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# A carbon nanoparticle and DNase I-Assisted amplified fluorescent biosensor for miRNA analysis

Hongfeng Li[a,1], Yang Li[a,1], Wenqin Li[a], Liang Cui[b,**], Guohua Huang[c], Jiahao Huang[a,*]

[a] School of Biomedical Engineering, Biomaterials Research Center, Guangdong Provincial Key Laboratory of Construction and Detection in Tissue Engineering, Southern Medical University, Guangzhou, 510515, China
[b] Department of Chemistry, Zhejiang Sci-Tech University, Hangzhou, 310008, China
[c] Chronic Airways Diseases Laboratory, Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China

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ABSTRACT

Nucleic acid-based biosensors have become powerful tools in biomedical applications. But the stability issue seriously limits their wide applications. Fortunately, the emergence of carbon nanoparticles (CNPs), which can effectively protect DNA probes from enzymatic digestion and unspecific protein binding, provides a good solution. In this work, a DNase I-aided cyclic enzymatic amplification method (CEAM) for microRNA analysis has been developed based on the coupling use of nucleic acid probes with specific molecular recognition ability as well as CNPs with excellent biostability. The method is simple and sensitive, with a detection limit down to 3.2 pM. Furthermore, satisfactory results are achieved for miRNA analysis in breast cancer cell lysate, demonstrating the applicability in disease diagnosis. The ingenious combination of CNPs and nucleic acid probes can open a new chapter in the development of versatile analytical strategies that holds great potentials for clinical diagnosis, food safety, and environmental monitoring.

## 1. Introduction

MicroRNAs (miRNAs) are 19–25 nt non-coding RNAs that are processed from longer endogenous hairpin transcripts by the enzyme Dicer [1]. Several hundred miRNAs are encoded in the human genome and dozens have now been shown to be closely associated with a variety of diseases, including human cancers [2,3]. Nucleic acid probes are widely used for the detection of miRNAs [4,5], but they suffer from limitations, such as poor stability and undesired sensitivity. Therefore, the sensitive quantitation of small amounts of miRNA in real biological samples still remains a challenging task. In order to build powerful RNA sensors, it is crucial to improve the stability of nucleic acid probes and also incorporate proper signal amplification principles into the assay methods.

To improve the biostability, nucleic acid probes have been modified with nuclease-resistant backbones, such as 2′-O-methyl RNA bases [6], L-DNA base [7,8], and locked nucleic acids [9]. Unfortunately, these strategies are not compatible with nucleases, which are widely used in signal amplification methods [10]. It is an intractable problem to fabricate nucleic acid-based biosensors with acceptable stability and enzyme-assisted signal enhancement effect.

In recent years, nanomaterials with unique properties have been used to create new types of analytical tools in life science and biotechnology. Gold nanoparticles, silver nanoparticles, quantum dots, graphene oxide, and molybdenum disulfide nanosheets have been widely used in many biosensing applications [11–13]. Additionally, carbon nanomaterials with fascinating characteristics also show promising potentials in these fields [14,15]. Special attention has been paid to carbon nanoparticles (CNPs), which are chemically inert, biologically compatible, and have low cytotoxicity [16]. However for biosensor development, CNPs are always responsible for signal generation yet play unimportant role in the molecular recognition part. To well incorporated into biosensing systems, CNPs should be coupled with other powerful components that exhibit satisfactory affinity with targets of interest. Fortunately, nucleic acid probes which possess unexceptional identification molecular recognition capability play a good complementary role in CNP-based sensors [17]. CNPs exhibit reversible and distinct binding affinities towards single-stranded nucleic acid probes versus double-stranded nucleic acid probes, which enables the use of CNPs into nucleic acid-based sensing platforms. Prof. Yang from Xiamen University used to report a promising sensing method for ATP

* Corresponding author.
** Corresponding author.
*E-mail addresses:* cuiliang@hnu.edu.cn (L. Cui), jhuangaf@connect.ust.hk (J. Huang).
[1] These authors contributed equally to this work.

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and kanamycin, in which carbon nanoparticles could protect aptamers from the digestion of nuclease and then enable the signal amplification due to the recycling use of target molecules [18].

With this regard, the incorporation of nucleic acid probes and CNPs into biosensors can significantly improve the sensor performances and effectively overcome most of the aforementioned limitations, such as poor stability, unsatisfactory recognition capability, and incompatibility with nucleases. In this work, an innovative method is proposed for effective detection of miRNA by using nucleic acid probes and CNPs. The stability of nucleic acid probes is effectively improved by CNPs and the detection sensitivity is greatly enhanced by the employment of DNase I. Nucleic acid probes are responsible for target identification and signal amplification mediated by DNase I, while CNPs make great contribution to the stability improvement and target-responsive signal production. Therefore, a cyclic enzymatic amplification method (CEAM) is developed for sensitive detection of miRNA in the cancer cell lysate. To our knowledge, this is the first report for miRNA determination by using CNPs as probe stabilizers and fluorescence quenchers as well as DNase I for target regeneration and signal enhancement.

## 2. Experimental section

### 2.1. Materials

DNase I, HPLC-purified RNA, RNase inhibitor, and DEPC-treated water were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). The DNA probes were synthesized on a PolyGen Column 12 DNA synthesizer and the reagents were purchased from Glen Research (Sterling, VA, USA). All DNA/RNA sequences are listed in Table 1. Carbon nanoparticles (CNPs) were purchased from Beijing DK nano technology Co. Ltd (Beijing, China). Cell lysate from breast cancer cell MDA-MB-231 was obtained by following the reported procedures with 5 mM $MgCl_2$ and 5 mM $CaCl_2$ included [19]. The total RNA kit, which was employed to extract the total RNA from breast cancer cells, was purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2. Fluorescence measurements

Fluorescence measurements were carried out on a RF-5301-PC Fluorescence Spectrophotometer (Shimadzu, Japan). For P7a, excitation and emission wavelengths were set at 560 and 582 nm, respectively, with 5 nm bandwidth. The emission spectra were obtained by exciting the samples at 560 nm and scanning the emission from 570 to 650 nm in steps of 1 nm. For molecular beacons, excitation and emission wavelengths were set at 490 and 520 nm with the same bandwidth. All experiments were conducted in 20 mM Tris-HCl (pH 8.0) buffer containing 5 mM $MgCl_2$ and 50 mM NaCl. The sensing system contained fluorescent single-stranded DNA probes, CNPs, and DNase I. All these species were dissolved in the buffer solution and kept at the concentration of 50 nM, 0.02 mg/mL, and 100 unit/mL, respectively. And the reaction time was fixed at about 30 min. For the sensitivity study, the fluorescence measurements were performed in 200 μl solution consisting of 50 nM fluorescent DNA probes, 0.02 mg/mL CNPs, 100 unit/mL DNase I, and varying concentrations of target miRNA at room temperature for 30 min. Furthermore, for the selectivity investigation, the signal recording was performed in 200 μl solution consisting of 50 nM fluorescent DNA probes, 0.02 mg/mL CNPs, 100 unit/mL DNase I, and perfectly matched and mismatched RNA targets of the same concentration at room temperature for 30 min.

**Table 1**
Sequences of oligonucleotides.

| Name | Sequence |
|---|---|
| P7a | 5' -AAC TAT ACA ACC TAC TAC CTC A -TMR -3′ |
| Molecular Beacon | 5' – FAM- CGC GAA CAA ACA CAC ACC ACA ATT CGC G – Dabcyl – 3′ |
| Let-7a | 5' - UGA GGU AGU AGG UUG UAU AGU U -3′ |
| Let-7b | 5′- UGA GGU AGU AGG UUG UGU GGU U -3′ |
| Let-7e | 5′- UGA GGU AGG AGG UUG UAU AGU -3′ |
| Mir 122 | 5′- UGG AGU GUG AC A AUG GUG UUU G -3′ |

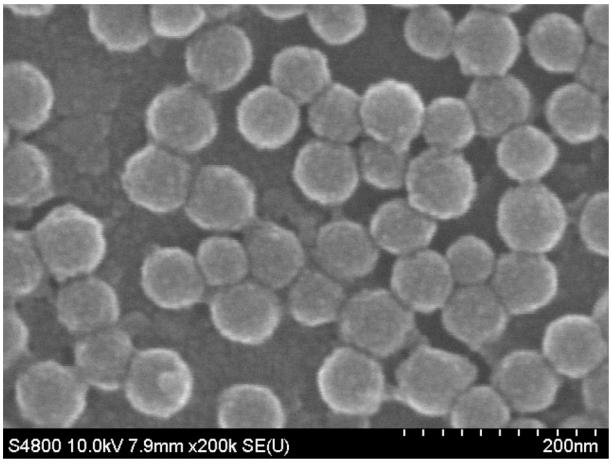


**Fig. 1.** SEM image of CNPs.

### 2.3. Gel electrophoresis

A 20% non-denaturing PAGE analysis of the products from the cyclic enzymatic amplification reaction was carried out in 1 × TBE (pH 8.3) at 1w power for about 2 h. After Stains-All staining, gels were scanned.

## 3. Results and discussion

### 3.1. Adsorption, desorption, and biostabilization of DNA on CNP surface

CNPs purchased from the company were characterized by a scanning electron microscope (SEM). As reveals in Fig. 1, the CNPs were nearly spherical in shape and the diameter was about 60 nm. To investigate the adsorption and desorption behaviors of DNA on CNP surface, the DNA/CNPs interaction was recorded by monitoring the fluorescence change of a TMR-labeled single-stranded DNA probe (P7a) upon incubation with CNPs. As shown in Fig. 2A, a strong fluorescence signal was observed from the solution containing P7a. Upon the addition of CNPs, an immediate decrease of the fluorescence intensity occurred. The result reveals that P7a effectively absorbed onto the CNP surface and CNPs exhibited excellent fluorescence quenching property. After the addition of five-fold excess complementary RNA (let 7a), the fluorescence increased (Fig. 2A, blue line), suggesting that the P7a hybridized with let 7a to form a RNA/DNA duplex, resulting in desorption of the P7a from CNP surface and subsequent fluorescence recovery. CNPs exhibit differential absorption forces with single or double-stranded nucleic acid probes, which forms the basis of most CNP and nucleic acid-based biosensors.

In order to investigate the biostability of CNP-protected DNA, a molecular beacon (MB, the sequence was listed in Table 1) was prepared. Firstly, the degradation reactions were carried out on a fluorospectrometric photometer with results shown in Fig. 2B. Because the quencher of the MB had already efficiently quenched the fluorophore when the MB assumed a hairpin structure, the background signal of the MB was extremely low regardless of the absence or presence of CNPs. However for the un-protected MB, the addition of DNase I instantaneously caused a rise in fluorescence intensity and the signal leveled off in less than 2 min. On the contrary, no apparent increase in fluorescence intensity was observed even after 15 min when the CNP-

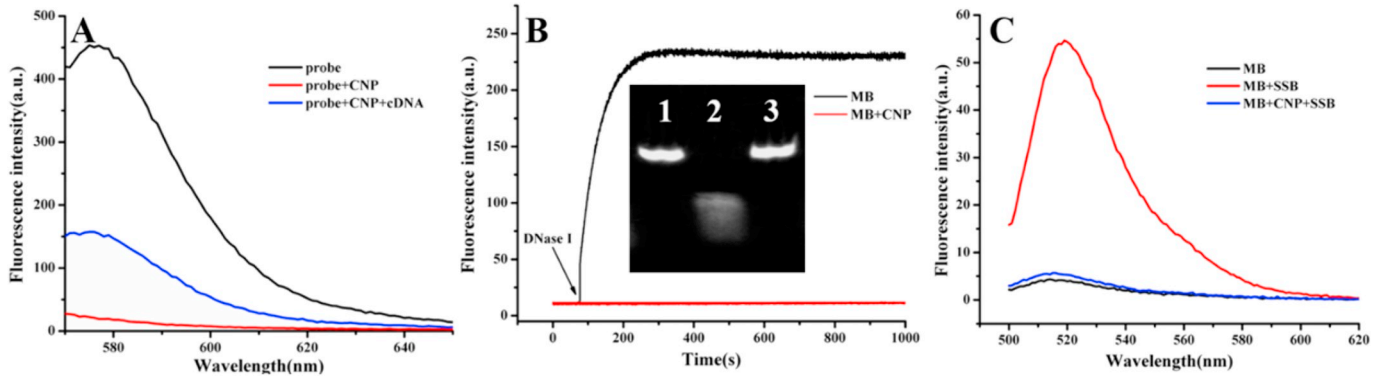


**Fig. 2.** (A) Fluorescence emission spectra of 50 nM P7a to the addition of 0.02 mg/mL CNPs with and without the introduction of 5 x cDNA. (B) Fluorescence response of MB and MB-CNP to DNase I digestion. Insert, gel electrophoresis analysis of MB and MB-CNP samples treated with DNase I. Lane 1: MB-CNP + DNase I; Lane 2: MB + DNase I; Lane 3: MB only. (C) Fluorescence response of MB and MB-CNP to SSB binding.

protected MB was incubated with DNase I. Further electrophoresis experiments were also performed to confirm the reactions. The MB was incubated with CNPs and was then treated with DNase I. As shown in the inset of Fig. 2B, there was no detectable hydrolysis of the MB in the presence of CNPs even after 60 min (lane 1). In contrast, no-protected MB was invisible, indicating effective enzymatic hydrolysis of the MB occurred. These results show that DNA adsorbed on the surface of CNPs was effectively protected from enzymatic digestion by DNase I. This finding is encouraging for many biomedical applications in which robust DNA probes and effective DNA delivery in complex biological samples are required.

Then we also studied the interactions of free MB and MB/CNPs with single-stranded DNA binding protein (SSB). In complex biological samples, normal DNA-MBs are subject to non-specific binding with proteins such as SSB. Such binding may cause MBs to open up unspecifically and give false positive signals. As shown in Fig. 2C, the black line represents the background signal of MB. After the addition of SSB with the same concentration, a remarkable fluorescence signal was observed (red line), and the signal-noise ratio reached up to 14. Interestingly, on the contrary, the signal of the MB/CNPs system only changed slightly upon the addition of the same amount of SSB (blue line), indicating an effective protection effect from interference from nucleic acid binding proteins. The excellent virtue makes it well suited to monitor miRNA or other biomarkers in vivo since the native biological environment abounds with these proteins.

The above experiment results together show that the CNPs/DNA probes not only exhibit excellent nuclease stability, but also resist binding of single-stranded DNA binding proteins. All of these properties are ideal for the assay of analytes existed in complex biological samples. Based on these interesting properties, we designed a cyclic enzymatic amplification method (CEAM) for sensitive detection of miRNA based on the employment of CNPs with protection effect and DNase I for target-activated enzymatic digestion.

### 3.2. The principle for the detection of miRNA

The working principle of the amplified fluorescent assay is illustrated in Fig. 3. In the absence of miRNA, a single-stranded dye-tagged DNA probe adsorbed on the CNPs produces a very weak fluorescence signal (path a). Upon the addition of complementary miRNA (path b), the DNA/RNA hybrid duplex detaches from the CNPs surface and the fluorescence signal is effectively recovered. More importantly, the DNA probe in DNA/RNA hybrid will immediately become a suitable substrate for DNase I digestion (path c and d) and then be efficiently cut into pieces by DNase I (path e). This subsequently causes the release of the target miRNA (path f), which can then bind to another probe on CNPs and initiate a second round of hybridization, recognition,

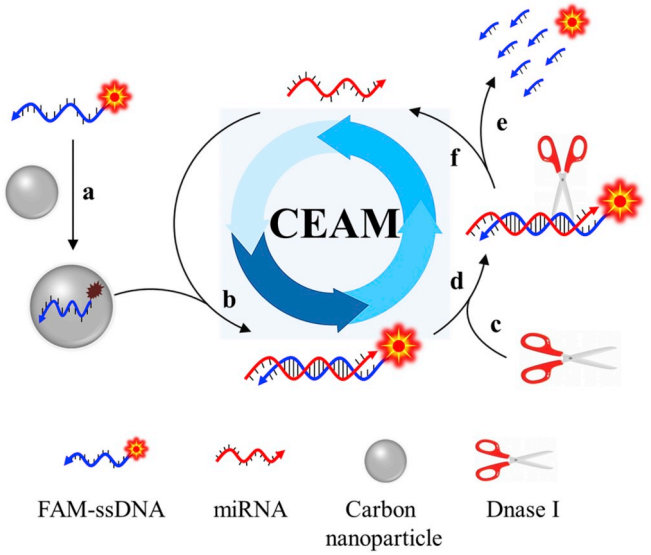


**Fig. 3.** Working principle of cyclic enzymatic amplification method (CEAM) for miRNA analysis. First, FAM-tagged single-stranded DNA (FAM-ssDNA) attached to the surface of the carbon nanoparticle (path a) causes a significantly quenched fluorescence signal. Upon the introduction of target miRNA, RNA/DNA hybrid is formed (path b). The subsequent addition of DNase I (path c) recognize its suitable substrate: RNA/DNA hybrid (path d). As a result, FAM-ssDNA is digested into small pieces and a strong fluorescence signal is yielded (path e). Furthermore, miRNA keeps intact and goes back to its initial state (path f), which is ready to launch another reaction cycle: hybridization, digestion, signaling, and release. The recycling use of target molecule makes it possible to effectively amplify the detection sensitivity.

cleavage, and liberation. This cyclic reaction will repeat continuously until all the captured probes are consumed and all fluorophores light up, thereby resulting in a significant fluorescent signal amplification.

### 3.3. Assay performances

In this work, let-7 miRNA family members are used as targets because their sequences are very similar and their expression levels are closely associated with cell fate and the development of human cancers[3]. A DNA probe P-7a complementary to let-7a was used. We examined CEAM at low miRNA concentration by monitoring the fluorescence responses. The solution containing P-7a/CNPs upon the addition of a low concentration of miRNA (i.e. 0.1 nM) is shown in Fig. 4A, and no apparent increase in fluorescence signal was observed. This shows that the target concentration was already below the detection limit of the hybridization assay. We then added DNase I to the

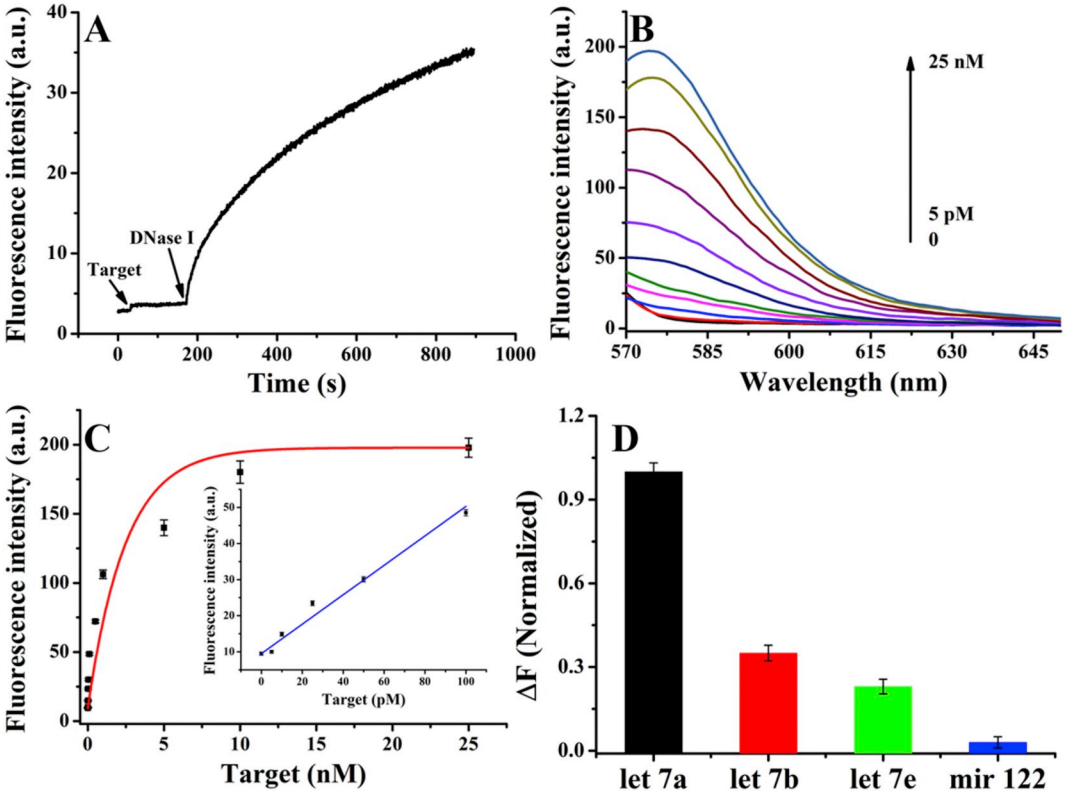


**Fig. 4.** (A) Time course study of CEAM at target concentrations of 0.1 nM. (B) The fluorescence responses when challenged with targets with different concentrations. (C) The relationship of the fluorescence signal with the concentration of target miRNA. (D) The detection specificity of the proposed method for different RNA sequences. Error bars represent the standard deviations in three independent experiments.

**Table 2**
The performance comparisons of nuclease-based methods for miRNA analysis.

| Enzyme | Advanced material | Detection limit | Ref. |
|---|---|---|---|
| Exonuclease III | AIEgen | 1 pM | [20] |
| Exonuclease III | AIEgen | 1 pM | [21] |
| Duplex-specific nuclease | Gold nanoparticle | 8.41 aM | [22] |
| Duplex-specific nuclease | Gold nanoparticle | 50 fM | [23] |
| Duplex-specific nuclease | Quantum dot | 0.16 nM | [24] |
| Duplex-specific nuclease | Quantum dot | 42 fM | [25] |
| Duplex-specific nuclease | Perylene derivative | 5 pM | [26] |
| Duplex-specific nuclease | Malachite green binding G-quadruplex | 1.03 pM | [27] |
| Duplex-specific nuclease and terminal deoxynucleotidyl transferase | Carboxylated microsphere | 0.1 pM | [28] |
| Duplex-specific nuclease and terminal deoxynucleotidyl transferase | Copper nanoparticle | 20 pM | [29] |
| T4 RNA ligase, amligase, and lambda exonuclease | Cationic conjugated polymer | 0.1 fM | [30] |
| DNase I | Polydopamine nanosphere and Gold Nanocluster | 3.6 pM | [31] |
| DNase I | Polydopamine nanosphere | 2.3 pM | [32] |
| DNase I | Carbon nanoparticle | 3.2 pM | This work |

reaction mixture, and a rise in fluorescence intensity appeared instantly, indicating a quick cleavage of the DNA probes. These results confirm that the cyclic enzymatic amplification worked very well as expected.

The fluorescence signals with different target concentrations were then measured. Fig. 4B shows the fluorescence intensity observed upon different concentrations of targets. The results reveal that the fluorescence intensity grew higher as the concentration of the targets increased. Fig. 4C shows the standard curve demonstrating the relationship between the fluorescence intensity and the concentration of target miRNA. The calibration plot is linear between 5 and 100 pM, and is well fitted by a formula: $y = 9.50 + 407.76\,x$ ($n = 3$, $R^2 = 0.9688$), where y is the fluorescence intensity and x is the let 7a concentration (nM). The limit of detection (LOD) was calculated as 3.2 pM by using three folds of the standard deviation of the blank sample (without the presence of target). The detection limit of this method is competitive with many nuclease-assisted signal amplification methods, with detail comparisons shown in Table 2 [20–32]. The high sensitivity is mainly achieved from the remarkable fluorescence quenching ability of CNPs and the efficient digestion activity of DNase I.

The high similarity detected between different sequences in the miRNA family poses severe challenges for detecting miRNAs. The detection selectivity of this method was also examined. As is shown in Fig. 4D, similar RNA sequences with tiny differences by 1–2 bases (let 7b, let 7f, and mir 122) were used to test the sensing performance. The selectivity seemed good, since the signal produced by the specific targets was much stronger than those caused by other mismatched RNA analogues. Actually, it is quite difficult to guarantee the sensing selectivity because the miRNA is usually very short and the sequences of mismatched targets are only one base different from that of the

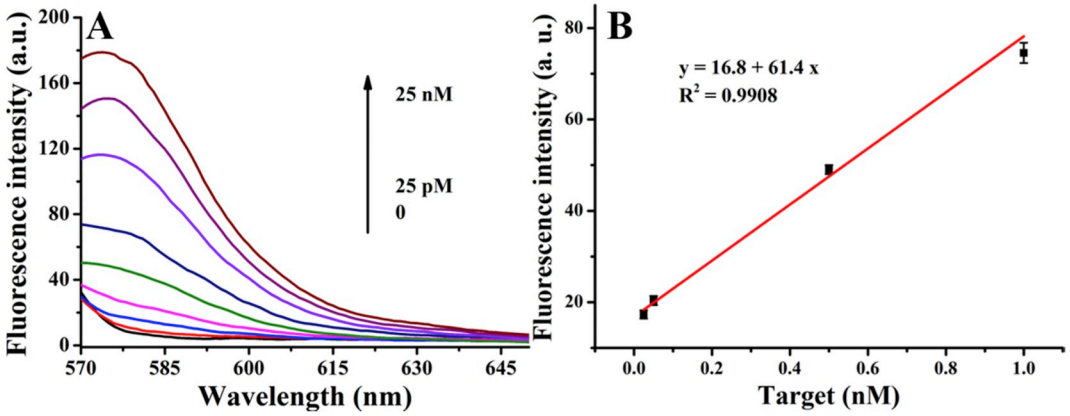


**Fig. 5.** (A) Fluorescence spectrum analysis of miRNA in the small amount RNA extracted from cultured cells. (B) The relationship of the fluorescence signal with the concentration of target miRNA.

perfectly matched target. It is noted that additional measures should be taken in order to further improve the detection selectivity, such as the employment of other powerful nucleic acid probes modified with artificial nucleic acid bases, including 2′-O-methyl RNA bases [6], L-DNA base [7,8], and locked nucleic acids [9].

### 3.4. Detection in biological matrix

The data demonstrates that this scheme can achieve sensitive detection of miRNA and is also featured with good selectivity. Considering the significance of miRNA analysis in complex biological samples, this new method was applied to detect miRNA in the lysate of the breast cancer cell line, MDA-MB-231, which has been reported with very low expression levels of let-7a. As demonstrated in Fig. 5A, the fluorescence intensity upon the addition of increasing concentrations of miRNA shows a proportionate increase. As shown in Fig. 5B, the calibration plot is linear within the range from 25 to 100 pM, and is well expressed by the following equation: $y = 16.8 + 61.4\ x$ ($n = 3$, $R^2 = 0.9908$), where y and x represent the fluorescence intensity and the let 7a concentration (nM), respectively. The minimum detectable concentration was found to be 23.1 pM, which is consistent with that obtained in pure buffer solution. The results indicate that the method can be used for the direct detection of miRNA in complicated biological matrices, and it is expected to provide meaningful diagnostic strategies for many miRNA-related diseases.

## 4. Conclusions

In conclusion, a novel fluorescent amplified sensing method for miRNA analysis is described by the coupling use of CNPs which show amazing enzymatic protection effect as well as DNase I which exhibits target-activated enzymatic digestion property. The assay is convenient, with only mix-and-measure steps that can be quickly accomplished. Besides, the strategy is highly sensitive and reliable, that can realize the accurate determination of miRNA with even one base variation. More appealingly, the method can be directly applied in complex biological matrix. Therefore, the present sensing method provides a new avenue to explore the interactions between nucleic acid probes and nanomaterials for applications in nanomedicine and bioanalysis.

## Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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