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Highly sensitive fluorescence multiplexed miRNAs biosensors for accurate clinically diagnosis lung cancer disease using LNA-modified DNA probe and DSN enzyme

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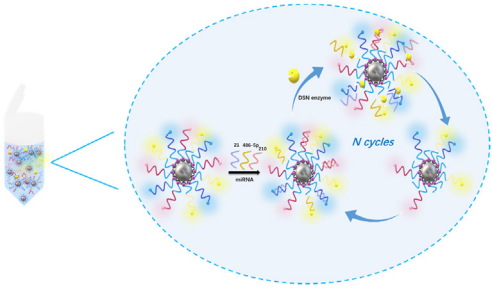
HIGHLIGHTS

- A multiplexed fluorescence-based biosensor combining DSN enzyme and LNA probe tethered MBs for microRNAs detection was settled.
- The developed method exhibited a high sensitivity and selectiovity for lung cancer (LC) diagnosis.
- The engendered limit of detection is equal to 98 aM, 120 aM, and 300 aM of mir-21, miR-210, and miR-486-5p, respectively.
- Via using this serum 3-microRNAs signature, we were able to distinguish LC disease patients from healthy volunteers.
- Our proposed method was validated by computed tomography and revealed a high correlation.

GRAPHICAL ABSTRACT



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ABSTRACT

With the emergence of microRNAs as key biomarkers for disease diagnosis such as lung cancer, various techniques have been settled for their detection. However, these current methods require different amplification steps since numerous challenges for detecting circulating miRNAs are attributable to their intrinsic properties accounting for tiny sizes, high sequence similarity, and low abundance. Duplex specific nuclease (DSN)-based microRNA amplification has recently gained interest in biosensing applications thanks to its catalytic activity based on target recycling. In this context, we designed a highly selective, sensitive, and multiplexed fluorescence-based biosensor combining DSN enzyme and magnetic

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beads to detect three distinct microRNAs, including microRNA-21, microRNA-210, and microRNA-486-5p. By exploiting the above approach, we were able to detect as low as 98 aM, 120 aM, and 300 aM of mir-21, miR-210, and miR-486-5p, respectively. Furthermore, this recommended strategy displays a high selectivity toward an entirely matched target than the off-target. These results are ascribed to the potent DSN enzyme activity and to the locked nucleic acid (LNA)-modified DNA probe that boosted the heteroduplex probe/target stability. Lastly, our proposed method was applied to detect microRNAs in the serum samples and displayed a high efficacy to discriminate between healthy controls and lung cancer patients. Furthermore, the analytical accuracy of the proposed strategy was validated with the computed tomography (CT) technique of the chest. Thus based on these findings, this strategy could open new directions for detecting microRNAs associated with several diseases.

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1. Introduction

Cancer is a general term that refers to an assembly of maladies typified by the frenzied development and spread of abnormal cells. Once the spread is not well monitored, it can induce death [1]. Among them, lung cancer (LC). The world health organization reported around 2.1 million new lung cancer diagnostic cases and approximately 1.8 million deaths triggered by lung cancer worldwide in 2018 [2]. These findings indicate that LC places a substantial burden on national welfare systems, health services, and families [3]. Hence, there is an ultimate need for an ultra-sensitive and reliable platform for cancer diagnosis and prognosis in its early stages using easy-to-detect biomarkers such as miRNAs.

MicroRNAs (miRNAs) are a category of endogenous small noncoding RNAs of about 18–25 ncts in length that monitor the gene expression in multicellular organisms [4]. During the post-transcriptional event, they contribute to the blockage or the messenger RNAs' degradation [5]. Researchers have recently had a tremendous and emergent interest in exploring their role in different diseases, including lung cancer [6]. The outcomes state that their expression dysregulations are typically amended in various biological processes such as cell proliferation, invasion, apoptosis, death, and in diverse development stages of the disease [7]. Thus, microRNAs emerge as potential biomarkers for disease detection, including lung cancer. However, their analysis remains challenging due to their ultralow level, tiny size, sequence homology among family members of microRNAs, and ability to degrade [5,8]. These limitations influence their clinical assays, thus inspiring researchers to implant new strategies for selective, sensitive, and expedient detection of miRNAs in real samples.

Currently, the “gold standard” northern blotting analysis and real-time PCR are the predominant methods to analyze miRNAs. However, they are suffering from time-consuming steps, reduced sensitivity, incapability of multiplexed detection, in addition to their expensive cost, which makes them inappropriate for clinical application [9,10]. Therefore, biosensor technology has become an increasingly attractive alternative to miRNAs detection to satisfy these unmet clinical needs. A wide number of electrochemical, electrical, and optical sensors combined with various amplification strategies have been developed [5]. It is necessary to be noticed that, in current years, fluorescent-based biosensors have gained more attention because of their considerable advantages, including their simplicity, short processing time, and their easy-to-use equipment [11]. Yet, this method lacks sensitivity. Therefore, scientists have taken advantage of the different enrichment and amplification strategies [9]. Among them is the use of a special bio-molecular immobilizing carrier like magnetic beads (MBs), considered a versatile tool for biosensing [12,13]. The combination of DNA probes and MBs was already used in various analytical applications, including miRNAs detection. MBs can be easily separated using a permanent magnet within seconds, thus generating an ultralow background. Moreover, we can widen the surface area, which increases the density of the immobilized ligand via the aid of magnetic beads [14,15]. In this context, streptavidin-coated magnetic beads are emerging as a particularly promising tool to conjugate the capture probe to magnetic beads due to many advantages, including the high binding capacity between biotin and streptavidin interaction with a disassociation constant (K_d) of 4×10^{-14} M [16].

This approach may be combined with other amplification strategies such as rolling circle amplification [17], hybridization chain reaction (HCR) [18], and more. Recently, duplex-specific nuclease-assisted target recycling opened new paths as an isothermally linear signal amplification method for microRNA detection [19]. Ye et al. were considered the scientists who took advantage of the DSN enzyme coupled with the Taqman probes to improve miRNAs detection [20]. Over since other researchers have further studied this amplification strategy. DSN enzyme in association with MBs has undeniably bettered the sensitivity of the miRNA assays. In this perspective, Wang's group developed an assay that engaged both magnetic beads and DSN enzymes to boost miRNA detection. They were able to detect as low as 0.3 fM of miRNA [21]. Previously, this combination (DSN/MBs) has also been successfully demonstrated for a single microRNA detection using a fluorescence-based approach by our group. In that report, we studied the effect of a DNA spacer and the base pair orientation on the DSN activity, in which we demonstrated that the sensitivity was boosted in the presence of triethylene glycol (TEG); DNA linker with LOD equal to 0.17 fM [22]. However, it has not yet been effectively applied multiplexed detection since the latest studies have revealed that the expression of a single miRNA is not enough to identify one specific disease [23]. For example, miRNA-21 is upregulated in approximately all the tumors [24,25].

In this context, we assessed an ultrasensitive, simultaneous, multiplexed, and effective fluorescence-based method for lung cancer diagnosis via the detection of three different microRNAs (miRNA-21, miRNA-210, and miRNA-486-5p). The recommended assay engaged magnetic beads and duplex-specific nuclease (DSN). Concerning the detection probe, we have employed an LNA-modified DNA probe due to its beneficial advantages. The design was based on our aforementioned report. In addition, we investigated the Triethylene glycol as a probe spacer since it enhances the DSN activity by creating a suitable microenvironment for the enzyme's activity. This approach displayed a great selectivity toward a single base mismatch with no cross-reactivity. Moreover, this method exhibited a high sensitivity even in the real sample.

2. Materials and methods

2.1. Materials

We obtained the streptavidin magnetic beads from New

England Biolabs (Beijing, China) (4 mg/ml, 1 μm). DEPC treated water (RNase-free), and all the oligonucleotides listed in table S1 were ordered from Sangon Biotechnology Co., Ltd (Shanghai, China). DSN kit was purchased from an Evrogen distributor in Guangdong, China. The RNase inhibitor (2000U) was provided by Thermo Fischer Scientific Co., Ltd (Shanghai, China). Human serum and Tween20 were bought from Sigma life science Co., Ltd (Jiangsu, China). Sodium hydroxide (NaOH), disodium dihydrogen phosphate dedocarbohydrate (Na_2 H_2PO_4. $12H_2O$), sodium dihydrogen phosphate dehydrate (NaH_2PO_4. $2H_2O$), and sodium chloride (NaCl) were purchased from KESHI, China. Tris-HCl was obtained from biosharp China. Healthy human serum samples were bought from Sigma life science Co., Ltd (Jiangsu, China).

All the outcomes were attained by Tecan infinite M1000 PRO, a monochromator-based microplate reader. The various spectrum of FAM, TAMRA, and Cy5 were collected from 515 to 600 nm, 570−650 nm, and 650−750 nm under different excitation wavelengths of 483 nm, 545 nm, and 625 nm, respectively. All of the measures have proceeded in triplicate.

2.2. LNA probe design

In the proposed work, we followed the probe design of our previous study wherein we optimized the use of a DNA spacer and the pair bases orientation. It was unveiled that the triethylene glycol (TEG) spacer exhibited a higher fluorescence intensity [22]. For further improvement of the multiplexed microRNA detection, we exploited the use of locked nucleic acid (LNA) as capture probes. LNA is a modified ribose comparable to 2′-*O*-methyl RNA wherein the sugar's 4′-C and 2′-O atoms are hinged via a methylene bridge. This covalent bridge efficiently restrains the flexibility of the ring and creates a rigid conformation (A-type) in the presence of the target oligonucleotides. This latter boosts phosphate backbone preorganization and base stacking, causing an amended affinity toward the target miRNA sequences and a greater melting temperature [26]. However, since the LNA displays an exonuclease resistance, we decided to work with DNA probes containing LNA tags in the middle of the DNA probes sequences [27]. The arrangement of bases within each probe was chosen according to several criteria: the melting temperature, the binding capacity between the probe and the target sequence, and the cross-reactivity between the different probes and between the probe and other microRNAs, where there is no target [28]. For that reason, we exploited the LNA Oligo Optimizer tools and the LNA Oligo Tm Prediction on the Qiagen website (LNA Technology (qiagen.com)) to design the right probe for each target microRNA. After several essays, the right probe was obtained, and they are presented in Table S1.

2.3. MiRNAs detection and the DSN cleavage reaction

Following the optimization of the probes' concentration and their immobilization into the magnetic beads surface via streptavidin/biotin interaction (supporting information), the reaction mixture of 20 μl in a sterilized PCR tube was loaded with 20 μg of magnetic beads conjugated to LNA probes, 2 μl of the different microRNAs each at different concentrations, 1 × DSN master buffer, 20 U RNase inhibitor, microRNAs, and 0.5 U DSN enzyme. The mixture was incubated at 55 °C for 120 min in a thermocycler. Afterward, the MBs carrying the unreacted DNA capture probes were separated by a permanent magnet, and the DSN cleavage process was stopped. The collected volume of the supernatant containing the cleaved fluorescent dyes was 10-fold diluted with DEPC water. Then, the spawned solution was poured into a 96-well microplate and underwent a fluorescence measurement. Finally, the spectrum was collected under the appropriate excitation wavelength for each dye. Before checking the sensitivity and the selectivity of the proposed assay, we optimized the DSN reaction conditions, counting the temperature, the time, and the DSN quantity, following the mixture preparation in the presence of 1 nM of each microRNAs.

1 × DSN master buffer (50 mM Tris-HCl (pH 8.0), 1 mM DTT and 5 mM $MgCl_2$)

2.4. MiRNAs detection in complex matrice

To check the feasibility of our proposed method for LC detection, we first applied it in a more complex matrice rather than a buffer. In this regard, we investigated the commercial serum sample. The latter was incubated at room temperature for 10 min and then heated to 95 °C for 5 min. Next, the serum was diluted in the DSN reaction mixture (1:100 v/v), prepared as described in sections 2.3 and 2.4, and containing various spiked microRNAs to desired concentrations (0.01, 1, and 10 pM). Afterward, the reaction was incubated at 55 °C for 2 h, followed by fluorescence measurement. Secondly, we applied the method for the detection of microRNAs extracted from healthy controls and lung cancer patients according to the Ethical Committee of Chongqing General Hospital approved this study (No. S2021-026-01) (supporting information).

3. Results and discussion

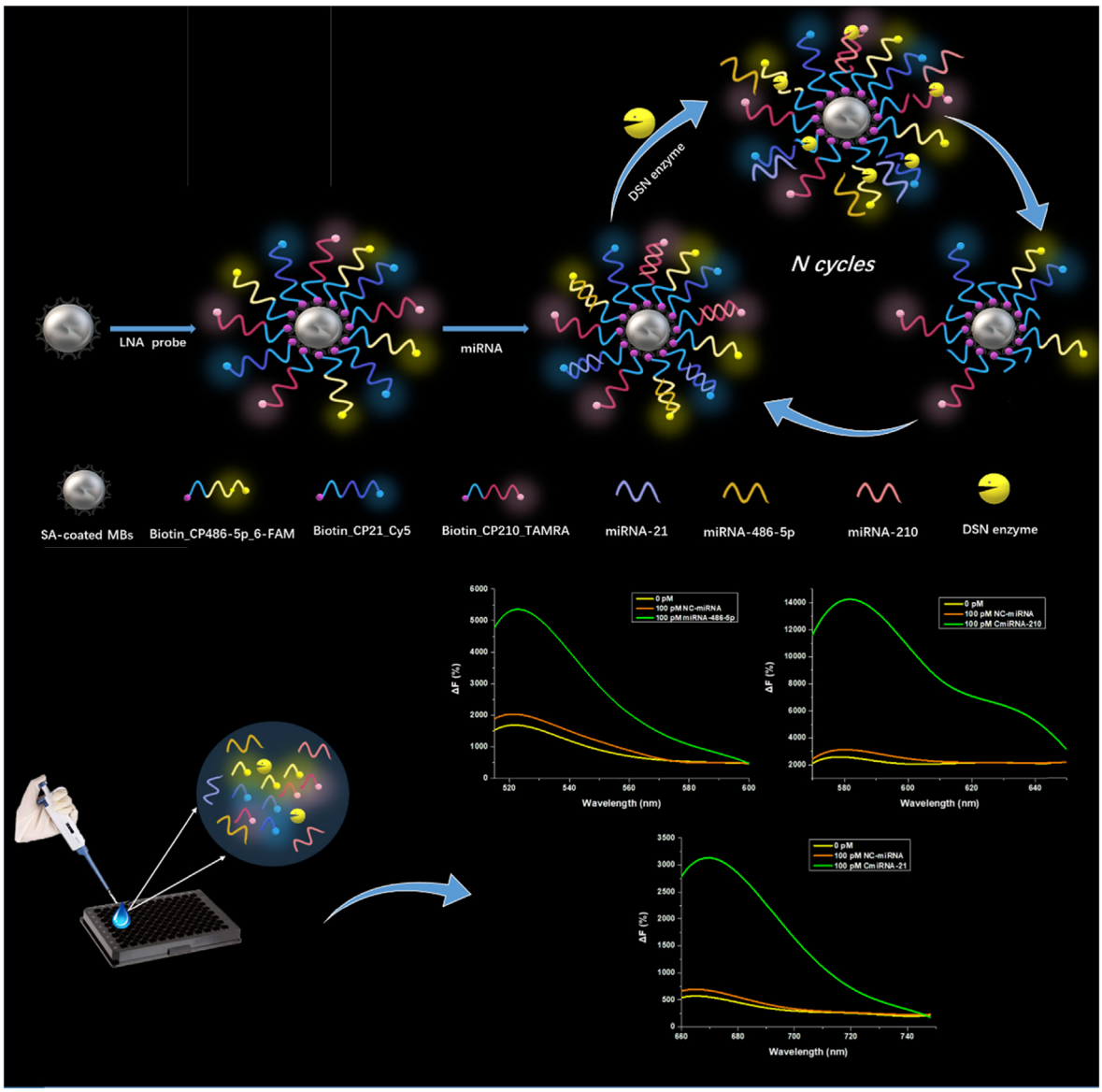
Our proposed assay is based on magnetic beads as probe carriers and the DSN enzyme as a signal amplification strategy. In addition, we used LNA-modified capture probes to improve the hybridization specificity and stability, which helps for temperature normalization.

This method is applied for the simultaneous detection of three distinct miRNAs, including microRNA-21, microRNA-210, and microRNA-486-5p, associated with lung cancer. The choice of these microRNAs is based on one study conducted by Shen and his colleagues wherein they could distinguish between benign and cancerous lung nodules using this microRNAs panel [29]. Moreover, each of these microRNAs has separately displayed high sensitivity and specificity toward a lung cancer diagnosis.

The LNA-modified probes were firstly coupled to the magnetic microbeads via a streptavidin/biotin interaction. Then, the DSN exercises its activity at the time of hybridization with the target microRNA and under the optimal conditions. The latter implicates probes hydrolysis, dyes releasing, and target liberation, which in turn hybridizes with another probe launching a new cycle. In the end, the magnetic beads were collected, and the supernatant carrying the dyes was aspired and underwent fluorescence measurements. The obtained signal from the proposed assay, termed ΔF%, was calculated as shown in the formula below.

$$\Delta F\% = (F_i\text{-}F_0)/F_0*100$$

Where F_0 refers to the background signal in the absence of microRNAs, and F_i refers to the recorded signal following the addition of miRNA to the mixture. As shown in Scheme 1, the fluorescence spectra were obtained in the presence of the three complementary microRNAs (miRNA-21, miRNA-210, and miRNA-486-5p), and in the presence of non-complementary miRNA (NC-miRNA) at 100 pM wherin the highest fluorescence intensities for the three dyes (cy5, TAMRA, and FAM) were recorded at 670 nm, 580 nm and at 521 nm respectively. These findings validated the principle of our method as described above.



Scheme 1. Illustration of the proposed method principle.

3.1. Optimization of the DSN reaction

The enzymatic activity is generally affected by several key parameters, including the temperature, the time, and the amount of the DSN enzyme. Thus, we optimized all these factors. Each microRNA was tested separately in this section, and the fluorescence signal was recorded using a target concentration of 1 nM. Since we opted to work with an LNA-modified DNA probe instead of an unmodified DNA probe, the melting temperature (Tm) was influenced [30]. To the best of our knowledge, it was proved that the substitution of a single DNA monomer by LNA monomer increased the Tm (2−10 °C) in the presence of complementary miRNA compared to DNA/miRNA duplexes [31]. Therefore, an optimization of the reaction temperature was required. As illustrated in Fig. 1A, the three targets have the same curve trend. For instance, an increase in the fluorescence response (ΔF%) was spotted as the temperature increased to 55 °C, and then a slight decrease was perceived. These findings were accredited to the influence of the melting temperature on the stability of the heteroduplex LNA/miRNA [32]. The obtained results were in good agreement with those of previous reports, where the researchers mentioned that the working temperature needs to be 10 °C—15 °C less than the Tm, and our three probe/target duplexes' Tm is nearly 75 °C. For that reason, 55 °C was chosen to be an optimal reaction temperature in this work. To further maximize the biosensor response, the DSN reaction time was optimized for all three probes. The fluorescence intensity ΔF% was recorded from 30 min to 150 min and the reaction was carried at 55 °C in the presence of 1 nM of the miRNA target and 0.5 U of the DSN enzyme. Based on Fig. 1B, the ΔF% value achieved the above-average point when over 2 h for the different reaction mixtures. Hence, the subsequent experiments fulfilled the miRNAs detection at 55 °C for 120 min. Along with the temperature and the incubation time, it was stated that the DSN quantity could also influence the DSN reaction functioning. In this regard, we have investigated the effect of the DSN amount on the response of 1 nM of the various miRNAs (Fig. 1C). The acquired outcomes disclose that the normalized signal increased substantially from 0.1 U to 0.5 U, then an insignificant

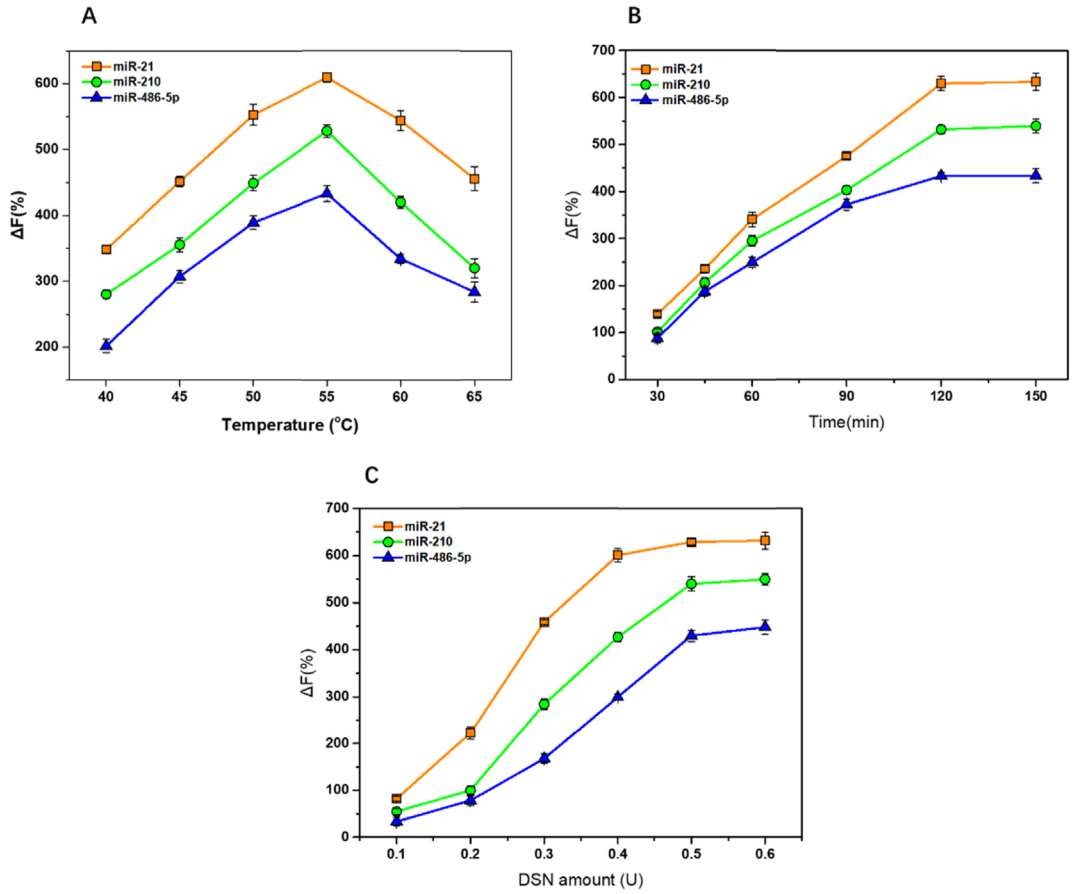


Fig. 1. Optimization of the DSN reaction parameters using 1 nM of microRNAs (each), 20 μg/mL MBs and 1 μM probes; A) temperature optimization with 0.5 U DSN enzyme and the reaction time is 2 h, B) time optimization with 0.5 U DSN enzyme and the reaction temperature is 55 °C, C) DSN amount optimization reaction conducted for 2 h at 55 °C.

increase was detected for 0.5 U (less than 10%). The same results were recorded for microRNA-21, microRNA-210 and microRNA-486-5p. Thus, 0.5 U was chosen as the optimal amount for further experiments.

3.2. Multiplexed detection of miRNAs

After the DSN reaction optimization, we investigated our proposed detection strategy in a multiplex format. We firstly explored the dependency of the fluorescence intensity upon the miRNAs concentration. Then, to gauge the sensitivity of the proposed method, we experienced the multiplex approach by raising the concentrations of each target miRNA from 0.5 fM to 10 pM. In order to evade wasting time in the successive experiments, the fluorescence intensities were recorded at 580 ± 5 nm, 670 ± 5 nm, and 521 ± 5 nm for miR-210, miR-21 and miR-486-5p, respectively. As illustrated in Fig. 2, linear correlations between the fluorescence intensity and miRNAs concentration on the logarithmic scale were obtained within a range from 0.5 fM to 10 pM with a regression coefficient equal to 0.998. The equations for the three different microRNAs were:

$$\Delta F\% = 35.23 \log([\text{miRNA-21}]) + 214.264$$

$$\Delta F\% = 31.21 \log([\text{miRNA-210}]) + 170.23$$

$$\Delta F\% = 24.16 \log([\text{miRNA-486-5p}]) + 173.778$$

Using the recommended method, we were able to detect as low as 98 aM, 120 aM, and 300 aM of microRNA-21, microRNA-210, and microRNA-486-5p, respectively (based on the 3σ approach/slope) [33]. The variation in the limit of detection (LOD) for the different biomarkers was ostensibly down to the stability/melting temperature of the probe/microRNA heteroduplex as well as to the total coverage of the MBs corresponding to the various probes used in this work. Our result exhibits a lower detection limit with better sensitivity than the preceding techniques in literature employed for simultaneous miRNAs detection. For instance, Yuan's group [34] reported a multiplexed electronic biosensor for microRNA detection using hairpin probes immobilized on Au electrode and duplex specific nuclease as a signal amplification strategy. Via applying this technique, they detected as low as 4.3 fM and 3 fM of miR-141 and miR-21, respectively, which are 2 magnitudes higher than our obtained LOD. In another report [28], a sandwich-type based Single Molecule Array (Simoa) procedure was investigated by Cohan et al. for the detection of various miRNAs without applying any amplification strategy. In their study, they used LNA as a capturing probe which was coupled to paramagnetic microbeads. They were able to detect miRNAs at femtomolar concentrations with LODs fluctuating between 1 and 30 fM. For more comparison, Jie and his co-workers [35] established a fluorescence-based platform for miR-21 and miR-144 detection using quantum dots as fluorescence tags and Au coated MBs as a probe carrier, in addition to two different enzymes (exoIII and T7 enzyme) in cascade to improve the sensitivity. Despite using two catalytic reactions, the LOD remained high (1.5 pM). More recently, Shen et al. [36] developed an electrochemical biosensor. To boost the sensitivity of their device, they immobilized DNA probes on gold-coated magnetic nanospheres and employed a

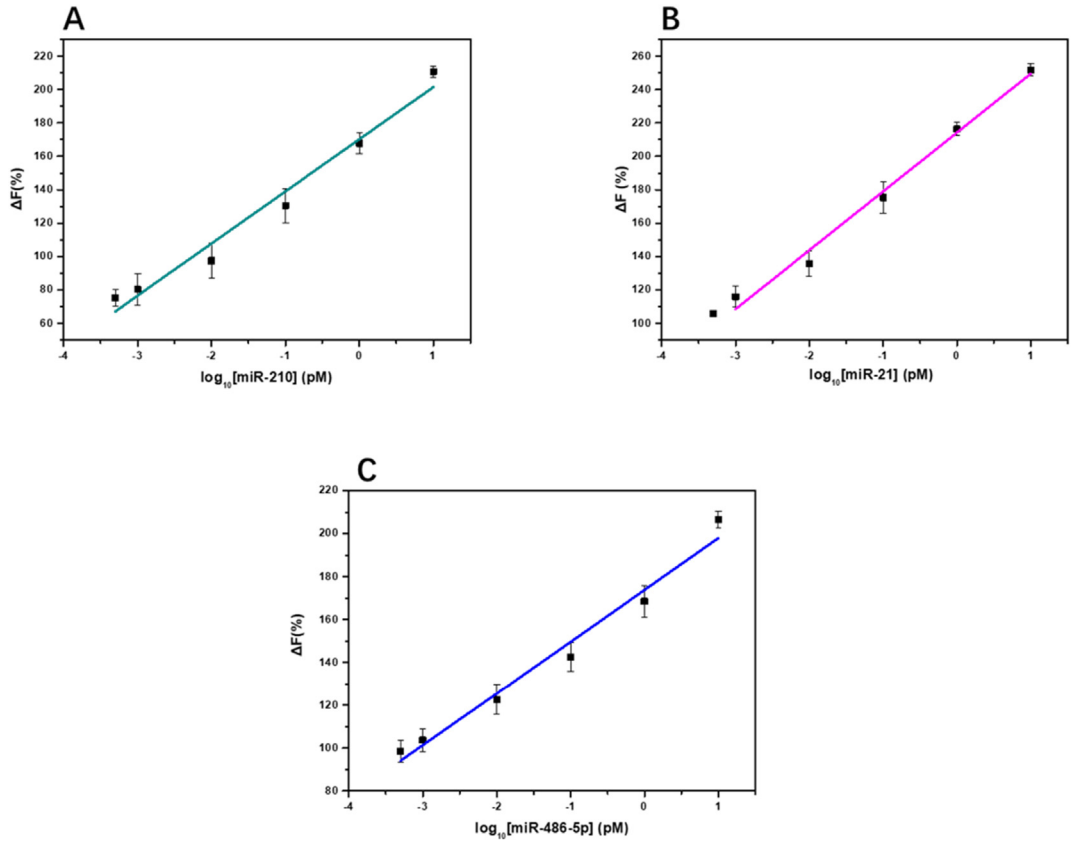


Fig. 2. The calibration curves of the three microRNAs A) miR-210, B) miR-21, and C) miR-486-5p, using different concentrations that range from 0.5 fM to 10 pM. The tests were carried out for 2 h at 55 °C with 0.5 U DSN enzyme.

hyperbranched hybridization chain reaction. The engendered LOD for miR-155 and miR-21 were 1.8 fM and 1.5 fM, respectively. Thus, these outcomes are accredited to the use of the DSN enzyme that has excellent enzyme stability and a great catalytic activity allowing a considerable enhancement in the signal via target recycling [37]. Moreover, the insertion of a DNA spacer (triethylene glycol (TEG), affects the local microenvironment surrounding the magnetic beads tethered probes and the steric hindrance, subsequently improving the DSN activity as reported previously by our group [22]. Despite investigating the same process in both works, the engendered limit of detection was further improved in this report, which decreased from 170 aM to 98 aM. Hence, these results are attributed to the contribution of the LNA-modified DNA probe through bettering the establishment of a resilient probe/target heteroduplex via boosting its thermo-stability. Even though the LNA monomers grant a relative level of nuclease resistance toward endo- and exonucleases, we proved that it is still possible to employ it with DSN enzyme for signal amplification, yet a suitable probe pattern is required. As described in a prior study, it was revealed that if numerous LNA insertions are located either at the 3′ or 5′ ends of the probe, the digestion by nucleases is lessened [27]. Likewise, Wahlestedt et al. [38] demonstrated that the arrangement of the LNA monomers along the probe sequence affects the nuclease resistance. Thus, via creating the appropriate probe design we were able to develop a highly sensitive biosensor for simultaneous miRNA detection.

3.3. The cross-hybridization, selectivity, and reproducibility of the proposed assay

For multiplexed assays, the presence of numerous targets in the same sample may cause a cross-reactivity, which may diminish the sensitivity of the method via increasing the false signals. Thus, we checked the cross-reactivity between the probes and the different miRNA biomarkers (miR-21, miR-210, and miR-486-5p) in the presence of 0.5 U DSN enzyme. Fig. 3A indicates the fluorescence intensity (ΔF%) collected from the various singleplex mixtures; mixture 1 (LNA probe-21, 1 nM miR-21, 1 nM miR-210 and 1 nM miR-486-5p), mixture 2 (LNA probe-210, 1 nM miR-210, 1 nM miR-21 and 1 nM miR-486-5p) and mixture 3 (LNA probe-486-5p, 1 nM miR-21, 1 nM miR-210 and 1 nM miR-21). The obtained results revealed that the probe-21 with two miRNA-210 and microRNA-486-5p produced a normalized signal that exhibited slight variation in ΔF% intensities 20% and 30%, respectively, compared to the target miRNA (590%). Similar results were recorded for mixtures 2 and 3, where the nonspecific miRNAs generated insignificant fluorescence intensities compared with the target microRNAs. Therefore, we demonstrated the absence of self-complementarity and cross-hybridization even at high concentrations by applying this strategy. These findings proved that our proposed detection method has high sensitivity and efficiency, which can be accredited to the DSN enzyme preference in the presence of a fully-matched target compared to an unspecific target.

Among the challenges for microRNAs detection is the capability to discriminate between the closely homologous miRNA sequences. This selectivity test is a crucial step to prove the efficacy of our technique in differentiating between the fully matched target and one or more mismatches along with non-complementary microRNAs (NC-miRNA). The test was carried at 55 °C for 2 h using 0.5 U of DSN enzyme and 1 nM of microRNA. Similar results were obtained for NC-microRNA and the three targets with one-base, double-base, and triple-base mismatches generating very low

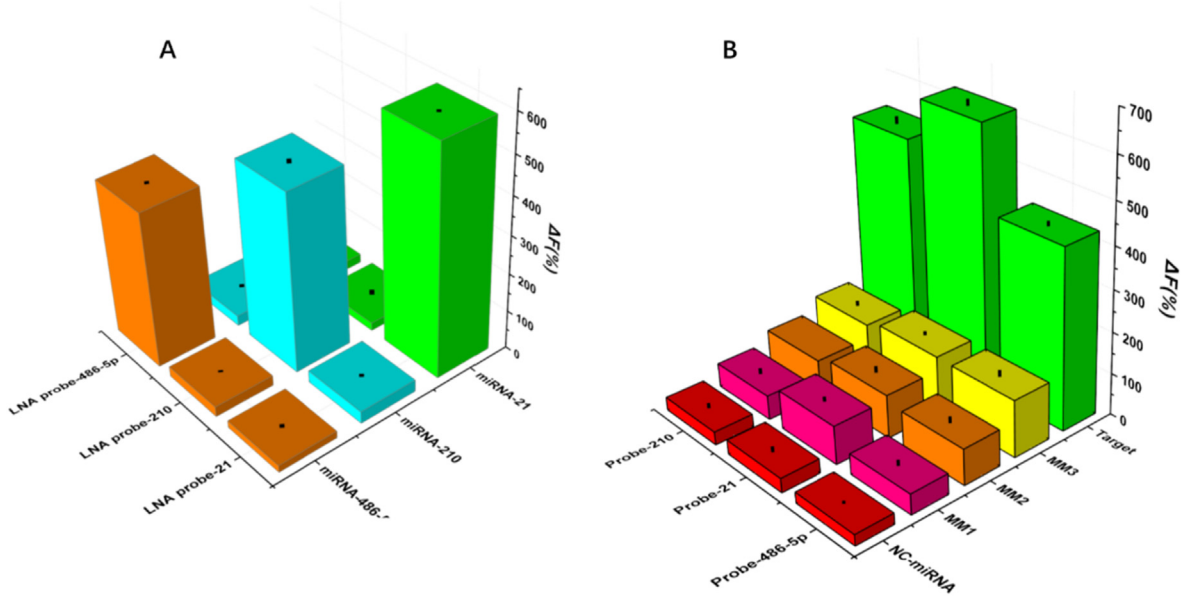


Fig. 3. A) The cross-hybridization response of the different probes toward 1 nM of the target and the irrelevant microRNAs, B) The selectivity test in the presence of 1 nM of one, two, and three bases mismatch and non-complementary miRNA compared to the target miRNA. The tests were carried out for 2 h at 55 °C with 0.5 U DSN enzyme.

signals, which range from 26 to 130% using probe-486-5p, from 35 to 134% using probe-21, and from 34 to 120% using probe-210. Whereas, in the presence of the target microRNAs, the signal increases 3 to 5-times compared to a single base mismatch. These findings endorse the excellent capability of the DSN enzyme to distinguish perfectly matched hetero-hybrids (miRNAs/DNA) from mismatched ones, in addition to the use of the LNA modified probe since it improves base stacking of fully matched base pairs and diminish the stabilization of mismatched base pairs stacking interactions [39]. (Fig. 3B). The reproducibility of our method was also evaluated by exploring the relative standard deviation (RSD) within-batch and between-batch. The intra-assay test (within-batch) was attained by running the reaction in the presence of 1 nM of the different targets and 0.5 U DSN enzyme with three simultaneous tests. The obtained intra-assay RSD value was equal to 3%. Whereas for the inter-assay (between-batch), three experiments were separately arranged under the same conditions. The engendered RSD value was 5%. Thus, these findings evoke great repeatability of our developed miRNA assay.

3.4. Real sample test

To validate the viability of our proposed technique for real-world samples, we performed the simultaneous detection of the three miRNAs mentioned overhead in human serum (bought from Sigma life Sciences, China). The detection was carried in 100-fold dilution of human serum samples spiked with 0.01, 1, and 100 pM of microRNAs and in the presence of 0.5 U DSN enzyme. As exposed in Table 1, the recoveries were measured for the spiked miRNA 21, miRNA-210, and miRNA-486-5p at 0.01, 1 and 100 pM are 101%, 95.4% and 96.4%, 88%, 102% and 101.2%, 92%, 100.5% and 89.9% ($n = 3$),respectively, and the engendered relative standard deviations (RSDs) ranges from 0.5 to 12%. These findings revealed that the performance of the designed fluorescence sensor in 100-fold diluted healthy human serum was comparable to PBS results, indicating that the detection of the microRNAs in real-world samples is also achievable. Consequently, this method may be of great value for multiplex miRNAs assay in clinical analysis.

Our assay's applicability in real clinical samples was checked by monitoring the different miRNAs expression in total RNA samples extracted from the serum of healthy volunteers and lung cancer (LC) patients. The expression profile of the three targets was recorded, and their discriminatory potential was investigated for its ability to differentiate between LC and matched control samples. For instance, as shown in Fig. 4, microRNA-21 was overexpressed in lung cancer patients compared to controls, and it unveiled the highest degree of differentiation with p values < 0.01. These results are in correspondence with the previous reports. For instance, miRNA-21 influences the expression level of tumor suppressor PTEN and induces cell proliferation and migration. Moreover, there is an inverse correlation between the level of miR-21 and the expression of maspin and PDCD4, which provokes the growth and the invasion of tumors by preventing apoptosis [40]. miR-210 behaved similarly as miR-21 with a statistically significant ($p \leq 0.05$) difference. These findings are attributed to the hypoxic condition that is an aspect associated with a solid tumor, which raises the level of microRNA-210 since it is correlated with the hypoxia-inducible factor- HIF-1a and HIF-2a [41]. Another interesting outcome was that microRNA-486-5p, contrary to miR-21 and miR-210, was found to be downregulated between LC patients and healthy controls ($p < 0.05$). Generally, the overexpression of miR-486-5p stimulates apoptosis and hinders cell proliferation and invasion. Therefore, it can be concluded that miR-486-5p may act as a tumor suppressor contributing to the progression of LC [42]. Hence,

Table 1
Determination of the spiked miRNA-21, miRNA-210, and miRNA-486-5p in real sample serum.

Target	Added (pM)	Found (pM)	Recoveries (%)	RSD (%)
MicroRNA-21	1	1.01 ± 0.2	101	1
	10	9.54 ± 1	95.4	4.6
	100	96.4 ± 0.02	96.4	3.6
MicroRNA-210	1	0.88 ± 0.4	88	12
	10	10.2 ± 0.05	102	2
	100	101.2 ± 2.83	101.2	1.2
MicroRNA-486-5p	1	0.92 ± 0.07	92	8
	10	10.05 ± 1.04	100.5	0.5
	100	89.9 ± 3.6	89.9	10.1

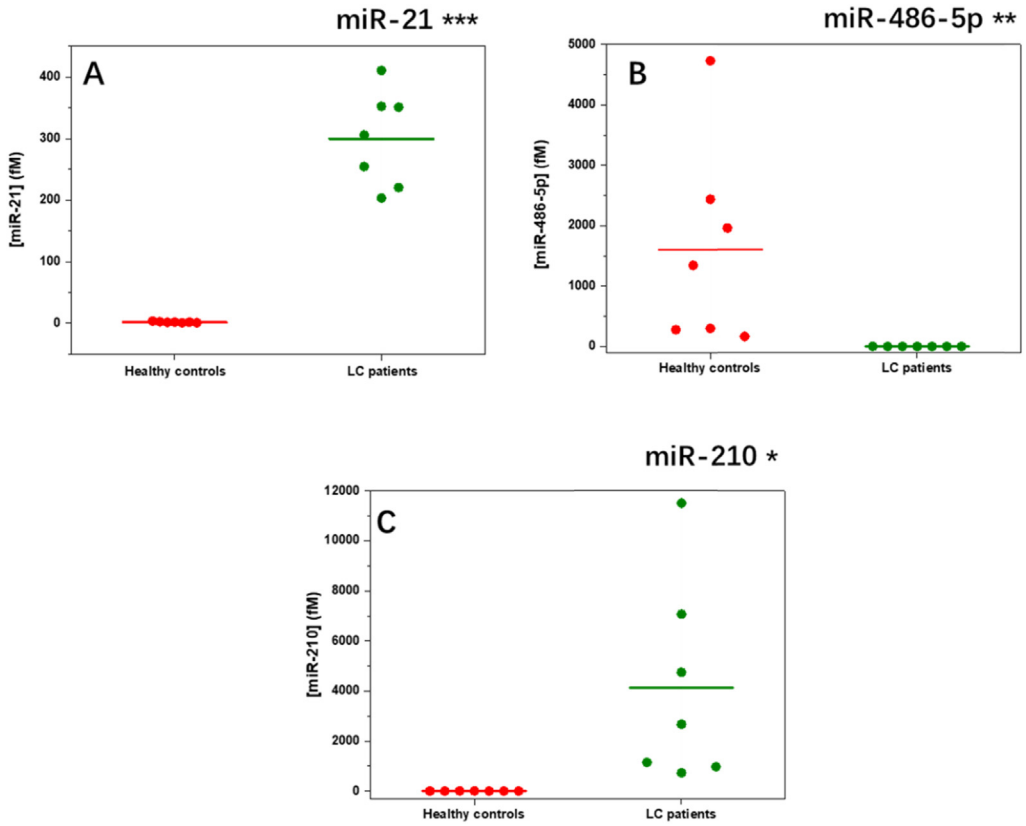


Fig. 4. Quantification of the different microRNAs (miR-21, mi R-210, and miR-486-5p) in the real clinical samples of healthy controls and LC patients using our proposed method.

this assay can be concluded that this method is suitable for miRNA profiling in real samples. These results were confirmed using a computed tomography (CT) technique, which agreed with our results and indicated that an individual carries a lung cancer disease (supporting information). Thus, the serum 3-microRNAs signature used in this work confirms their diagnostic value in distinguishing lung tumors from healthy volunteers with high accuracy.

4. Conclusion

This present work established a novel fluorescence-based strategy for sensitive, selective, and multiplexed LC miRNAs detection. The designed biosensor is based on the engagement of DSN enzyme-induced reuse of targets in cooperation with magnetic beads as a probe carrier. Their combination with the LNA–modified DNA probes boosted the probe/target duplex stability and improved the fluorescence response. Furthermore, the developed biosensor exhibited a high selectivity even in the presence of a single base mismatch with a slight false positive signal issued from a cross-hybridization event. Predominantly, our proposed method displayed a high efficiency for the multiplexed detection of various miRNAs extracted from human serum samples. The panel of three miRNAs confirmed their diagnostic value in distinguishing between healthy controls and LC patients. Our results illustrated that this approach proffers an alternative amplification strategy with a single catalytic reaction, which could open new paths for concurrent miRNA detection and clinical diagnosis.

CRediT authorship contribution statement

Khouloud Djebbi: Conceptualization, Investigation, Methodology, Writing – original draft. **Juanxia Xing:** Resources, Methodology. **Ting Weng:** Resources, Writing – review & editing. **Mohamed Bahri:** Software. **Mohamed Amin Elaguech:** Software. **Chao Du:** Investigation. **Biao Shi:** Resources. **Li Hu:** Validation. **Shixuan He:** Formal analysis. **Pu Liao:** Supervision, Project administration. **Chaker Tlili:** Supervision, ensuring that the descriptions are accurate and agreed by all authors, Writing – review & editing. **Deqiang Wang:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wang Deqiang has patent #202111442305.4 pending to China National Intellectural Property Administration; CNIPA.

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Appendix A. Supplementary data

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