S/N ratio decreased, revealing that the GO at a high concentration would cause excessive quenching effect on the cleavage-produced short FAM-labeled

oligonucleotides. According to this, a concentration of GO at 3.0 μg mL^{-1} is selected for the following analysis procedure.

Next, we investigated the influence of the amount of DSN used in the sensing experiment. The fluorescence response was measured upon addition of 0.025 U, 0.05 U, 0.075 U, 0.10 U, and 0.20 U DSN in the presence of 1 nM T_1, respectively. The control samples were treated in the same way without miRNA, correspondingly. As shown in Fig. 2B, the fluorescence intensity around 520 nm increased gradually with the increasing amount of DSN from 0.025 U to 0.075 U and then leveled off as the DSN's amount was over 0.075 U. The fluorescence intensities corresponding to those control samples were broadly the same, which was even similar to the signal of blank sample without addition of DSN, indicating negligible nonspecific background fluorescence is produced by DSN on probes. Reason for this phenomenon is probably DSN's strict cleavage preference. In the sensing system, it hydrolyzes only probe DNA in DNA-RNA hybrid duplexes, while DNA free in buffer solution is left intact and then adsorbed onto the surface of GO, thus no fluorescence enhancement is observed in control samples. Therefore, the optimal amount of DSN is 0.075 U in 20 μL reaction volume.

(Fig. 2)

3.4 The sensitivity of the sensing system

Under the above optimal experimental conditions, we investigated the sensitivity of this proposed method. Fig. 3 illustrates the fluorescence emission spectra observed upon addition of different concentrations of T_1. The fluorescence

intensities increased remarkably with the increasing T_1 concentrations from 0 to 100 nM. The plot of the fluorescence intensity change ($\Delta F=F-F_0$) *vs* the logarithm value of T_1 concentration displayed a good linear relationship in the range from 500 fM to 1 nM. The regression equation is ΔF=20.13log(miRNA)+22.56, with a regression coefficient (R^2) of 0.9759. The limit of detection (LOD) based on 3σ method was calculated to be 160 fM. Table 2 summarizes the LOD & experimental complexity level of some recently-reported signal amplification miRNA assays. The comparison clearly shows that the proposed method has a couple of advantages compared to others. First, our proposed strategy does not need either complex modification or quencher labeling which would be cost-effective and easy to operate. The experimental procedure is just a process of "mix-and-detect", therefore it was quite simple. Second, our method is more sensitive than that based on GO-protected DNA probes and cyclic enzymatic amplification method (CEAM), in view of the similar complexity level. The good sensitivity of our proposed method is attributed to the highly effective catalytic cleavage activity of DSN as well as the "postmixing" strategy of GO addition. Fan and coworkers ever reported that the "postmixing" strategy was more rapid and sensitive, compared with the conventional "premixing" method of GO adddition.[20] Although the LOD values reported by the methods based on GO fluorescence quenching coupled with isothermal strand-displacement polymerase reaction (ISDPR),[28] and the electrochemical biosensor with DSN-aided amplification,[38] respectively, are lower than that we obtained. However, the methods used in these literatures are much more complex.

What is more, a series of repetitive measurements with 10 pM T_1 was used for evaluating the precision of the proposed amplification miRNA detection method and a relative standard deviation (RSD) of 4.9% was obtained, demonstrating a satisfactory reproducibility of the assay. These results indicate that the sensing system can be used for accurate quantification of miRNA. In addition, it can be applied to all miRNAs by easily changing the specific DNA probe sequence, because DSN has no requirement on recognition of sequence.

(Fig. 3)

(Table 2)

3.5 The specificity of the sensing system

It is notable that some miRNA sequences only differ one or two nucleotide(s), which is a great challenge for miRNAs analysis in distinguishing miRNA family members with high homology. To evaluate the sequence specificity of the assay, three artificially synthesized miRNAs, let-7b (T_1, complementary), let-7c (T_2, one-base mismatched), and miR-21 (T_3, non-complementary) were selected as the detection model. Fig. 4 shows the comparison of the fluorescence signals' response to different miRNAs. When 1 nM miRNAs were added into the sensing system, the T_1 showed significant fluorescence intensity change ($\Delta F=F-F_0$), but neither of T_2 and T_3 could induce distinct fluorescence increase. Even at high concentration of miRNAs (10 nM), the ΔF value for T_1 was 3.43 times than that for T_2, and the ΔF for T_3 was still negligible. The high sequence specificity is due apparently to the additional mismatch discrimination ability brought to the system by the DSN.

(Fig. 4)

4 Conclusions

In summary, we have developed a simple, highly sensitive and selective miRNA detection method based on the difference in affinity of GO with ssDNA containing different numbers of bases in length as well as the DSN-induced target recycling. By coupling DSN to the sensing system, the recycling of target miRNA is realized, which leads to the amplified detection of the miRNA (LOD: 160 fM). Good sequence selectivity is also achieved due to the great sequence discrimination ability of DSN. Furthermore, this mix-and-detect assay format is simple and can be finished within 40 min. Such a novel amplified sensing strategy is expected to offer a new platform for highly sensitive and specific detection of miRNA and shows promising application in early clinical diagnosis and biomedical research.

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Table 1 DNA probe and miRNAs sequences used in this work

Name	Sequence (5′-3′)	Number of bases
P-7b (P_0)	FAM-AACCACACAACCTACTACCTCA	22
let-7b (T_1)	UGAGGUAGUAGGUUGUGUGGUU	22
let-7c (T_2)	UGAGGUAGUAGGUUGUAUGGUU	22
miR-21 (T_3)	UAGCUUAUCAGACUGAUGUUGA	22

Table 2 Comparison of LOD & experimental complexity level of different signal amplification-based miRNA assays

Method	LOD	Complexity level	Ref.
Duplex-specific nuclease signal amplification (DSNSA)	100 fM	Complex (dual labeling)	36
Backbone-modified molecular beacons (MBs)	400 fM	Complex (dual labeling & backbone-modified MBs)	37
Electrochemical biosensor with DSN amplification	1.0 fM	Complex (chemical modification)	38
GO-based ISDPR method	2.1 fM	Complex (primer design)	28
GO-protected cyclic enzymatic amplification method (CEAM)	9 pM	Easy (single labeling)	29
GO-based DSN amplification method	160 fM	Easy (single labeling)	This study

Figure captions:

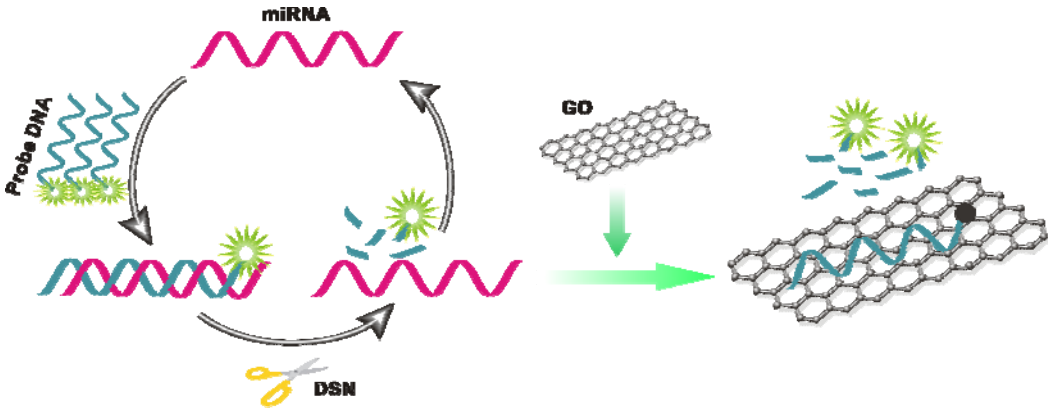
Scheme 1 Schematic representation of GO-based fluorescent sensing system for the detection of target miRNA with DSN-aided signal amplification strategy.

Fig. 1 Fluorescence emission spectra of P_0 (20 nM) at different conditions: (a) P_0 in Tris-HCl buffer; (b) P_0+DSN+(1 nM) T_1+GO; (c) P_0+(1 nM) T_1+GO; and (d) P_0+DSN+GO.

Fig. 2 (A) The effect of GO concentration on the fluorescence response of the sensing system. The concentration for DSN was fixed at 0.10 U. (B) The effect of the amount of DSN on the fluorescence response of the sensing system. The concentration for GO was fixed at 3.0 μg mL^{-1}. F and F_0 are the fluorescence signals in the presence and the absence of T_1 (1 nM), respectively.

Fig. 3 Fluorescence emission spectra of P_0 (20 nM) after DSN amplification reaction with varying concentrations of T_1 (a to j: 0, 500 fM, 1 pM, 5 pM, 10 pM, 50 pM, 100 pM, 1 nM, 10 nM, 100 nM, respectively), and then addtion of GO (3.0 μg mL^{-1}) for 5min. Inset: Scatter plot of the fluorescence intensity change ($\Delta F=F-F_0$) *vs* logarithm of T_1 concentration.

Fig. 4 Specificity evaluation of the method for miRNA detection. Bars represent the fluorescence intensity change ($\Delta F=F-F_0$) *vs* the different miRNAs targets.



Scheme 1

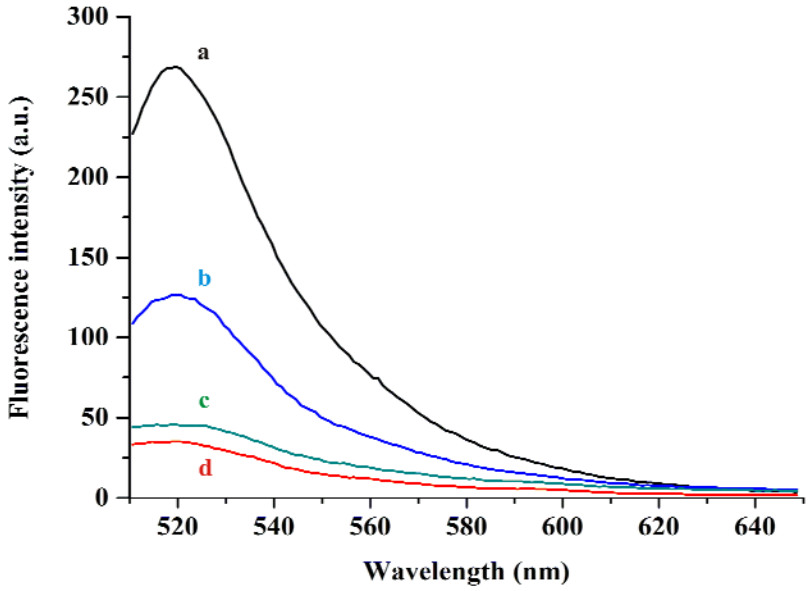


Fig. 1

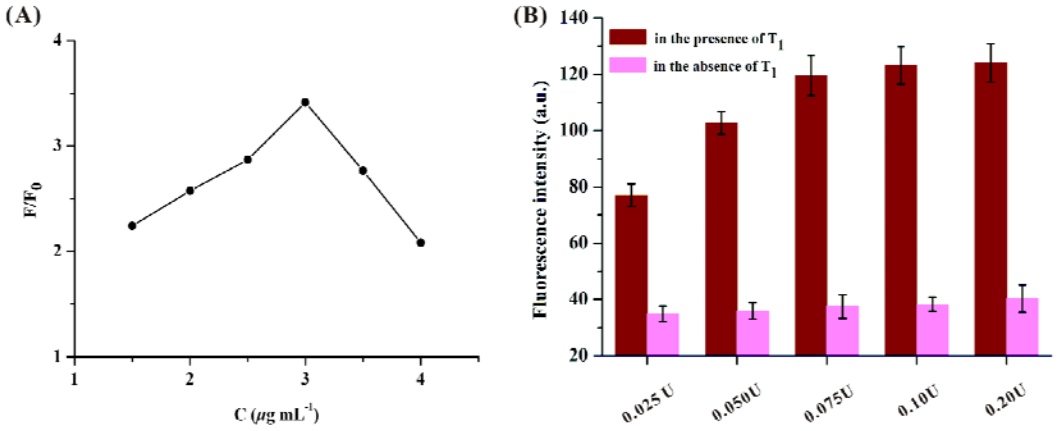


Fig. 2

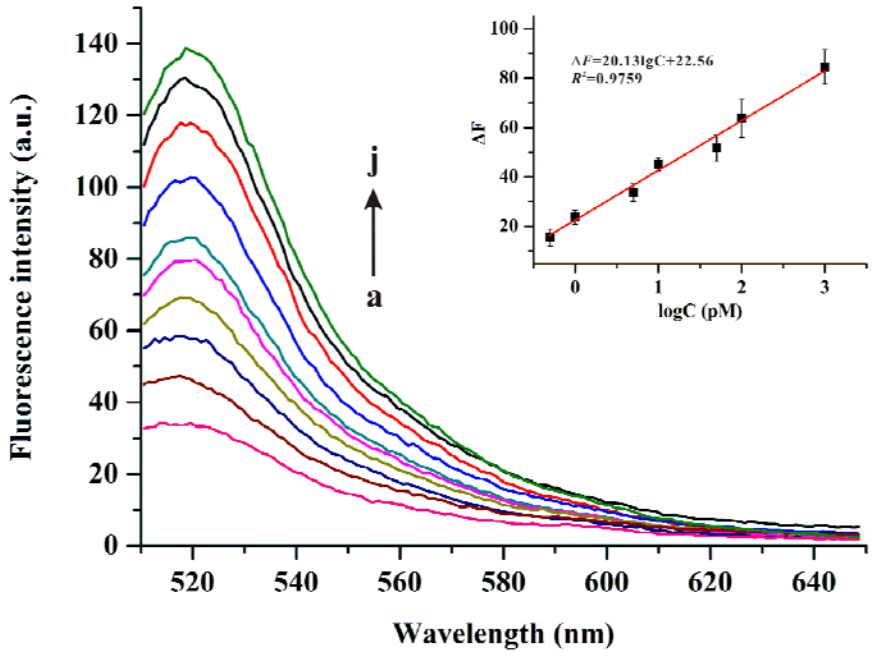


Fig. 3

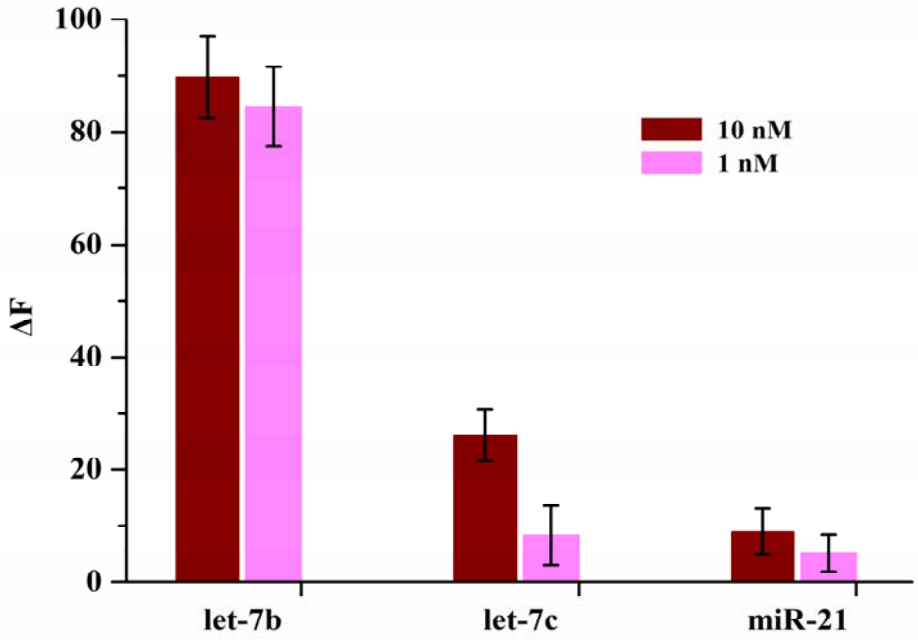


Fig. 4