Journal of Fluorescence (2022) 32:2297–2307
https://doi.org/10.1007/s10895-022-03023-z

RESEARCH

Design and Fabrication of a DNA-copper Nanocluster-based Biosensor for Multiple Detections of Circulating miRNAs in Early Screening of Breast Cancer

Samira Sadeghi[1] · **Mahdi Rahaie**[1]

Received: 28 March 2022 / Accepted: 25 August 2022 / Published online: 16 September 2022

Abstract

Breast cancer is one of the most prevalent cancers and a significant cause of fatalities in women. Diagnosis of breast cancer faces substantial challenges, owing to the multi-factored nature of the illness. Thus, the necessity for an inexpensive, rapid, and non-invasive diagnostic method that enables early detection with high sensitivity and specificity is of primary significance. In this study, a biosensor based on the fluorescence emission of DNA-templated Cu nanoclusters was designed to simultaneously detect and quantify three significant biomarkers of breast cancer (circulating microRNAs, miR-21, miR-195, and miR-155). Fluorescence spectroscopy, FESEM and TEM microscopy, and DLS confirmed the validation of the biosensor. A detection limit of 1.7 pM with a linearity range of 500 nM to 3 µM was obtained. In conclusion, the innovative selection of three biomarkers, the utilization of the HCR process, and an the elaborated design of probes are considered to be among the most important advantages of this biosensor, enabling it a simultaneous triple diagnosis of the blood specific circulating microRNAs without a need for any enzymes, thermo-cycles, expensive or complex facilities, linkers, fluorescent tags, and time-consuming methods.

Keywords Fluorescent biosensor · Copper nanocluster · Multiple biomarkers · miRNAs · Breast cancer

Abbreviations

miRNA	Micro RNA
Cu Nanocluster	Copper Nanocluster
FE-SEM	Field Emission Scanning Electron Microscope
TEM	Transmission Electron Microscope
DLS	Dynamic Light Scattering
qRT-PCR	Quantitative Real-Time PCR
HCR	Hybridization Chain Reaction
NGS	Next-generation sequencing
RT	Room Temperature
LOD	Limit of Detection
NCs	Nanoclusters
CuNCs	Cu Nanoclusters
SPR	Surface plasmon resonance

✉ Mahdi Rahaie
mrahaie@ut.ac.ir

[1] Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran 14399-57131, Iran

Introduction

Breast cancer, as the most frequent malignancy in women, is the most common cancer with an ~ 2.3 million new cases (equal to 11.7%) and a high cancer mortality rate (6.9%) in 2020 [1], which is due to tumor metastasis as the main reason [2].

Early detection is one of the crucial steps in breast cancer survival, as more than 90% of women diagnosed with the first stage of the disease survive at least five years, compared with about 15% for women with the advanced stages of diagnosis [3]. Currently, mammography is considered to be the most credible screening technique for the diagnosis of breast cancer. Although, its shortcomings include false-positive results, cancer misdiagnosis for other non-cancer diseases, and low sensitivity for tumor diagnosis in breast tissues with high density [4]. Therefore, there is a significant need for the factors that could replace biomarkers in an early and highly sensitive breast cancer screening.

MicroRNAs, especially those detectable in serum or blood (circulating microRNAs), are the primary biomarkers in cancer detection. These small RNAs with a length of 18–25 nucleotides play a vital role in different biological

processes in the human body, such as gene regulation [5–7]. Most of the miRNAs associated with breast cancer have been identified and play one of the oncogenic or tumor-suppressive roles in breast cancer [8]. Specifically, miR-21, exhibiting a significant overexpression associated with many types of cancers, is considered a critical element based on numerous experimental studies [9]. Because of its high sensitivity and specificity at early stages, miR-21 has attracted the most attention among miRNAs in the early detection of breast cancer [3]. Another miRNA related to breast cancer is miR-155. Both miR-21 and miR-155 have mostly been targeted in detecting breast cancer due to their presence in serum makes screening less invasive [10]. Studies show that the serum levels of circulating miR-21 and miR-155 increase in blood samples of patients with breast cancer [11]. Studies report a sensitivity of 73% with a specificity of 81% for miR-21 and a sensitivity of 78% with a specificity of 75% for miR-155 [12]. Because of the general dysregulation of miR-21 and miR-155 in several malignancies, there is a requirement to apply another miRNA as a more specific biomarker for breast cancer. High levels of circulating miR-195 have been observed in blood samples of only breast cancer patients [13]. It has been shown that miR-195 individuated blood samples of breast cancer patients from other cancers and normal controls with a sensitivity and specificity of 88% and 91%, respectively [14]. The most common conventional methods for circulating miRNAs detection include Northern blot, qRT-PCR as the golden standard, microarray, next-generation sequencing, and single-molecular sequencing. The classic method, Northern blot, is a particular and moderately sensitive approach with some disadvantages, such as the requirement for large sample amounts, long execution time, and complicated equipment [15]. Another method with moderate specificity and high sensitivity is qRT-PCR. This method, widely considered the golden standard, has some disadvantages such as the difficulty of primer designing for small RNAs, a short length of primers that decreases the efficiency of PCR due to low melting temperature, being time-consuming and medium-throughput, and the lack of new miRNAs detection ability [15, 16]. The low specificity and sensitivity microarray is another semi-quantitative method [17]. Next-generation sequencing (NGS), with high specificity and moderate sensitivity, is expensive and time-consuming [15, 18]. Therefore, there is a need for a low-cost, simple, rapid method with high sensitivity and specificity, capable of quantitatively and simultaneously detecting multiple miRNAs. Nanotechnology-based approaches seem to be a better alternative to address the conventional methods' limitations, as they tend to be more sensitive and specific in miRNAs detection [19]. Several DNA-based nanostructures have been identified and applied to improve and develop the efficiency of miRNAs detection methods [20]. In this approach, miRNAs analysis relies mainly upon the hybridization of the miRNAs with a probe which is its complementary nucleic acid, followed by transduction reactions to provide quantifiable signals. The special physiochemical features of nanostructures lead to signal production and enhancement. This tends to be the major advantage of nanotechnology over conventional methods in miRNAs' detection [21]. Nanoclusters are a novel class of nanomaterials that fill the gap between metallic organic structures and crystalline metal nanoparticles. They are noticed due to their innovative properties such as sub-nanometer size, biocompatibility, optical properties, photo-stability, and ease of synthesis [22]. Because of the metal nanoclusters' unique specifications, such as optical and plasmonic features and molecule-like properties, they are an appreciable option in biosensing applications [23]. Fluorescence-based sensors have attracted the most attention due to their high sensitivity and fast response [24]. Currently, the use of Cu nanoclusters has accelerated due to their excellent properties. These properties include good optical and chemical stability, being nontoxic and economical, high luminous efficiency, long fluorescence lifetime, large stokes displacement, and the ability to self-assembling on double-stranded DNA. Also, Copper nanoclusters show no desire to form single-stranded DNA, which provides a worthwhile opportunity to offer label-free fluorescent probes for molecular sensing programs. DNA-templated copper nanoclusters indicate emission near 600 nm (590 nm) wavelength by exciting with 340 nm UV wavelength. This property leads to the ability to detect nucleic acids such as miRNAs by designing a complementary probe [25, 26].

Dirks and Pierce introduced an efficient, low-cost DNA amplification technique known as hybridization chain reaction (HCR), prevalent in nucleic acid biosensing applications [27]. There is no need for primer designing, enzymes, thermocyclers, and complicated equipment in HCR. Being enzyme-free, isothermal, highly efficient, and straightforward make HCR an appropriate tool in multiplexed miRNAs detection approaches [28, 29].

In this study, a low-cost, label-free, and enzyme-free optical Copper nanocluster-DNA-based biosensor was designed and introduced as a novel technique for multiple detections of circulating microRNAs in the prognosis of breast cancer. The combination of circulating miR-21, miR-155, and miR-195 was selected as multiple biomarkers for early and specific detection of breast cancer (significantly up-regulated microRNAs). HCR was applied to enhance the signal. Also, DNA-templated Cu nanoclusters were used as fluorescent signal producers.

Materials and Methods

Materials

The oligonucleotides (Table1) used in the experiment were purchased from Macrogen (South Korea). Chemical materials including $CuSO_4.5H_2O$, KCl, Na_2HPO_4, KH_2PO_4, and NaCl were purchased from Merck (Germany), and Sodium-L-Ascorbate was purchased from Sigma-Aldrich chemical co (USA). To prepare buffers and solutions, it was used ddH_2O.

Apparatus

Fluorescence excitation and emission spectra were read on Cary Eclipse from Agilent Technologies (USA). Transmission electron microscopy (TEM) was carried out on CM200 from Philips (the Netherlands), and field emission scanning electron microscopy (FESEM) was carried out on an SEM microscope (S4160) from Hitachi (Japan). The size-frequency of CuNCs was analyzed by a Nano sizer DLS from Malvern (Japan).

Hybridization Chain Reaction (HCR) and Amplification

At first, different concentrations (0, 0.5, 0.7, 1, 1.5, and 3 µM) of target miRNAs (miR-21, miR-155, and miR-195) and 3 µM hairpin probes (P1 and P2), which were specially designed, were mixed in 20 mM phosphate buffer saline (PBS) at pH 7.5. The solution was incubated at 95 ℃ for 15 min and then at room temperature (RT) for about 2 h to promote HCR. Agarose gel electrophoresis was used to confirm the HCR process and investigate its product.

DNA-templated CuNCs Formation and Fluorescence Assessment

After two hours, $CuSO_4$ (Cu/DNA 200:1), and Ascorbate (Asc/Cu 20:1) were added to the HCR solution and gently shacked in the dark. Different parameters including kind of buffer, pH, and the effect of excitation wavelength on Cu nanocluster emission were evaluated. Eventually, to confirm the synthesis of self-assembly CuNCs on dsDNA (HCR product), the reaction solution was analyzed through UV visible and fluorescence spectroscopy, TEM, FESEM, and DLS analysis.

The Analytical Parameters of Biosensor

The sensitivity and selectivity of the biosensor were determined by using different concentrations of target microRNAs and non-target sequences, respectively. Upon the data, linearity response range and LOD were determined.

Results and Discussion

Target Selection and Probe Designing

Among circulating miRNAs used as biomarkers, miR-21, miR-155, and miR-195 were chosen as multiple targets in this study for early and specific detection of breast cancer for the first time. They were selected due to their high specificity and sensitivity. Table 2 shows some references and documents as reasons for selecting microRNA biomarkers. They have indicated a specificity of 81% and sensitivity of 73% for miR-21, specificity of 75% and sensitivity of 78% for miR-155, specificity of 91%, and sensitivity of 88% for miR-195 in the detection of breast cancer [12, 14]. Combining these three miRNAs as multiple biomarkers makes the detection process more specific, sensitive, and validated for clinicians.

To improve the limit of detection of the biosensor, the synthetic complementary deoxyribonucleic acid probes named P1 and P2, which have hairpin structures were specially designed. By hybridizing with targets, they initiate the HCR. As shown in Table 1, the same color on targets and probes are complementary sequences. The secondary structures of used sequences in this study are shown in Fig. 1. As shown in this figure, all sequences have hairpin second structures which contain a single-stranded DNA loop and a middle double-stranded part which helps to prevent

Table 1 The sequences of oligonucleotides were used in this study

Oligonucleotides	Sequences 5'→ 3'
miR-21	CAACACCAGTCGATGGGCTGT
miR-155	TTAATGCTAATCGTGATAGGGGTT
miR-195	TAGCAGCACAGAAATATTGGC
P_1	AACCGGTTGTGGTCAGCTACCCGACAAATT
P_2	ACGATTAGCACTATCCCCAAATCGTCGTGTCTTTAT

The same color highlights on targets and probes are complementary sequences

Table 2 miRNAs used in this study and their role as breast cancer biomarkers

miRNA	Specificity (%)	Sensitivity (%)	Ref. no.
miR-21	High (81)	Middle (73)	[11]
miR-155	Middle (75)	High (78)	[11]
miR-195	Extremely high (91)	High (88)	[13]
Combination of miR-21, miR-155, and miR-195	Extremely high	Extremely high	-

non-target hybridizations and non-target bindings before the HCR process and also one or two single-stranded tails.

HCR Process and the Biosensor Structure

The schematic of the biosensor is shown in Fig. 2. In this figure, P1, which is complementary to the last part of miR-195, the whole sequence of miR-21, and the primary part of miR-155, starts the HCR process by hybridization to miR-21 and changes into a linear form from the hairpin structure. After that, miR-155 is hybridized to its complementary sequence, which is partially on P1 and partially on P2. P2, which is complementary to the last part of the miR-155 sequence and the primary part of miR-195, is changed to linear form by hybridization to its targets. Sticky ends lead the process to continue.

This nicked double-stranded DNA (HCR product) acts as a template for copper nanoclusters (CuNCs) construction and then fluorescent assessment in the biosensing strategy. Since Cu nanoclusters are synthesized in a relatively cheap and practical manner compared with Au or Ag NCs or other fluorescent nanoparticles and they have a size < 2 nm which shows unique physicochemical property (between nanoparticles and atomic/molecular structures) [30], it was used CuNCs as a stable fluorescence reporter in this work.

For qualitative analysis of HCR, gel electrophoresis was conducted. The results are shown in Fig. 3. Samples including a concentration of 1 μM of probes and targets were electrophoresis. Since the length of single probes and targets are < 50 bp (Table 1) and regards the position of HCR-resulted amplicons (second, third, and fourth band from left to right) on the gel in ~ 200 bp, it was confirmed that the HCR process had been done, successfully.

Cu Nanocluster Formation and Characterization

To observe the synthesized copper nanoclusters, an HCR sample with a concentration of 2 μM of the target sequences was prepared for scanning electron microscopy. In the images (Fig. 4) obtained from field emission scanning electron microscopy (FE-SEM), clusters of these particles were visible, which corresponded to the images presented in previous work [31]. The regularity and similarity in shapes of the particles under the microscope indicate the integrity of the formation of Cu nanoclusters. The larger-sized particles are predicted to be due to the stacking of copper nanoclusters resulting from the high density of double-stranded DNA molecules carrying the Cu nanoclusters on their backbones. As an explanation, it should be said that because the gel electrophoresis confirmed the formation of double-stranded DNA and also the fluorescence spectroscopy showed the CuNCs peak (at 590 nm) which proved that CuNCs are formed (which can be formed only on double-stranded DNA backbones and not on single-stranded DNA) the larger sized particles could be as a result of stacking of CuNCs on the double-stranded DNA backbones. In addition, due to the HCR process, the double-stranded DNA should be extended and have an increase in length which is confirmed by gel electrophoresis.

Synthesized copper nanoclusters with the HCR sample containing a concentration of 2 μM of the target sequences were observed by TEM.

The results are indicated in Fig. 5. The synthesized copper nanoclusters were visible in the form of single, scattered, and numerous particles with a size of less than 10 nm and about 2-5 nm in the image, which corresponded with previous reports [32, 33]. The stacking of Cu nanoclusters is responsible for observing the larger particles that the current TEM could not distinguish. As seen in Fig. 5, nanoclusters tend to be accumulated, staying together and forming a more oversized shape which may be seen as a single particle. However, by focusing on particles, their distinct peripheries would be clear.

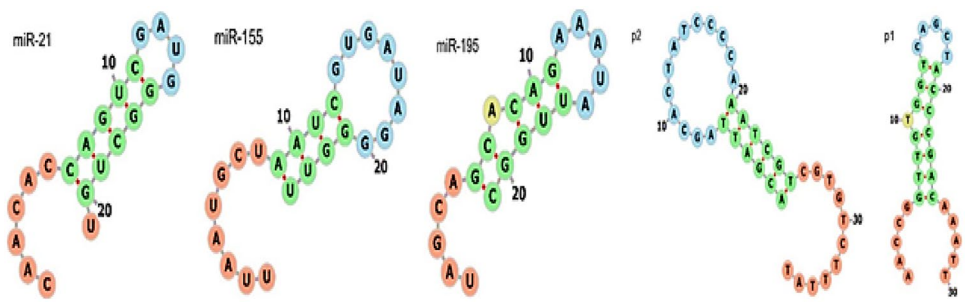


Fig. 1 The second molecular structure of used sequences (Table 1) in this study

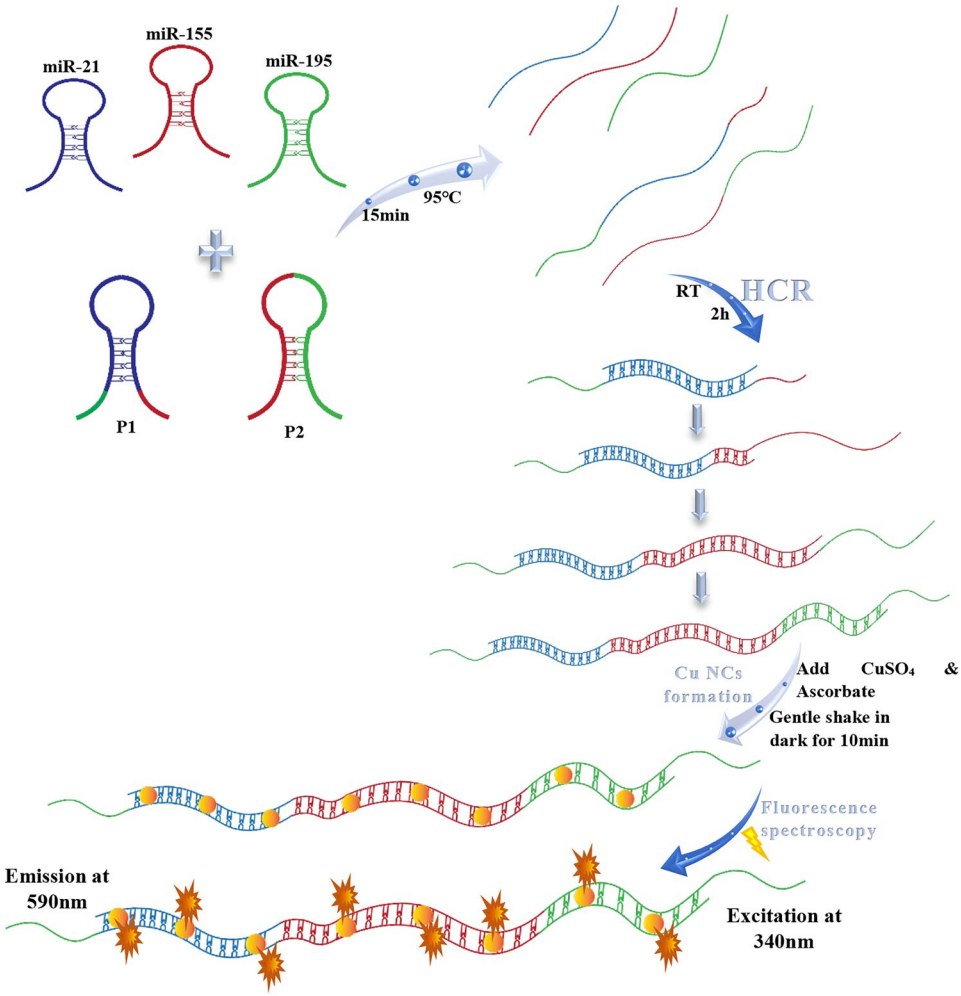


Fig. 2 A schematic of the present biosensor

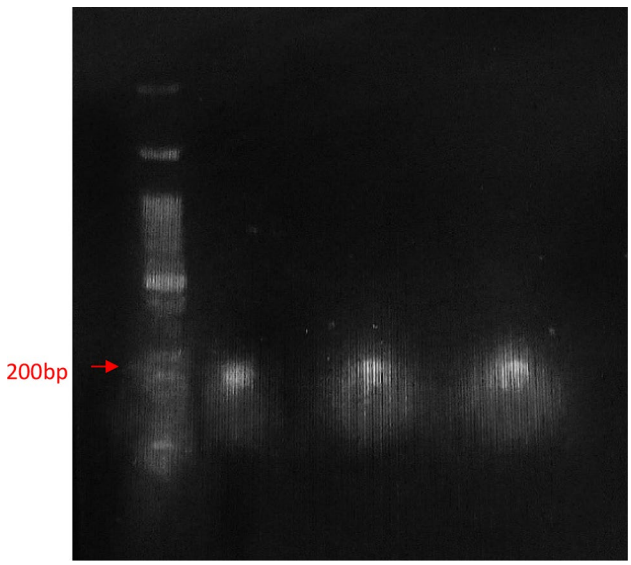


Fig. 3 Gel electrophoresis for analysis and confirmation of HCR products. The first line (left) is a 1 kb DNA size marker, and the others are HCR Products

To investigate the particle size with dynamic light scattering (DLS), an HCR sample with a concentration of 0.2 µM of the target sequences was prepared and then analyzed with DLS analysis. The size of copper nanoclusters synthesized in this study was about 2 nm on average (Fig. 6). The observed peak size of ~ 2 nm precisely corresponded to DLS reports for copper nanoclusters in previous studies [34, 35]. The result indicates that the Cu nanoclusters were formed in an integrated size in which all the particles are between 1 to 10 nm in length, and most of them were about 2 nm.

Optimization of Biosensor

Biosensing Reaction Solution and pH

To select an appropriate buffer for the biosensing reaction, three buffers, including PBS, MOPS, and potassium phosphate (KP), were analyzed to select an appropriate buffer for the biosensing reaction. As reported, Cu nanoclusters have an emission wavelength of about 600 nm (590 nm).

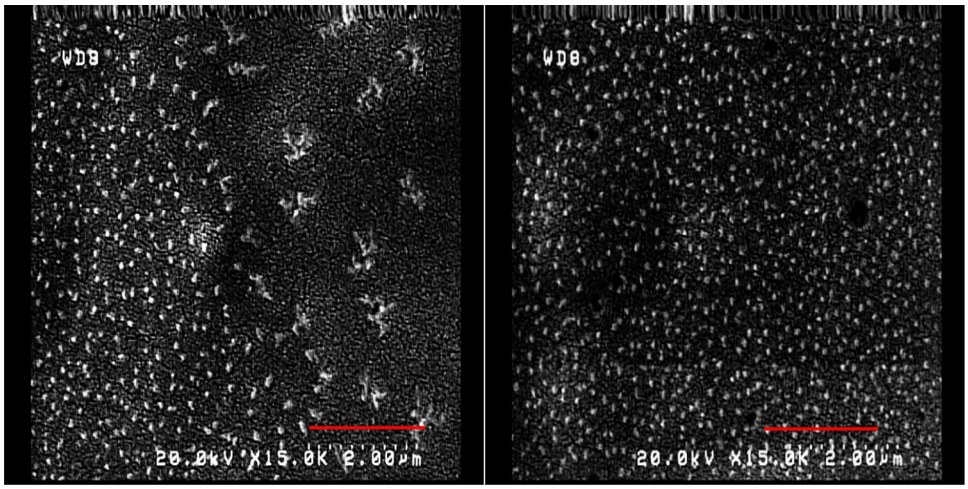


Fig. 4 The FE-SEM images of synthesized Cu NCs

This wavelength is associated with the wavelength of orange color in the visible light spectrum [36]; samples with these three different buffers were analyzed under UV light for color change. Only the samples containing PBS buffer indicated the strong orange color under UV light. The results are shown in Fig. 7.

In this figure, Samples 1–4 are negative controls with deionized water, PBS, MOPS, and KP buffers without Cu NCs, respectively. Others show the different concentrations of target sequences (miRNAs) with probes (HCR products) and Cu NCs in PBS buffer (samples 5 to 10), MOPS buffer (samples 11 to 16), and potassium phosphate (KP) buffer (samples 17 to 22) under UV light. According to the results, PBS buffer was selected for all analyzes.

For qualitative optimization of pH and to select the efficient pH for Cu NCs formation, five different pHs (6, 7, 7.5, 8, and 9) were selected and analyzed into PBS buffer. The sample with a pH of 7.5 indicated the sharpest orange color, samples with a pH of 7 and 8 showed medium orange, and the colors of samples with a pH of 6 and 9 did not change under UV light. It seems that the hybridization of single-stranded DNAs in the HCR process occurred only in the samples with a pH range between 7 to 8, as was indicated in previous studies [37], and the most efficient pH for CU nanoclusters formation is a pH of 7.5. Also, the impact of the presence of Mg^{2+} ion was analyzed. Due to the sharpest orange color of the sample without Mg^{2+} ions, it was concluded that the absence of Mg^{2+} ion is more appropriate for Cu NCs formation. It is assumed that the presence of Mg^{2+} ions distracts the reduction reaction of Cu^{2+} ions by Ascorbate and consequently disrupts the Cu nanoclusters formation. Also, it seems the Mg^{2+} ion in the buffer can induce a lifetime-varied emission state of Cu NCs as seen in Ag and Au nanoclusters, respectively in the works of Ma et al. and Basu et al. Bivalent Mg^{2+} have a nonspecific interaction with the DNA backbone, and due to its small size (compared to other used divalent cations), it can contribute to the ionic linkage. However,

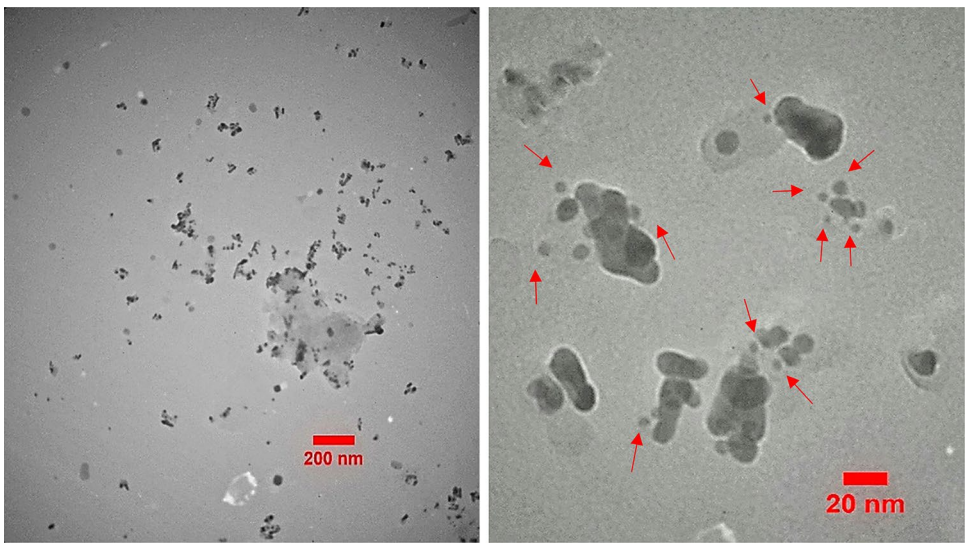


Fig. 5 The TEM images of used Cu NCs

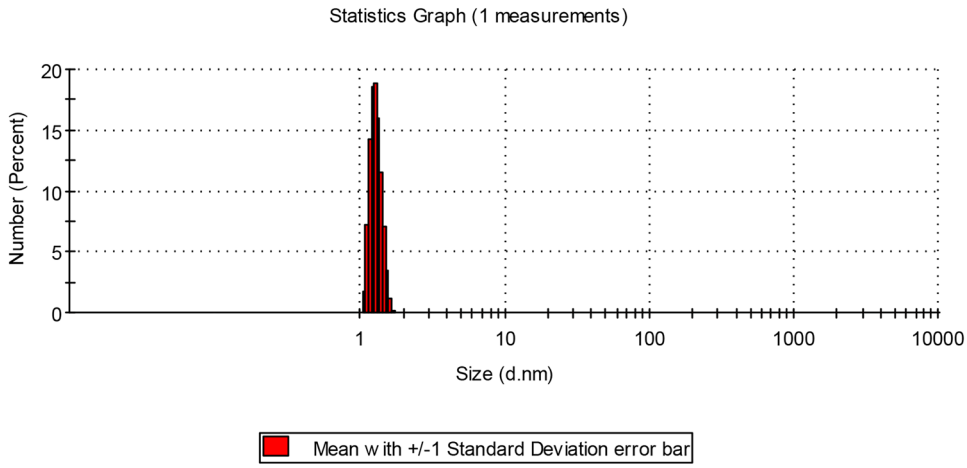


Fig. 6 DLS analysis for Cu NCs

the modulated emission still is associated with the DNA sequence and length-dependent emitting manner [38, 39]. The results are indicated in Fig. 8.

Excitation and Emission Wavelengths

To obtain an optimized excitation wavelength to excite Cu NCs, four wavelengths (300, 320, 340, and 400 nm) were selected, and the emission of the sample with 1 μM of target sequences and probes (HCR) with Cu NCs were analyzed. The significant emission in 590 nm was obtained by excitation wavelength of 340 nm. The results are shown in Fig. 9.

It has been found that different parameters, including the size of the nanocluster, charge, ionic strength and pH of the buffer, surface ligands, the temperature of the reaction solution, and molecular structure of them, affect the fluorescence of Cu nanoclusters [26].

Biosensor Analytical Parameters

The sensitivity of the biosensor was analyzed by fluorescence spectroscopy. Samples (HCR products) with different concentrations (0, 0.5, 0.7, 1, 1.5, and 3 μM) of target sequences (miR-21, miR-155, and miR-195) and probes

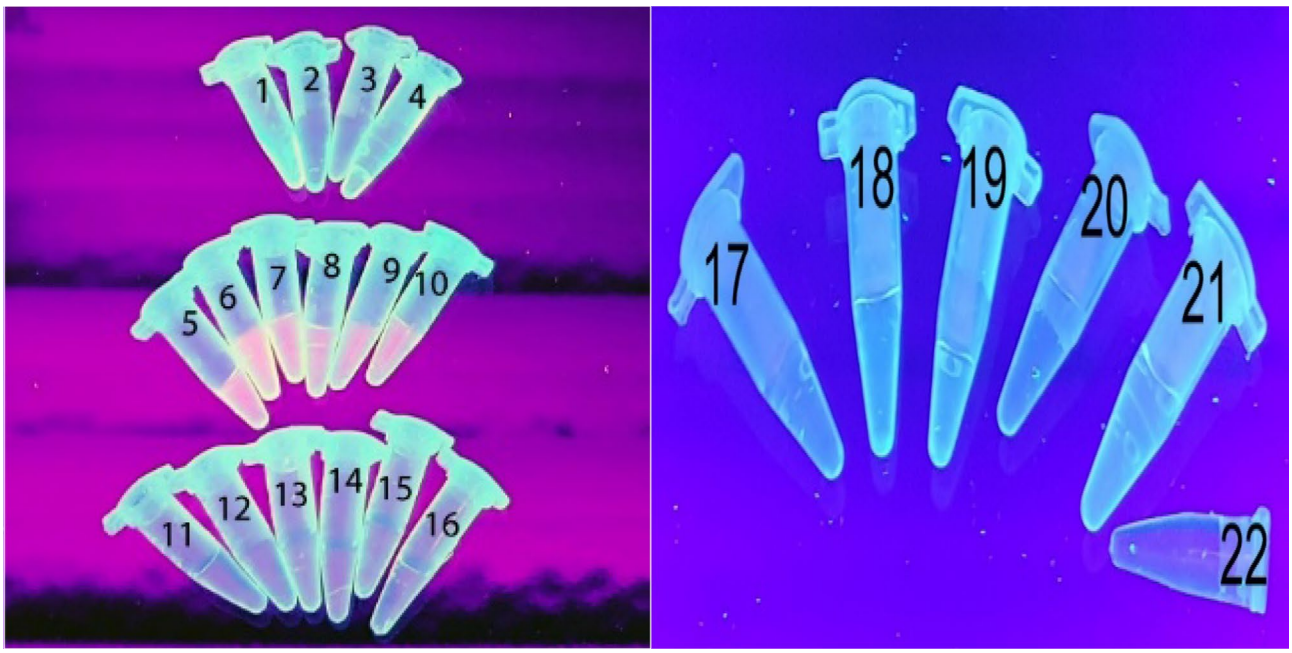


Fig. 7 Buffer selection and qualitative optimization of the biosensor. Left: 1–4 are negative controls with deionized water, PBS, MOPS, and KP buffers without Cu NCs, and 5–10 are different concentrations of target sequences with probes (HCR products) and Cu NCs in PBS buffer, respectively. 11–16 show the samples with MOPS buffer, and right: the 17–22 samples in KP buffer under UV light

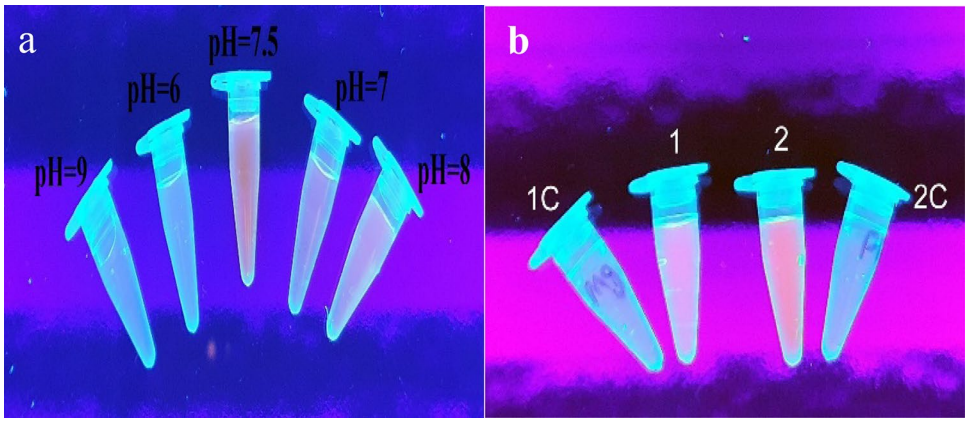


Fig. 8 A: pH optimization with different pHs of PBS buffer. B: the presence (1) and absence (2) of Mg^{2+} ion in the buffer. Sample 1C is target-free control with Mg^{2+} ion, and 2C is target-free control without Mg^{2+} ion

containing Cu NCs were excited in 340 nm wavelength (in three replications). The results are indicated in Fig. 10. The linear range and R^2 were calculated, and the detection limit of the biosensor was obtained based on the below formula.

$$DL = \frac{F \times SD}{b} = 0.0017nM$$

Which SD is the blank (0 nM) standard deviation, b is the slope of the line, and F is equal to 3.3.

This LOD (1.7 pM) was significant due to the unique and novel design of the probes and the absence of any linker or enhancer sequences in the present work. This is the first report which proves the use of a fluorescent and label-free biosensor for triple detection of miRNAs. In work done by Rahaie and Khayat Noroozi, a LOD of 82 pM was obtained for dual detection of microRNAs involved in Alzheimer's disease [40]. Vaisocherová et al. fabricated a low-fouling SPR-based biosensor that detected multiple microRNAs in a parallel format (not together) with a detection limit of 0.5 pM [41]. Regarding low concentrations of the biomarkers in the blood (from nM to fM) [29], it seems the present work provides a new optical and accessible approach for clinicians to do multi-detections of breast cancer biomarkers with a significant LOD.

The specificity of the biosensor was analyzed by fluorescence spectroscopy (Fig. 11). To investigate the specificity of the biosensor, probe sequences were prepared in the absence of target sequences and the presence of three other non-target sequences at concentrations of 2 and 3 µM (with three replications). A fluorescence spectrophotometer checked emission. No significant emission peaks were observed at ~ 600 nm (590 nm) with non-target sequences. Since there are different circulating miRNAs in patients' blood samples, the result is essential for a clinician to discriminate cancer biomarkers from other miRNA molecules.

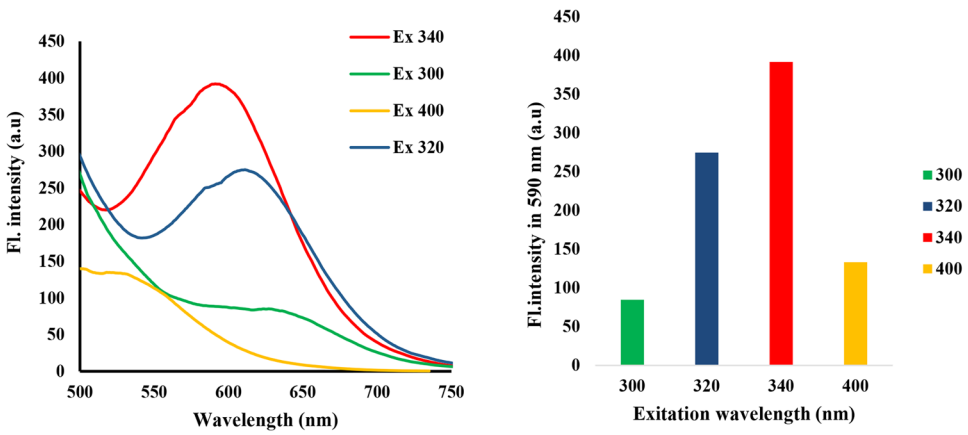


Fig. 9 The optimization of excitation wavelength to excite copper nanoclusters with different excitation wavelengths from 320 to 400 nm

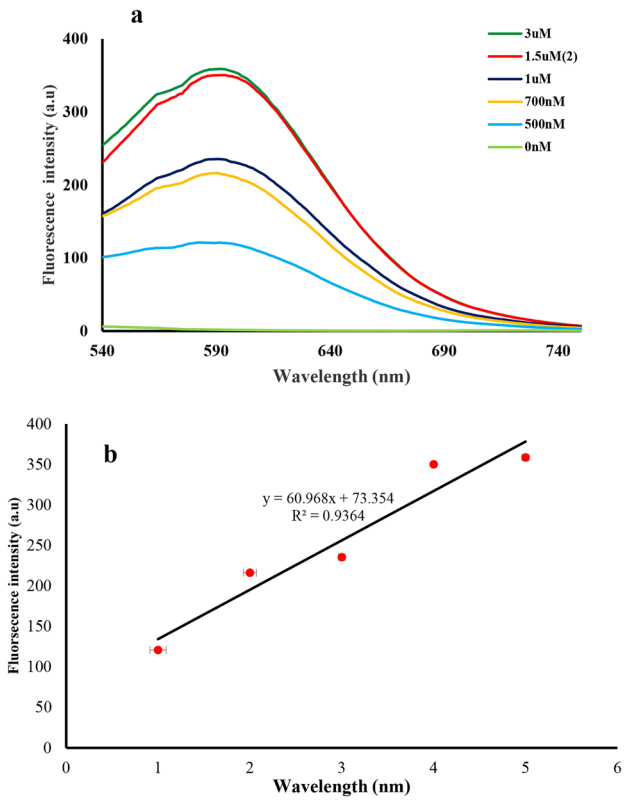


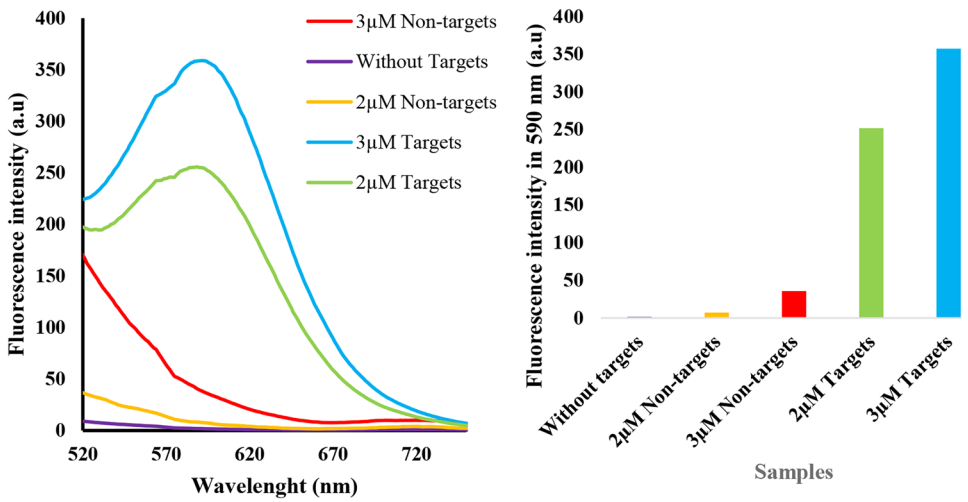
Fig. 10 A: fluorescence spectroscopy of different concentrations of target sequences (with probes and Cu NCs). B: linear response range of the biosensor. Numbers 1 to 5 on X-axis are associated with target sequences concentrations of 500 nm, 700 nm, 1 µM, 1.5 µM, and 3 µM, respectively. Error bars show three replications

Conclusions

In summary, in the absence of integrative approaches for diagnosing breast cancer biomarkers in an early stage and precise manner, this study introduces a novel biosensor for this purpose. It was designed and fabricated based on DNA-templated copper nanoclusters as fluorescence reporters. It enables triple biomarkers detections and analysis of three of the most significant circulating miRNAs (oncomirs), simultaneously for the prognosis of breast cancer. The multiple miRNAs screening enables early detection of breast cancer with high accuracy and specificity, without enforcing any limitations or side effects. The optical biosensor allows a non-invasive diagnosis approach to be possible. Also, the implementation of a label-free, enzyme-free method, only with a specific probe design together with implementing HCR, enables a potentially highly accurate result for clinical applications with a significantly low LOD (1.7 pM) within the target molecules (microRNAs) concentrations range of 500 nM to 3 µM, and minimum signal disruptions.

The positive implications of this novel approach are not limited to the technical side. Economically, by eliminating the necessity for sophisticated molecular structures, PCR, and fluorescent labels, alongside reducing the use of complicated experimental equipment for the test procedure, the costs of undertaking the proposed biosensor seem to be significantly lower than the conventional methods. The reduction of costs, in this case, by no means generates lower effectiveness or efficiency. On the contrary, the efficiency

Fig. 11 The specificity assay of the biosensor with/without target and non-target sequences



of the process is maximized due to PCR test elimination, and its efficiency for application in clinical labs is ensured by undertaking an integrative, multiple screening approach (multi-targets detection in one test). The present work has the practical potential to detect multiple nucleic acids in clinical samples (blood), especially miRNAs, due to the lack of need for extraction, purification, labeling, and enzymes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10895-022-03023-z.

Acknowledgements The authors acknowledge the instrumental and financial support of the University of Tehran and the Iran National Science Foundation (INSF) in this work.

Authors' Contributions Both authors contributed to the study's conception and design. Material preparation, data collection, experimental works, data analysis, and writing were performed by S. Sadeghi and M. Rahaie. M. Rahaie edited the initial and mature draft of the manuscript.

Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflicts of Interest/Competing Interests The authors declare that there are no conflicts of interest.

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