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Cost-effective monitoring of microRNA-205 applied as a biomarker using G-quadruplex DNAzyme and 1,1'-oxalyldiimidazole chemiluminescence

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ABSTRACT

Trace levels of microRNA-205, known as a biomarker of lung cancer, in human serum was quantified for the first-time using G-quadruplex DNAzyme linked to detection complementary probe and 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL). First, capture complementary probes immobilized on the surface of paramagnetic bead selectively bound with microRNA-205 existing in human serum. Then, with the addition of detection complementary probe linked to hemin aptamer, a complex linked to hemin aptamer was formed with the completion of hybridization between microRNA-205 and two complementary probes. With the addition of hemin in the solution, finally, a complex linked to G-quadruplex DNAzyme was formed from the interaction of hemin aptamer and hemin. Resorufin, luminescent dye, was formed from the reaction of Amplex Red and H_2O_2 in the presence of the complex linked to DNAzyme acting as a horseradish peroxidase (HRP)-mimicking enzyme. The concentration of resorufin formed from the reaction was dependent on the concentration of microRNA-205 in human serum. Thus, the brightness of resorufin emitted in ODI-CL reaction was enhanced with the increase of microRNA-205. The limit of detection (LOD) of the biosensor with ODI-CL detection, capable of sensing microRNA-205 (dynamic range: 0.4–62.5 nM), was as low as 0.13 nM. It was confirmed that the biosensor can quantify trace levels of microRNA-205 with statistically acceptable accuracy, precision, and recovery.

1. Introduction

Since the discovery of microRNAs existing in human serum, they have been widely applied as new biomarker for the early diagnosis and accurate prognosis of human cancers [1–3]. This is because the identity of microRNAs is related to neoplastic transformation [4], tumor progression [5], and tumor-initiating cells [6]. Thus, recently, various types of biosensors, capable of accurately sensing specific microRNAs in a sample, have been reported [7,8].

In order to precisely and reliably analyze microRNAs, which are small endogenous noncoding RNAs (~22 nt) [9], analytical methods, designed based on the hybridization between a target microRNA and a complementary probe, a single-stranded DNA (ssDNA), have been developed [10,11]. In order to quantify trace levels of microRNAs exiting in human fluidic sample, chemiluminescence [12], colorimetric [13], electrochemical [10], and fluorescence [11] were applied as detection methods. Additionally, in order to enhance the sensitivity of biosensor, advanced enzyme assays for quantifying microRNAs using horseradish peroxidase (HRP) [14], alkaline phosphatase (AP) were reported [15].

Recently, various biosensors with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) have been developed for the early diagnoses of several human diseases [16–19] and rapid monitoring toxic materials for food and environmental safety [20–22]. The reports noted that the sensitivity of ODI-CL detection is better than those of other optical sensors such as conventional chemiluminescence using 1,2-dioxetane or luminol derivatives, colorimetric, and fluorescence [16,17]. ODI-CL was used as an advanced detection method for enzyme immunoassays operated with AP [17,23], HRP [17,23–25], or Neuraminidase (NA) [26]. Additionally, the enzyme assay with ODI-CL detection and two enzymes (e.g., AP, HRP) was able to simultaneously quantify two cancer biomarkers in a sample [17,23]. Also, ODI-CL was used as a detector of biosensor operated with HRP-mimicking DNAzyme, a G-quadruplex formed from the binding reaction of hemin aptamer and hemin [27]. ODI-CL detection has also been applied to quantify mutated DNA genes using a hybridization method for the diagnosis of mad cow disease [28].

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Table 1
MicroRNAs and complementary probes.

Material	Sequence
MicroRNA-205	5'-UCCUUCAUUCCACCGGAGUCUG-3'
Capture complementary probe conjugated with biotin	5'-GGAATGAAGGATTTTTTTTT-biotin-3'
Detection complementary probe linked to hemin aptamer	5'-AAAGGGTAGGGCGGGTTGGGTAAATAAAA A CAGACTCCGGT-3'
MicroRNA-15a	5'-UAGCAGCACAUAAUGGUUUGUG-3'
MicroRNA-17	5'-CAAAGUGCUUACAGUGCAGGUAG-3'
MicroRNA-21	5'- UAGCUUAUCAGACUGAUGUUGA-3'
MicroRNA0155	5'UUAAUGCUAAUCGUGAUAGGGGUU-3'

Based on the previous reports, we designed and developed for the first time a cost-effective biosensor with ODI-CL detection for the quantification of microRNA-205, a biomarker of lung cancer, in human serum. First, two ssDNA complementary probes (capture complementary probe and detection complementary probe linked to hemin aptamer) selectively bound with microRNA-205 in human serum. With the addition of hemin in the solution containing the complex linked to hemin aptamer (5′-AAAGGGTAGGGCGGGTTGGGTAAA-3') from the hybridization, DNAzyme linked to the complex was formed. Then, luminescent dye (resorufin) formed from the reaction of Amplex Red and H_2O_2 in the presence of the DNAzyme emitted bright red light with the addition of ODI-CL reagents. Details are described below.

2. Materials and methods

2.1. Chemicals and materials

As shown in Table 1, microRNA-205, capture complementary probe conjugated with biotin, detection complementary probe linked to hemin aptamer, and the rest of the microRNAs were purchased from Alpha DNA. Magnetic bead conjugated with streptavidin (Dynabeads MyOne streptavidin T1, 1 μm), Amplex Red were purchased from ThermoFisher Scientific. Hemin was purchased from Sigma Aldrich. Bis (2,4,6-trichlorophenyl) oxalate (TCPO) and 4-methylimidazole (4MImH) were purchased from TCI America. 3% and 30% Deionized H_2O (HPLC grade), Ethyl acetate, Isopropyl alcohol, and high concentration of PBS (pH 7.4, 20×) and TBS (pH 7.4, 10×) were purchased from EMD. Four different Tris-HCl buffers (pH 7, 7.5, 8, and 8.5, 1 M each), $MgCl_2$, NaCl, and ethylenediaminetetraacetic acid (EDTA) were purchased from VWR. Pooled normal human serum was purchased from Innovative Research.

2.2. Methods

2.2.1. Quantification of microRNA-205 in a sample

Fig. 1 shows that procedures for quantification of microRNA-205 using a biosensor designed with a capture complementary probe immobilized on the surface of paramagnetic bead, a detection complementary probe linked to hemin, and ODI-CL detection. Details are described below.

2.2.1.1. Immobilization of capture complementary probes on the surface of magnetic bead. Paramagnetic beads (2 μg/μl) in 2× binding buffer (0.5 ml, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl) was mixed with the capture complementary probe conjugated with biotin (5 μM, 0.5 ml) in water in a 1.5-ml microcentrifuge tube. The mixture (1 ml) in a borosilicate tube was incubated for 15 min using a rotor (18 rpm) at room temperature. After the incubation, the capture complementary probe immobilized on the surface of paramagnetic beads was separated with a magnetic separator (Bioclone, Inc). The capture complementary probes on the paramagnetic beads were washed 3 times with the binding buffer. Then, the final particles in PBS (1 ml) containing 0.1% bovine serum albumin (BSA) were stored in a refrigerator.

2.2.1.2. Hybridization of microRNA-205 using the capture and detection complementary probes. The capture complementary probe immobilized on the surface of paramagnetic bead in Tris-HCl (pH 8.5, 50 μl) was mixed with mcroRNA-205 (e.g., 0 ĩ100 nM) in 10-fold diluted human serum with Tris-HCl (pH 8.5, 50 μl) in a 1.5-ml microcentrifuge tube. Then, the mixture in the microcentrifuge tube was incubated for 30 min using the rotor at a laboratory oven (VWR, 37 °C). After the incubation, the complexes, formed from the hybridization of microRNA-205 and the capture complementary probe, immobilized on the paramagnetic beads were washed 3 times with 1 × TBS buffer with the magnetic separator. Then, the detection complementary probe linked to hemin aptamer (100 μl) was mixed with the complexes in the microcentrifuge tube. The mixture was incubated for 30 min using the rotor and the laboratory oven at 37 °C. After the incubation, the final complexes linked to hemin aptamer, formed from the final hybridization, immobilized on the surface of paramagnetic beads were washed 3 times with 1 × TBS (pH 7.4) using the magnetic separator.

2.2.1.3. DNAzyme reaction for the quantification of microRNA-205. 25 μM hemin (100 ul) in TBS was added in the microcentrifuge tube containing the complexes linked to hemin aptamer formed the hybridization proceeded in Section 2.2.2. The mixture was incubated for 15 min at room temperature to produce the HRP-mimicking DNAzyme formed from the binding reaction of hemin aptamer linked to the detection complementary probe and hemin. After the incubation, the final products immobilized on the surface of paramagnetic beads were washed 3 times with 1 × TBS. After the washing, 1 × TBS (100 μl) containing 2.5 μM Amplex Red, **1**, and 0.1 mM H_2O_2 was added in the microcentrifuge tube. The mixture was incubated for 15 min at room temperature to produce resorufin, **2**, from the DNAzyme reaction as shown in Fig. 2(B). After the incubation, the TBS solution containing resorufin (20 μl) was transferred into a borosilicated tube (12 × 75 mm). The tube was inserted into the detection cell of a luminometer with two syringe pumps (Lumat 9507, Berthold, Inc.). 100 mM H_2O_2 (25 μl) dissolved in isopropyl alcohol was dispensed through the first syringe pump of the luminometer. Then, the relative CL intensity immediately emitted with the addition of ODI (25 μl) using the second syringe pump was measured for 2 s.

Fig. 2(A) shows that the brightness of light emitted in ODI-CL reaction was enhanced with the increase of DNAzyme based on the ODI-CL reaction mechanism of Fig. 2(B). Resorufin is formed from the DNAzyme reaction. Then, resorufin, as an emitter, receives energy from the high-energy intermediate, **X**, formed from the reaction of ODI, **4**, and H_2O_2. Finally, the resorufin under the excited state, **3**, formed through the energy transfer between **X** and resorufin under the ground state, emits a bright red light.

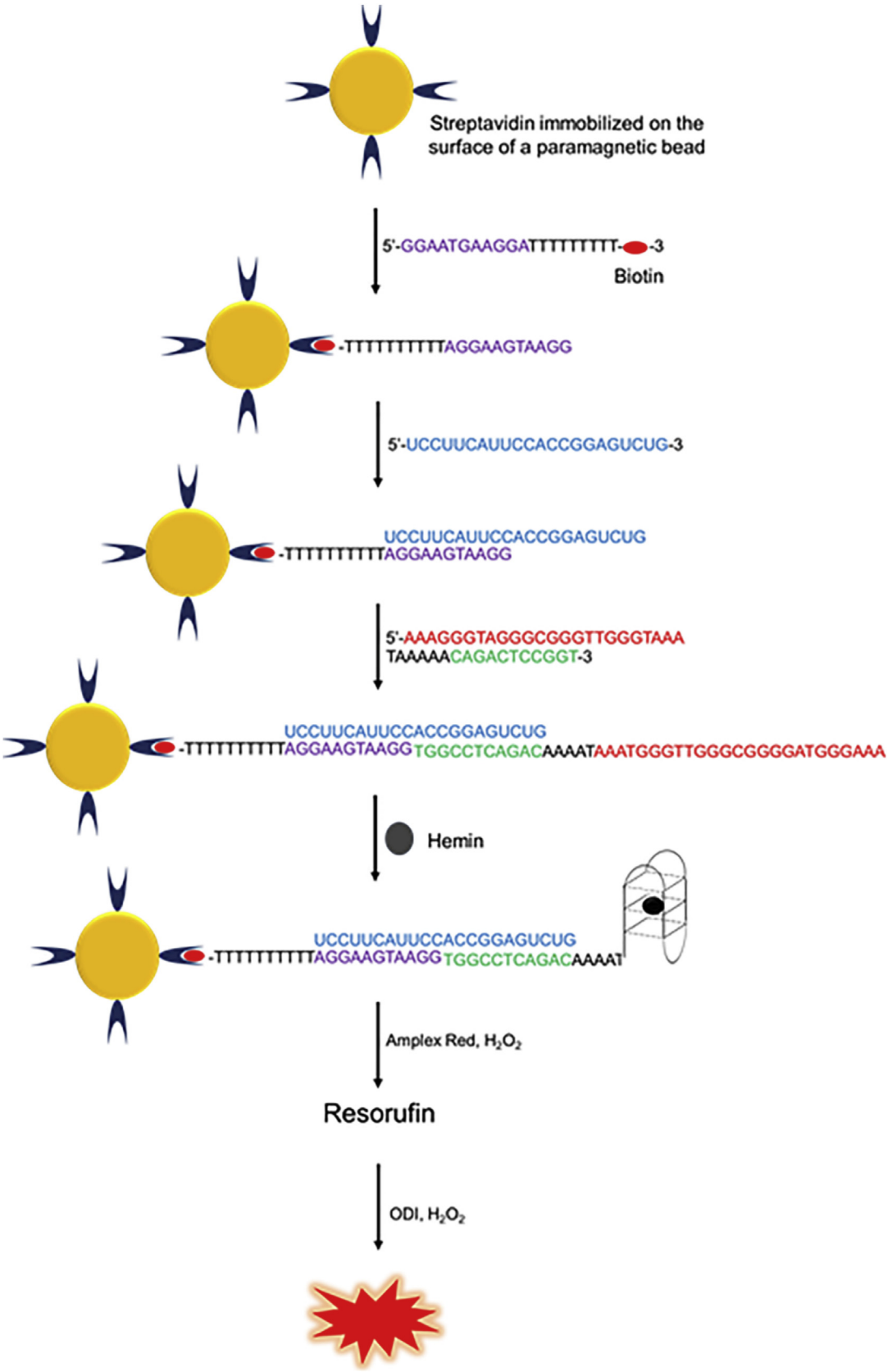

Fig. 1. Procedures for the quantification of microRNA-205 with a capture complementary probe immobilized on the surface of paramagnetic bead, a complementary probe linked to hemin aptamer and ODI-CL detection.

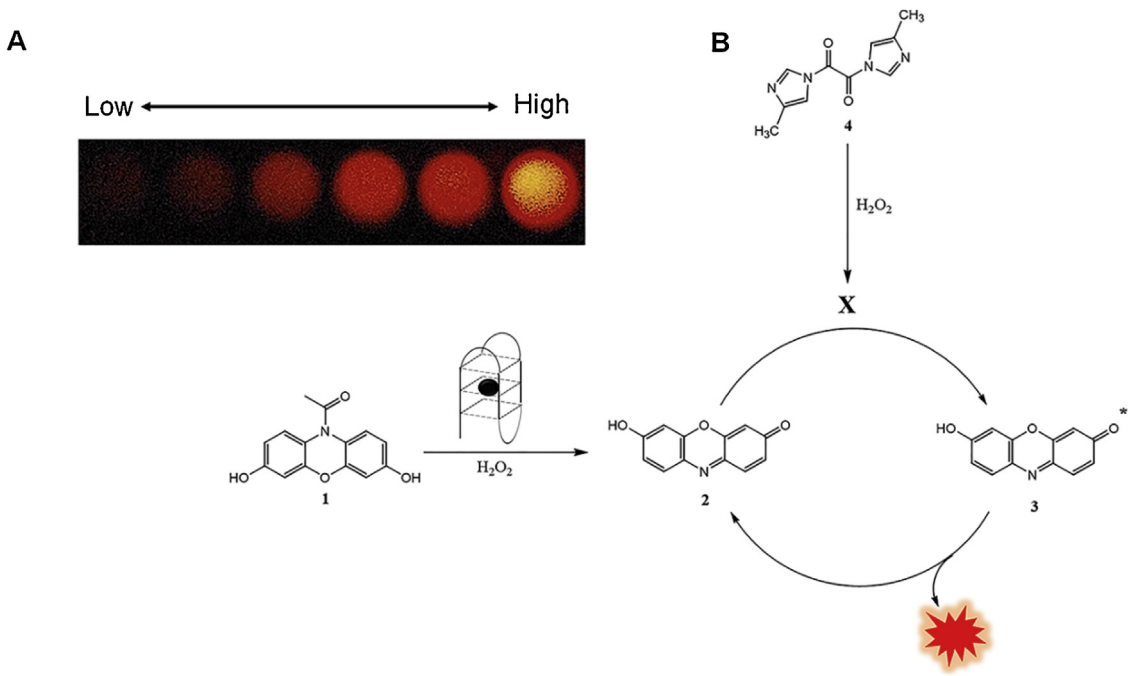

Fig. 2. (A) Effect of G-quadruplex formed with the addition of hemin in ODI-CL reaction (Concentration of hemin from the left: 2.5, 5, 10, 20, 40, 80 μM), (B) Possible ODI-CL reaction mechanism in the presence of resorufin formed from the reaction of Amplex Red and H_2O_2 with the G-quadruplex, formed with hemin aptamer and hemin, as a DNAzyme. 1. Amplex Red, 2. resorufin under the ground state, 3. resorufin under the excited state, 4. ODI, X. high-energy intermediate formed from the reaction of ODI and H_2O_2.

2.2.2. Specificity of the biosensor capable of sensing microRNA-205

In order to determine the selectivity of biosensor, 20 μM microRNA-205 and 100 nM four different microRNAs (e.g., microRNA-15a, microRNA-17, microRMA-21, microRNA-155), also known as cancer markers, were prepared in 10-fold diluted human serum with Tris-HCl (pH 8.5). Each sample was treated based on the procedures shown in Fig. 1 to study whether the biosensor can quantify microRNA-205 in a sample with acceptable selectivity.

3. Results and discussion

3.1. Optimization of variables to develop a biosensor with ODI-CL detection

Fig. 3(A) indicates that a biosensor, operated with DNAzyme reaction with ODI-CL detection, can be developed with 2.5 μM Amplex Red. With the increase of Amplex Red up to 2.5 μM, the relative CL intensity was enhanced. Then, in the presence of Amplex Red higher than 2.5 μM, the relative CL intensity was weakened due to the self-quenching of excess resorufin excited by the high-energy intermediate formed in ODI-CL reaction [27]. Thus, the relative CL intensity in the presence of 20 μM Amplex Red was the lowest.

As shown in Fig. 3(B), the relative CL intensity was enhanced with the increase of hemin concentration bound with hemin aptamer immobilized on the surface of magnetic beads. The relative CL intensities observed in the presence of hemin between 3.2 and 25 μM were linearly enhanced. However, the relative CL intensities in the presence of hemin higher than 50 μM were lower than those we expected with the linear curve due to the self-quenching of excess resorufin that was formed from the reaction of Amplex Red and H_2O_2 in the presence of G-quadruplex produced with hemin higher than 50 μM, under the excited state in ODI-CL reaction. Based on the preliminary experimental results shown in Fig. 3(B), 25 μM hemin was selected for the development of the biosensor for the quantification of microRNA-205.

Fig. 3(C) and (D), the best buffer solution for the DNAzyme reaction is different from that for the HRP enzyme reaction. As shown in Fig. 3(C), TBS (pH 7.4) was the best for the DNAzyme reaction, whereas Tris-HCl (pH 7.5) was the best for the HRP enzyme reaction as shown in Fig. 3(D). Thus, TBS (pH 7.4) was selected as a buffer solution for the DNAzyme reaction of Amplex Red and H_2O_2 in the presence of G-quadruplex formed from the binding reaction between hemin aptamer and hemin.

3.2. Optimization of hybridization for the development of a biosensor for the quantification of microRNA-205

As shown in Fig. 4(A), the efficiency of hybridization of microRNA-205 and capture & detection complementary probes in Tris-HCl (pH 8.5) was the better than those in the binding and enzyme buffers. Based on the results, Tris-HCl (pH 8.5) was selected for the development of the biosensor for the analysis of microRNA-25.

Based on the results shown in Fig. 4(A), the complex formed from the hybridization of microRNA-205 and two complementary probes was obtained in Tris-HCl based on the procedures shown in Fig. 1. The complex immobilized on the surface of magnetic bead (100 μg/ml) was diluted to study the effects of the complex concentration and incubation time for the DNAzyme reaction. As shown in Fig. 4(B), the relative CL intensity was dependent on the complex concentration bound with magnetic beads. Thus, the relative CL intensity was enhanced with the increase of magnetic beads. In addition, the brightness of CL emission was dependent on the incubation time. The relative CL intensity measured after 16-min incubation was constant within the statistically acceptable error range (± 5%). Based on the results shown in Fig. 4(B), 100 μg/ml magnetic beads bound with the complex and 15-min incubation for the DNAzyme reaction were selected for the design of the biosensor.

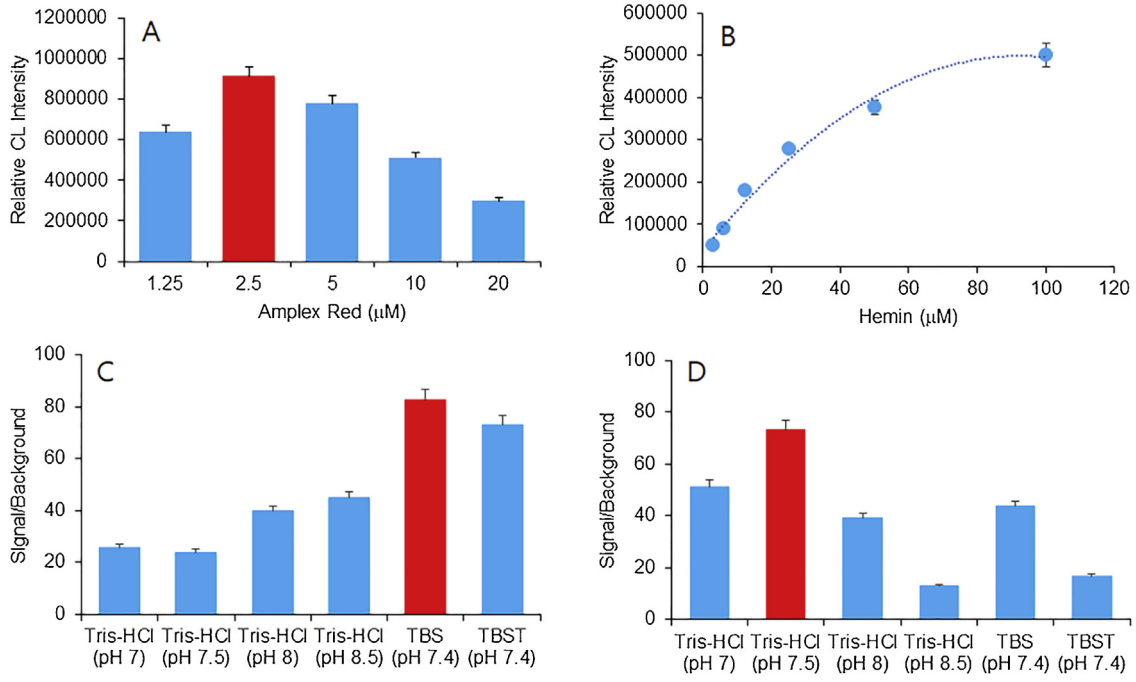

Fig. 3. (A) Effect of Amplex Red concentration to form resorufin in the HRP-mimicking reaction (B) Effect of hemin concentration in the presence of hemin aptamer (200 nM) to generate DNAzyme, (C) Selection of the best buffer for the reaction of Amplex Red and H_2O_2 in the presence of DNAzyme formed from binding reaction of hemin and hemin aptamer, (D) Selection of the best buffer for the reaction of Amplex Red and H_2O_2 in the presence of HRP for the conventional enzyme reaction.

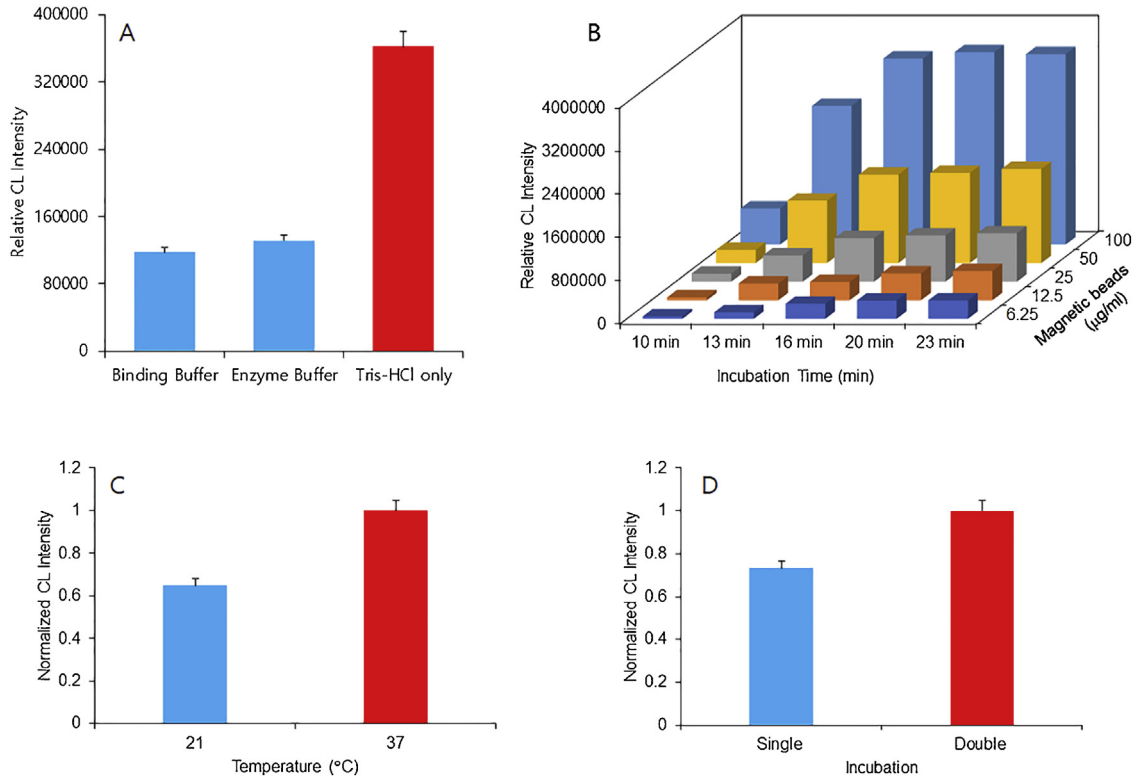

Fig. 4. (A) Selection of the best buffer solution for the hybridization of microRNA-205 and capture & detection complementary probes, (B) Effect of magnetic bead concentration under the different incubation time for the DNAzyme reaction. The error range of each bar was 4 7%. (C) Effect of temperature for the hybridization of microRNA-205 and complementary probes, (D) Differences between the single and double incubations for the hybridization of microRNA-205 and capture & detection complementary probes. Single incubation was performed for 60 min after simultaneously mixing microRNA-205 and capture & detection complementary probes, and double incubations. Double incubations for the hybridization were completed based on the procedures shown in Fig. 1.

Fig. 3(C) indicates that the incubation to produce the complex immobilized on the surface of magnetic bead at 37 °C is better than that at room temperature. Additionally, Fig. 4(D) shows that the double incubations (30-min each) to produce the complex bound with the magnetic beads, based on the procedures of Fig. 1, is better than the single incubation (60-min) to produce the complex formed

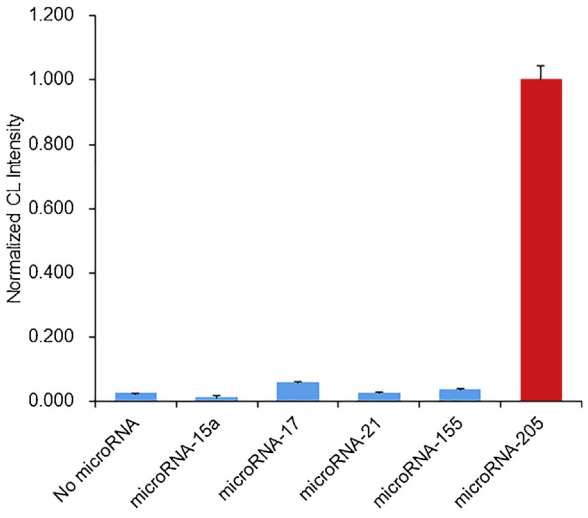

Fig. 5. Specificity of the biosensor capable of sensing microRNA-205 in human serum.

with the simultaneous additions of microRNA-205 and capture & detection complementary probes in Tris-HCl. Based on the experimental results shown in Fig. 4(C) and (D), the biosensor capable of sensing microRNA-205 was designed and developed with the complexes formed from the double incubations at 37 °C.

3.3. Development of a biosensor with ODI-CL detection using the hybridization and DNAzyme

Fig. S1 shows the linear calibration curve of the biosensor developed based on the preliminary research results shown in Figs. 3 and 4. The dynamic range of the linear curve for the analysis of microRNA-205 in human serum was 0.42–62.5 nM. Due to the shelf-quenching effect, the relative CL intensity in the presence of more RNA-205 than 62.5 nM was lower than that computed with the first order equation of the linear calibration curve. Duan and Chen [29] monitored the correlation between microRNA-205 and transforming growth factor-beta (TGF-beta) proteins to study the development of tumors. The concentration range of microRNA-205 in this research was covered with the dynamic range of the biosensor developed in this research The previous report [29] indicates that the dynamic range of the biosensor can be applied to early diagnose human cancer. The limit of detection (LOD = 3S/slope) was as low as 0.13 nM. S is the standard deviation of backgrounds measured 20 times. Normal Fe_3O_4 magnetic nanoparticles is also reported as a HRP-mimicking catalyst [30]. However, the background measured in the presence of Fe_3O_4 magnetic beads only without microRNA-205 was very low. The result indicates that activity of DNAzyme used in this research is much stronger than that of Fe_3O_4 magnetic beads in this condition. The LOD of the biosensor capable of sensing microRNA-205 was compared with those of other methods used to quantify various microRNAs as shown in Table S1. The results indicate that the biosensor can cost-effectively and rapidly quantify microRNA-205 in a sample.

As shown in Fig. 5, the biosensor was able to quantify microRNA-205 in human serum with good selectivity. This is because the capture complementary probe immobilized on the surface of magnetic bead can selectively bind with microRNA-205 in human serum. Thus, the relative CL intensity of the biosensor in the presence of other microRNAs, applied as cancer biomarkers, was similar to that (background) in the absence of any microRNA within the error range.

Table 2 indicates that the biosensor can quantify microRNA-205 with good accuracy, precision, and recovery. We have used three different serum samples for the research. The accuracy of the biosensor was 4–9 % in Table 2. The precision of the biosensor was 5–8.8 %. Also, the recovery range of the biosensor was 96–109 %.

Table 2
The recovery of the biosensor for the quantification of microRNA-205 in human serum (n = 5).

Sample	Added (nM)	Measured (nM)	Recovery (%)
A	10	9.6 (±0.6)	96.0
B	20	21.8 (±1.1)	109.0
C	40	42.9 (±3.8)	107.3

Fig. 5 and Table 2 indicates that the biosensor can be used as a new method for the quantification of microRNA-205 to early diagnose lung cancer.

4. Conclusions

Based on the experimental results shown in Figs. 3–5, a cost-effective and simple biosensor, capable of quantifying trace levels of microRNA-205, was developed for the first time using the hemin aptamer linked to the detection complementary probe and ODI-CL detection. It is expected that the cost to quantify microRNA-205 in a sample is less than US 10 cents. Additionally, we confirmed that the biosensor can analyze microRNA-205 with good selectivity for the accurately and early diagnose lung cancer.

As future work, we expect that a biosensor modified with a capture and a detection complementary probes matched with a specific microRNA can be used for the early diagnosis of human disease such as cardiac diseases, cancers, and infectious diseases. Also, we expect that the new concepts applied in this research can be used for the analyses of matched and mismatched single-stranded DNA, instead of microRNA, in a sample.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.112780.

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