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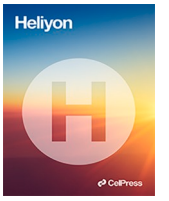

Research article

Sensitive fluorescence detection of miRNA-124 in cardiomyocytes under oxidative stress using a nucleic acid probe

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs of 18–25 bases. miRNAs are also important new biomarkers that can be used for disease diagnosis in the future. Studies have shown that miR-124 levels are significantly elevated during acute myocardial infarction (AMI) and play a key role in the cardiovascular system. A variety of methods have been established to detect myocardial infarction-related miRNAs. However, most require complex miRNA extraction and isolation, and these methods are virtually undetectable when RNA levels are low in the sample. It may lead to biased results. Thus, it is necessary to develop a technique that can detect miRNA without extracting it, which means that intracellular detection is of great significance. Here, we improved the traditional silicon spheres and obtained a biosensor that could effectively capture and detect specific noncoding nucleic acids through the layer-by-layer assembly method. The sensor is protected by hyaluronic acid so it can successfully escape the lysosome into the cell and achieve detection. With the help of a full-featured microplate reader, we determined that the detection limit of the biosensor could reach 1 fM, meeting the needs of intracellular detection. At the same time, we prepared an oxidative stress cardiomyocyte infarction model and successfully captured the overexpressed miR-124 in the infarcted cells to achieve in situ detection. This study could provide a new potential tool to develop miRNAs for sensitive diagnosis in AMI, and the proposed strategy implies its potential for biomedical research.

1. Introduction

MicroRNAs (miRNAs) are sequences of small noncoding RNA molecules with lengths of approximately 22 nucleotides that can

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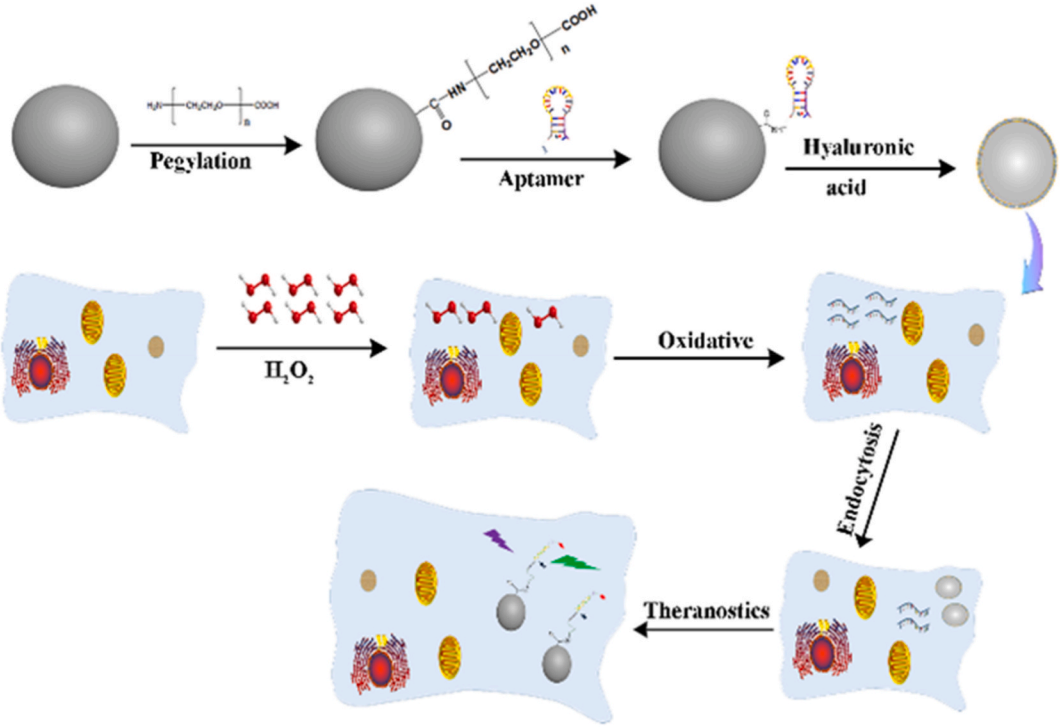

Scheme 1. The assembly of biosensors and the application of in-situ non-coding nucleic acid detection in cells.

effectively regulate gene expression [1–3]. The inappropriate expression of miRNAs plays a role in the prognosis and pathogenesis of many types of diseases [4–7]. In recent years, many researchers have proven that the occurrence and development of cardiovascular disease is accompanied by changes in specific miRNA expression [8–12]. Therefore, miRNAs as biomarkers are of great significance in the diagnosis of cardiovascular diseases [13–15]. Acute myocardial infarction (AMI) can induce a large amount of myocardial cell apoptosis, and subsequent ischemia reperfusion and oxidative stress can induce changes in a variety of apoptosis-related genes, proteins and pathways. The further expansion of cardiomyocyte apoptosis aggravates cardiovascular disease and increases mortality [16–19]. Studies have shown that miRNAs play a key regulatory role in the oxidative stress and myocardial infarction process [20,21]. The latest studies found that miR-124 levels are significantly increased when AMI occurs, and miR-124 is strongly expressed in all smoking individuals and is associated with the risk of subclinical arteriosclerosis caused by related increases in altered single-cell phenotypes [22]. The median level of relative expression of miR-124 in the blood of patients with acute myocardial infarction is about 1.5 times of that in normal people [23]. Randomized clinical trials have shown that serum miR-124 levels may be a useful prognostic indicator of the outcome after cardiac arrest [24]. Circulating miR-124 is highly expressed in patients with acute coronary syndromes and can distinguish meningitis that requires emergency coronary vascular occlusion [25]. Although clinical and preclinical studies suggest that miR-124 may play a key role in the cardiovascular system, miR-124 is a biomarker for early myocardial infarction detection [26,27]. Traditional microRNA quantification procedures are limited. Therefore, the development of rapid, selective, highly sensitive, effective and cost-effective diagnostic methods is an urgent need to identify biomarkers for the early diagnosis of various diseases [28–33]. At present, RT-qPCR is primarily used in miRNA expression research. RT-qPCR is the gold standard for miRNA, it is well recognized and RT-qPCR can keep a better balance between precision and sample size [34,35]. But it still has some limitations, for example, the specific primer/miRNA double chain melting temperature interval of less than 55° may reduce the sensitivity [34]. Northern blotting is recognized as the most reliable method for detecting miRNA, but the disadvantage is that the required sample size is large and the sensitivity is low [3]. Microarray chips can measure multiple samples at the same time but cannot avoid false-positives [3]. These methods still require the extraction and separation of miRNA, which is complicated and tedious. Additionally, when detecting miRNA in plasma, serum and tissue, if the miRNA content in the sample is extremely low, it is almost impossible to detect by the above methods or the detection method will have very low reproducibility [36]. As it is necessary to develop reliable detection techniques that do not require the extraction of miRNA, intracellular in situ analysis is of great significance. To analyze and detect intracellular miRNA, the capture probe adapter needs to be transported into the cell. Commonly used vectors include viral [37] and nonviral [38] vectors. Viruses used for gene delivery have disadvantages such as difficulty in achieving local delivery, low efficacy at the target site, and poor or unstable transduction efficiency. Nonviral vectors mainly include cationic liposomes, high molecular weight polymers and some nanomaterials. We selected inorganic nanomaterials that are easy to functionalize, have good biocompatibility, and have good targeting ability and cell permeability [39]. Due to the limitations of conventional methods, researchers have developed other strategies, such as using sensors and biosensors in techniques including colorimetry, electrochemistry, surface

Table 1
Sequence information of the oligonucleotides used in this study.

Annotation	Sequence	Modification
HPN CP-124-dabcyl	CCCACCAACCAT-Dabcyl-CTAATCGTGATAGG GGTA ATCAAGGTCCGCTGTGAACACGTACCCCTATCACGATTAGCATTAA-FAM	3′- FAM 5′-Amino C6
miR-124	CGUGUUCACAGCGGACCUUGAU	–
miR-124-mis1	CGUGUCCACAGCGGACCUUGAU	–
miR-124-mis2	CGUGUCAACAGCGGACCUUGAU	–
miR-124-mis3	CGUGUCAGCAGCGGACCUUGAU	–
miR-19a	UGU GCA AAU CUA UGC AAA ACU GA	–
miR-145	GUC CAG UUU UCC CAG GAA UCC CU	–
miR-197	CGG GUA GAG AGG GCA GUG GGA GG	–
miR-186	CAAAGAAUUCUCCUUUUGGGCU	–
miR-223	UGUCAGUUUGUCAAAUACCCCA	–
miR-134	UGUGACUGGUUGACCAGAGGGG	–
miR-23a	GGGGUUCCUGGGGAUGGGAUUU	–
miR-30a	GGACCCUUUCAGUCGGAUGUUUG	–
miR-21	GGACCUUAGCUUAUCAGACUGAUG	–

plasmon resonance, surface-enhanced Raman spectroscopy (SERS), electrochemiluminescence and fluorescence, to measure miRNA [40–50]. Among these technologies, fluorescent biosensors are more widely used to detect miRNA due to their advantages, which include low cost, fast response time, easy operation, and suitability for biomedical applications [28,51–53].

In previous research, we designed a biosensor material based on nanosilica spheres to identify and quantitatively detect miRNA and successfully applied it to the detection of noncoding nucleic acids in plasma [54]. Here, we made further improvements to the biosensor, using hyaluronic acid encapsulation to protect the biosensor, and successfully obtained a biosensor that can detect microRNA in situ in cells (Scheme 1). We have known that the sequence information of miR-124 is CGUGUUCACAGCGGACCUUGAU, and our specific aptamer is designed according to the target microRNA, we added 3′- FAM and 5′-Amino C6 based on miR-124. The probe aptamer's sequence is CCCACCAACCAT-Dabcyl-CTAATCGTGATAGG GGTA ATCAAGGTCCGCTGTGAA-CACGTACCCCTATCACGATTAGCATTAA-FAM(Table 1). With the help of a typical myocardial infarction cell model, the ability of the biosensor to detect miR-124 in situ was tested.

2. Experimental

2.1. Chemicals and materials

Chemical compounds such as hexadecyl trimethyl ammonium bromide (CTAB) and tetraethyl orthosilicate (TEOS) were purchased from Aladdin Reagent Company. 3-Aminopropyl triethoxysilane (APTES), N-ethyl-N′-(3-dimethylaminopropyl) car-bodiimide (EDC), diethyl pyrocarbonate, sodium cyanoborohydride and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were obtained from Sigma–Aldrich. Glutaraldehyde, silicone oil and NH2-PEG-COOH (MW = 2000) were purchased from Macklin Inc. Various reagents for RT–PCR were purchased from TaKaRa. Milli-Q® water was used in all experiments (resistivity 18.2 MΩ cm). Synthetic miRNA targets, the aminated oligonucleotide CPA, and the fluorescent group-labeled oligonucleotide (Table S1) were custom-made by Beijing Liuhe Huada Gene Technology Co., Ltd.

2.2. Synthesis of MSN-PEG-COOH

The synthesis method of MSN-PEG-COOH was similar to our previous biosensor synthesis method [54]. Briefly, MSN-OH was first synthesized by the template method, followed by further amination using 3-aminopropyl triethoxysilane (APTES) , MSN-OH was added to the ethanol solution of APTES and slowly shaken for 2–6 h, and the samples were washed with absolute ethanol three times after the reaction was completed, and dried in a 110-degree oven for 10–16 h. The glutaraldehyde solution was further carbonylated, the reaction products of the step before last step were added to the glutaraldehyde solution and slowly shaken for 2–6 h, and the samples were washed with water three times after the reaction was completed, and dried in vacuum at room temperature for 6–12 h. And finally, the carboxy-PEG-amine synthesis reaction of MSN-PEG-COOH occurred. The reaction product of the previous step was added to the carboxyl-polyethylene glycol-amino solution, and the reaction was slowly shaken for 6–12 h, and the sample was washed with water three times after the reaction was completed, and dried at room temperature for 6–12 h.

2.3. Assembly of biological sensor

The capture probe adapter (CPA) and MSN-PEG-COOH were dissolved in 5 × saline sodium citrate (SSC) solution (pH 7) (This is a solution of sodium chloride and sodium citrate, where the concentration of sodium chloride is 0.75 M and the concentration of sodium citrate is 0.08 M), the assembly reaction was carried out under dark conditions, and the biosensor was obtained by centrifugation after the reaction.

2.4. Preparation of aminated hyaluronic acid

First, hyaluronic acid was dissolved in 75 % ethanol and stirred for 30 min to swell, and then 50 mM hydrochloric acid was added to continue the reaction for 1 h. After the reaction, the precipitate was obtained at rest, washed with 75 % ethanol and ddH_2O_2, and finally dehydrated and dried with absolute ethanol. Then, hyaluronic acid was dissolved with formamide, and a certain amount of N-ethyl-N′-(3-dimethylaminopropyl) car-bodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was added. Then, 50 mM arabic acid dihydrazide was added and incubated in an ice bath. After the reaction was completed, the product was precipitated with excess ice ethanol, dialyzed and freeze-dried to obtain ammoniated hyaluronic acid.

2.5. Hyaluronic acid-coated biosensor

First, the biosensor was dissolved ultrasonically, and 5 mM N-ethyl-N′-(3-dimethylaminopropyl) car-bodiimide (EDC) and 10 mM N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were added and reacted for 30 min. Then, transparent aminated acid was added and reacted for 36 h under dark conditions. After the reaction, the product was precipitated with excess ice ethanol and then lyophilized after dialysis to obtain a hyaluronic acid-encapsulated biosensor.

2.6. Preparation of samples for characterization

The biological sensor and Hyaluronic acid-coated biosensor at a concentration of 0.1 mg/mL were sonicated for 15 min. Sixty microlitres of the dispersed samples was then dropped onto a Cu200 mesh (Dajikeyi, Beijing, China) for TEM analysis. Samples were also freeze-dried after dialysis and analyzed and characterized by ultraviolet and fluorescence spectroscopy, infrared spectroscopy, XPS analysis, and nuclear magnetic and mass spectrometry.

2.7. Target microRNA detection curve

Target miRNAs with different concentration gradients from 1 fM to 100 μM were prepared and dissolved in 5 × SSC. A target sample of 10 μL was added to 90 μL of the biosensor. After mixing and incubating for 30 min, the samples were washed with 1 × SSC 3 times and then washed with 5 % ethanol once. Finally, the cells were resuspended in 100 μL of 1 × SSC and tested with a full-featured microplate reader (BIO-TEK, USA). To use it, we first need to turn on the microplate reader and select the fluorescence measurement mode. We select the appropriate wavelength and filter according to the needs of the experiment, and put the head in contact with the instrument to avoid light interference. Then the sample RNA is aliquoted and placed in the microplate plate, and the immune reaction reagent is added in it, and the fluorescent probe is added after the reaction for labeling. Finally, the microplate is placed into the microplate reader, and the parameters of the reading fluorescence data are selected to measure the fluorescence intensity of the sample. Each sample concentration was tested 5 times in parallel to make a standard curve.

2.8. Detection of target sequence mismatches

Different miRNAs (Table S1) at concentrations of 100 nM were prepared and dissolved in 5 × SSC. Ten microliters of the miRNA sample were added to 90 μL of the biosensor and reacted for 30 min at room temperature. Then, the cells were washed with 1 × SSC 3 times and washed once with 5 % ethanol. Finally, the cells were resuspended in 100 μL of 1 × SSC and tested with a full-featured microplate reader (BIO-TEK, USA). Each sample was tested in parallel 5 times, and the test results were compared.

2.9. Cell culture

We used the H9C2 cell line, a cardiomyocyte cell line, to prepare an oxidative stress cardiomyocyte infarction model to confirm our experimental results. The H9C2 cell line was derived from the Holistic Gold Cell Bank (Beijing, China), and the H9C2 cells were cultured in DMEM (Sigma) and 10 % FBS (Gibco). H9C2 cells were incubated with 95 % air and 5 % carbon dioxide at 37 °C. After starvation in serum-free medium for 12 h, 200 μM hydrogen peroxide was used for 0 h, 2 h, 4 h, 6 h, 8 h, and 10 h to prepare oxidative stress models. Meanwhile, after 8 h of hydrogen peroxide treatment, the biosensor was added and incubated for 6 h for detection.

2.10. Cell counting Kit-8 (CCK8) assay

Since the whole part of our biosensor is composed ofnanosilica spheres, hyaluronic acid accounts for a very small part of the sensor and has no toxic effect on cells, so we conducted a toxicity experiment of nanosilica spheress on cells. To detect the cytotoxicity of nanosilica spheres, the cell viability was determined after the treatment with different concentrations of nanosilica spheres (0,10,20,40,60,80,100,120 pM) for 24 h, 48 h.0.1 mL H9C2 cells (8 × 104 cells/mL) were plated in 96-well plates following by exposure to nanosilica spheres. 10 μL of CCK-8 (Life-iLab, Shanghai, China; AC11L054) was added into each well and incubated for 2 h at 37 °C. Then the absorbance was detected at 450 nm based on microplate reader ((BioTek, USA). Data were statistically normalized to control group.

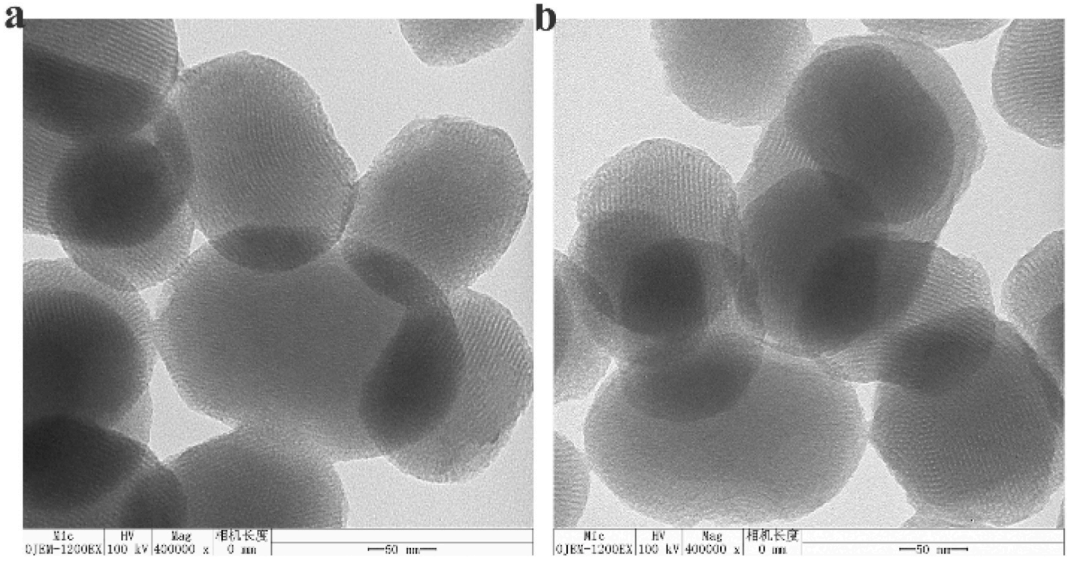

Fig. 1. TEM characterization of probe material morphology: MSN-OH (a), MSN-HA (b).

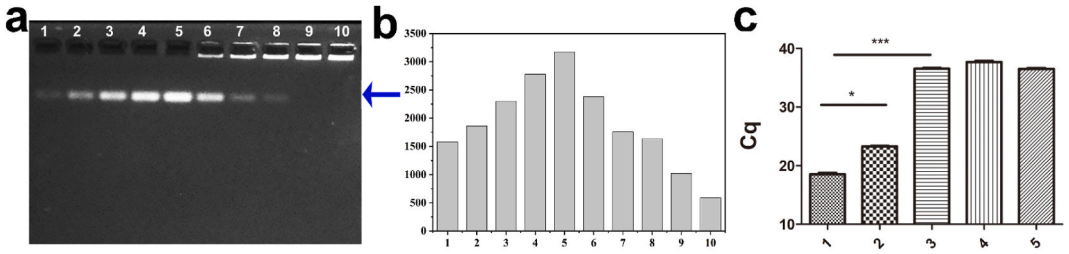

Fig. 2. Nanobiosensor assembly results of nucleic acid aptamers and analysis of RNA stability. Fig. 2a shows the results of an agarose gel. Lanes 1–5 are the results of different concentrations of aptamers in agarose gel electrophoresis. The concentrations of aptamers from lanes 1 to 5 were 20 pmol, 40 pmol, 60 pmol, and 80 pmol, 100 pmol, respectively. Lanes 6–10 are 80 pmol aptamers mixed with nanobiosensors of different masses and electrophoresed in an agarose gel. The masses of biosensors from lane 6–10 nm were 0.1 μg, 0.2 μg, 0.4 μg, 0.8 μg, and 1.0 μg, respectively; Fig. 2b shows the analysis software of Quantum CX5 to analyze the brightness of the band at the arrow mark; it shows that the nucleic acid aptamer can be assembled into the nanobiology On the sensor, Fig. 2c shows the stability experiment of nano-biosensor for miRNA: 1 is fresh miRNA with no treatment, 2 is miRNA encapsulated with nanobiosensor mixed with culture medium and processed overnight, and 3 is miRNA mixed with culture medium and processed overnight. 4 is to mix the nano biosensor and culture medium and then overnight treatment, 5 is pure medium to do overnight treatment. The Cq value of fluorescence quantitative PCR shows that the miRNA loaded by the nano-biosensor is almost not degraded and maintains good stability. ***$p < 0.001$.

2.11. RT-qPCR

The total RNA was extracted from the cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis was quantified with SYBR Premix Ex *Taq*II (Takara), and 50 ng cDNA and 500 nmol/L primers were quickly detected by an ABI 7500.

3. Results and discussion

3.1. Probe characterization

We used transmission electron microscopy to characterize the topography of the biosensor before and after packaging, as shown in Fig. 1. It can be seen from the figure that the size of the biosensor is approximately 110 nm, which is basically in line with our previous biosensors. The whole of our sensor is determined by a nanosilica spheres, which is only coated with a layer of hyaluronic acid on its surface. The captured microRNA is present in hyaluronic acid layer, both microRNA and hyaluronic acid have tiny forms, so it can be seen that hyaluronic acid has little effect on the morphology of the biosensor (Fig. 1, Fig. S2).

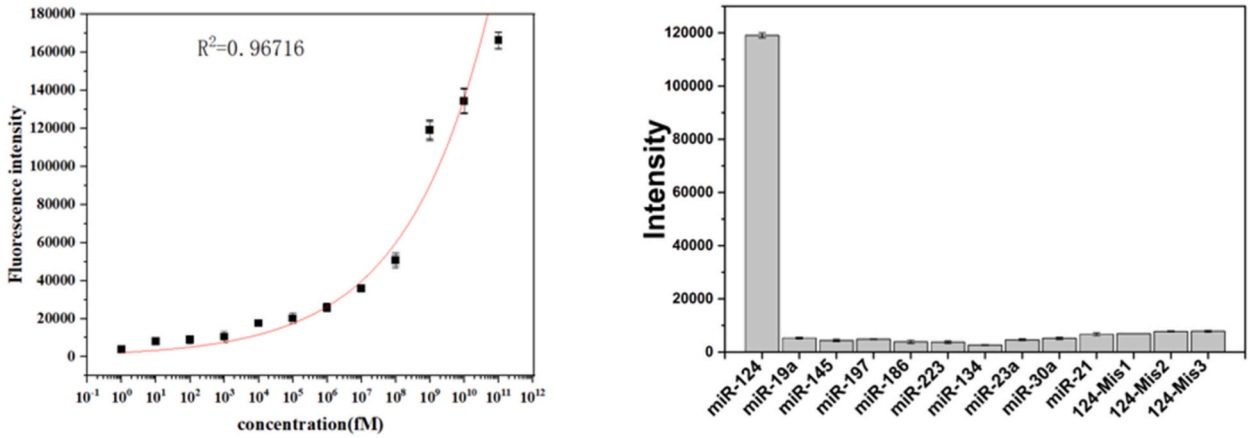

Fig. 3. The sensitivity and specificity analysis diagram of nano-biosensor for detecting miRNA miR-124. Fig. 3a is the standard curve for detecting miR-124 by biosensor material, and Fig. 3b is the comparison result of biosensor material for different small RNA detection.

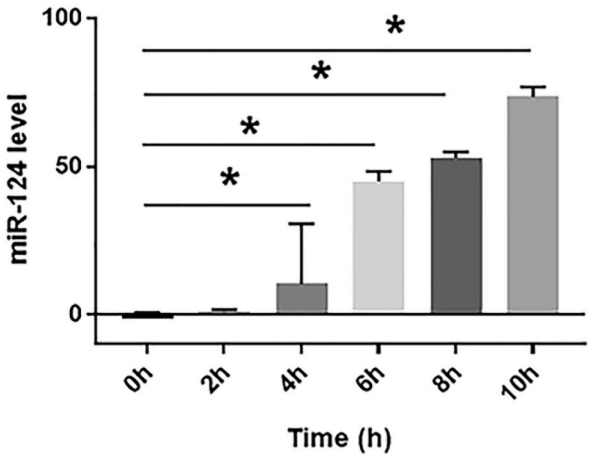

Fig. 4. Changes in the expression level of miR-124 in H9C2 cells after H_2O_2 treatment for different time conditions.

3.2. Biosensor and nucleic acid assembly characterization and the investigation of RNA stability

After mixing the aptamer with the nanobiosensor, we tested the condition of the aptamer by agarose gel experiments. Fig. 2a and b indicate that the aptamer was assembled on the nanobiosensor and remained in the loading hole of the agarose gel. We also mixed the miRNA with the nanobiosensor and added a certain volume of culture medium to incubate overnight in a carbon dioxide incubator and set up the miRNA-only group and the nanobiosensor group as the control group. After taking the overnight sample for reverse transcription, we performed a fluorescent quantitative PCR experiment to test whether the nanobiosensor can effectively protect miRNA from degradation. As shown in Fig. 2c, nanobiosensors played a role in stabilizing miRNAs and could protect miRNAs from degradation.

3.3. Biosensor detection range and mismatch analysis

To more accurately determine the concentration of miRNA to be detected, we first used the biosensor materials to detect miRNAs of different concentrations and made a detection standard curve, as shown in Fig. 3a. The results suggested that the biosensor could be used for the detection of miRNA. There was a linear relationship with the fluorescence intensity of the biosensor in the concentration range of 1 fM to 10^7fM (Fig. S3), and the detection limit was 1 fM (Fig. 3a). In addition, we also tested several other common miRNAs and miR-124 mismatches, and the test results are shown in Fig. 3b. Among the numerous miRNAs with a concentration of 10^8fM, the biosensor had a particularly high detection specificity for the target miR-124 but was not undetectable to other miRNAs. This also illustrated the specificity of the detection of the aptamer-type biosensor material we designed.

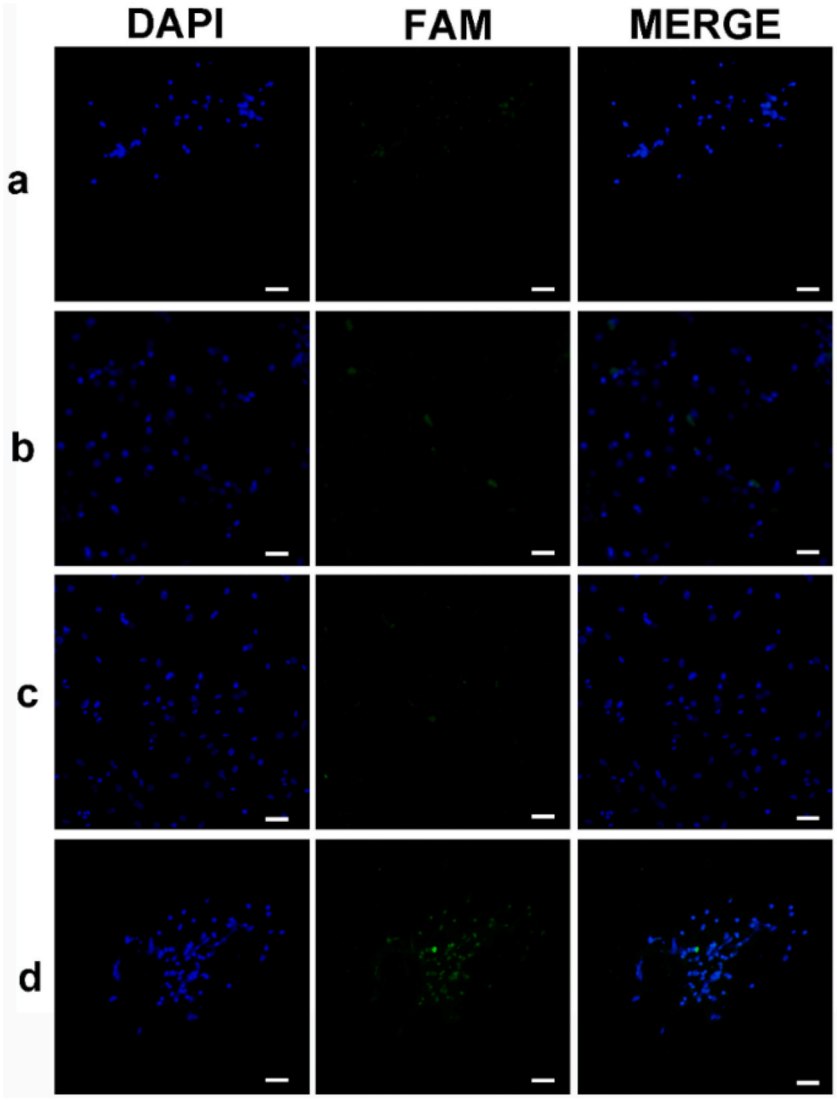

Fig. 5. The results of in-situ detection of nano-biosensors in cell. Fig. 5 a is the result of adding FAM-labeled nucleic acid aptamers to cells treated with H2O2, and 5b is the result of adding FAM-labeled nucleic acid aptamers to normal cells. 5c is to add nanoparticles to H2O2-treated cells, d is to assemble FAM-labeled aptamers to nanoparticles to form nanoprobes, and then add probes to H2O2-treated cells. The results show that nano-biosensors can transport FAM-labeled aptamers into cells and perform imaging analysis on specific miRNAs. (Scale bar, 50 μm).

3.4. Oxidative stress myocardial cell infarction model

H_2O_2 is usually used to treat H9C2 cardiomyocytes with oxidative stress to construct a model of cardiomyocyte infarction. After treatment with H_2O_2 for different times, the cells were collected to extract the total RNA, and then the expression levels of miR-124 in the cells were analyzed by fluorescence quantitative PCR. As shown in Fig. 4, under the induction of H_2O_2, the expression levels of miR-124 in cardiomyocytes increased continually.

3.5. In situ imaging of miR-124 in live cells

To demonstrate the performance of our biosensor in biomedical applications, we used nanobiosensors to detect myocardial infarction models prepared by oxidative stress. The cytotoxicity test has shown us that our biosensor is basically non-toxic to cells (Fig. S4). After the nanobiosensor was introduced into the cells for 6 h, the cells were washed 5 times with PBS, and then 4 % formaldehyde in PBS was added to fix the cells for 10 min at room temperature. After that, the formaldehyde solution was removed and washed with PBS, and the cells were stained with DAPI. Finally, we used a fluorescence microscope to image the cells, as shown in Fig. 5. Fig. 5b shows that in normal cardiomyocytes, due to the low expression of miR-124, the fluorescence of the biosensor was almost

Table 2
Comparison of miRNA detection between our study and other assays in the literature.

Type	LOD range	Sensitivity	Specificity	In situ	Cost	Evaluation	Disadvantage	Reference
Our biosensor	1 fm	Relatively high	Single-nucleotide	Yes	Low	intracellular detection	Lack of wide application	–
3D hydrogel biosensor	0.03 fM	Relatively high	Relatively high	Yes	–	intracellular detection	Lack of wide application	[52]
RT-qPCR	To attomolar range	High	Single-nucleotide	No	Relatively low	Gold-standard	Quantitative bias may be introduced	[34,35,55]
Northern blotting (NB)	nM–pM	low	low	No	–	Standard method	Complex and time-consuming; not fit for low abundance miRNA	[34, 56–58]
Microarrays chips	nM–pM	low	Low for highly homologous miRNAs	No	Relatively high	Parallel analysis of many miRNAs	Can not absolute quantification	[34,56,59, 60]

invisible under the fluorescence microscope; however, in the oxidative stress cardiomyocyte infarction model, the overexpression of miR-124 in the biosensor enhanced the fluorescence of the biosensor, and the fluorescence could be clearly seen. Similarly, if the aptamer was directly added to the oxidative stress myocardial cell infarction model (Fig. 5a), the aptamer could neither reach the cell nor function as a biosensor. By imaging cells in situ, we found that the biosensors could be used as probes for detecting oxidative stress in cells.

In addition, we compared other technologies to our biosensors, and we have tabulated it (Table 2). We compared them form seven aspects: LOD range, sensitivity, specificity, in situ, cost, evaluation and disadvantage. Then we have tabulated it (Table 2). Through Table 2 we can notice some advantages of our sensor. Our biosensor can be used to detect target RNA in cells. The nucleic acid probe has a high degree of specificity for the target miR-124, and the detection limit of this method is 1 fm. When using the probe, it will maintain good stability of RNA, preventing it from being degraded.

4. Conclusion

In summary, we designed and developed an enzyme-free self-assembled biosensor that could be used for noncoding nucleic acids. The detection limit of the sensor for noncoding nucleic acids reached 1 fM. In addition, the sensor was successfully used for the in situ detection of miR-124, which is an important biological surface molecule for myocardial infarction. This shows that the biosensor we designed is a potential sensor for the in situ diagnosis of myocardial infarction *in vivo*. In view of the characteristics of the corresponding specific and high expression of noncoding RNA caused by different diseases, our sensors can also be designed as biosensors corresponding to different noncoding nucleic acids for the *in vivo* in situ determination of the location of the disease. The biosensor does not need to extract and separate miRNA, so it is suitable for extremely low miRNA contents in samples such as plasma, serum, and tissue. This also opens new ideas for future precision medical diagnosis.

Ethics approval and consent to participate

No ethics approval was required for this article that did not involve patients or patient data.

Consent for publication

All authors consent to publication.

CRediT authorship contribution statement

Shuo Li: Writing – review & editing, Methodology, Investigation, Data curation. **Xiang-Yu Pei:** Writing – review & editing, Methodology, Data curation. **Xin-Yi Liu:** Methodology, Data curation. **Shu-Liang Wang:** Investigation, Data curation. **Wen Xu:** Methodology. **Jing-Jing Wang:** Methodology. **Zhen Feng:** Methodology. **Han Ding:** Writing – original draft, Supervision, Resources, Project administration, Data curation, Conceptualization. **Yin-Feng Zhang:** Writing – review & editing, Supervision, Project administration. **Rui Zhang:** Writing – review & editing, Validation, Supervision, Funding acquisition.

Declaration of competing 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33588.

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