Hybrid biosensor for the early diagnosis of breast cancer

Kelly Cho, Stephanie Chong, Edward Park, Ji Hoon Lee

Luminescent MD, LLC, Hagerstown, MD 21742

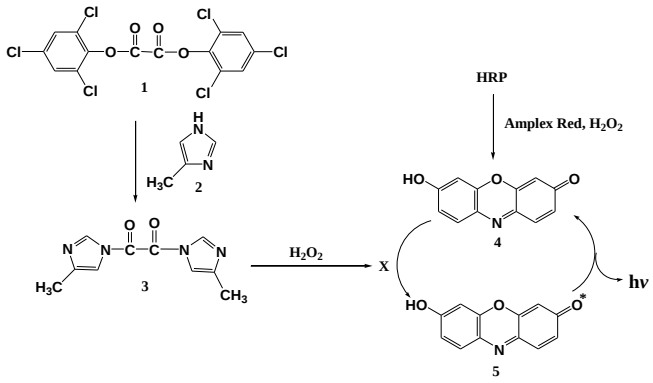
ABSTRACT

A cost-effective and novel hybrid biosensor using the combination of antibody and DNA aptamer was developed for the early diagnosis of breast cancer. 1,1'-oxalyldiimidazle chemiluminescence (ODI-CL) was applied as a detector of the hybrid biosensor. The antibody immobilized on the surface of polystyrene strip-well rapidly captured the carcinoembryonic antigen (CEA), which is a breast cancer marker, within 20 min at 37 °C. DNA aptamer-linked hemin aptamer was added in the strip-well washed with PBST. After a 1-hr incubation of mixuture in the strip-well at 37 °C, hybrid complex (antibofy-CEA-DNA aptamer-linked hemin aptamer) was formed. Hemin was added in the strip-well to form horseradish peroxidase (HRP)-mimicking DNAzyme (complex of hemin and hemin aptamer) for a 5-min incubation at 37 °C. Substrates (e.g., Amplex Red, H_2O_2) of HRP-mimicking DNAzyme added in the strip-well after the washing with PBST was incubated for 10 min at room temperature (21 ± 2 °C) to produce resorufin, which is a luminophore. Finally, CL emission was observed when H_2O_2 and ODI were consecutively added in the strip-well. The brightness of CL was proportionally enhanced with the increase of CEA. The limit of detection (LOD = 3 /slope) of hybrid biosensor was as low as 0.55 ng/ml.

Keywords: 1,1'-Oxalyldiimidazole, chemiluminescence, Aptamer, breast cancer, hemin, Immunoassay

Introduction

In general, enzyme-linked immunosorbent assays (ELISAs) with colorimeter detection using horseradish peroxidase (HRP) or alkaline phosphatase (AP) are widely used to quantify trace levels of carcinoembryonic antigen (CEA) known as a breast cancer marker.(*1-3*) In order to enhance the sensitivity of enzyme immunoassay (EIA), several types of chemiluminescence detection system have been applied.(*4-6*) For example, Scheme 1 shows the reaction mechanism of 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) applied as a detection system of EIAs using HRP enzyme.(*7-8*) In addition, EIAs with ODI-CL detection were faster and more sensitive than those with other CL detections such as 1,2-dioxetane and luminol.(*7*) Also, ODI-CL detection can be applied as a detection method in EIAs with AP as well as HRP. (*8*)



Scheme 1. 1,1'-oxalyldiimidazole chemiluminescence applied as a detection method of EIAs using HRP. **1** Bis(2,4,6-trichlorophenyl) oxalate, **2** 4-methylimidazole, **3** 1,1'-oxalyldiimidazole, **4** resorufin under the ground state, **5** resorufin under the excited state, **X** high-energy intermediate.

As shown in Scheme 1, ODI is formed from the reaction of bis(2,4,6-trichlorophenyl) oxalate and 4-methylimidazole. High-energy intermediate (**X**) formed from ODI and H_2O_2 transfers energy to resorufin formed from the reaction Amplex Red and H_2O_2 in the presence of HRP. Then, resorufin under the excited state emit red light.(*7*) The color of light emitted in ODI-CL reaction is determined the property of chemiluminescent dye such as resorufin.(*4, 7-8*)

Recently, cost-effective and easy-to-use DNA aptamers capable of capturing cancer markers such as CEA (*9*) and prostate specific antigen (PSA) (*10*) were developed. Using DNA aptamers having the same function as antobodies, several biosensors have been developed for the quantification of cancer markers.(*9, 11-13*)

HRP-mimicking DNAzyme, hemin aptamer-bound hemin, has been widely used to develop enzyme free biosensor using DNA aptamer.(*14-16*) This is because HRP-mimicking DNAzyme, instead of HRP, is acting as a catalyst in substrate solution used in HRP enzyme reaction. Thus, it was reported that the sensitivity of biosensor using HRP-mimicking DNAzyme is as good as that of EIA using HRP.

In general, CEA in human serum is quantified using sandwich EIAs using a primary antibody and a detection antibody conjugated with HRP.(*4*) If CEA aptamer conjugated with HRP-mimicking DNAzyme is used instead of the detection antibody conjugated with HRP, it is possible to develop a hybrid biosensor for the early

diagnosis of breast cancer. Also, if ODI-CL is used as a detection system of hybrid biosensor, the sensitivity of hybrid biosensor may be as good as that of EIA with ODI-CL detection. Based on the hypotheses described above, I developed an enzyme free hybrid biosensor with ODI-CL detection using HRP-mimicking DNAzyme.

Experimental

Chemical and materials

Three different types of CEA aptamer linked to hemin aptamer (CA) and hemin aptamer, as shown below, were purchased from Alpha DNA. CEA aptamer was linked to hemin aptamer using a linker composed of 5 adenines (AAAAA).

CA-1:
AAAGGGTAGGGCGGGTTGGGTAAATAAAAAATACCAGCTTATTCAATT

CA-2:
AAAGGGTAGGGCGGGTTGGGTAAATAAAAAAGGGGGTGAAGGGATACCC

CA-3:
ATACCAGCTTATTCAATTAAAAATAAAGGGTAGGGCGGGTTGGGTAAAT

Hemin aptamer: AAAGGGTAGGGCGGGTTGGGTAAAT

Hemin and bovine serum albumin were purchased from Sigma Aldrich. Bis (2,4,6-trichlorophenyl) oxalate (TCPO) and 4-methylimidazole (4MImH) were purchased from TCI America. 3 and 30 % H_2O_2 were purchased from VWR. Amplex Red was purchased from Cayman Chemical. Deionized H_2O, Ethyl acetate, and Iospropyl alcohol were purchased from EMD. CEA diagnostic kit for ELISA and 0 calibrator were purchased from Monobind, Inc. CEA antigen (25 µg) was purchased from Lee Biosolutions. 8-well EIA/RIA strip-well plate was purchased from Costar.

Development of hybrid biosensor

In order to develop hybrid biosensor capable of quantifying CEA in serum, the strip-well of ELISA kit (Monobind, Inc.) was used. As shown in Fig. 1, primary antibodies were bound with streptavidin immobilized on the surface of strip-well. Also, the strip-well was coated with BSA.
CEA standard or sample (50 µl) added in the strip-well was incubated for 20 min at 37 °C. CEA aptamer linked to hemin aptamer (100 µl) was inserted into the strip-well washed after the incubation. The strip-well containing aptamer was incubated for 1-hr at 37 °C. After the washed strip-well containing CEA immobilized with hybrid captures (e.g., primary antibody, CEA aptamer linked to hemin aptamer), 500 nM hemin (100 µl) was injected to the strip-well. After a 5-minute incubation, the strip-well was washed. Then, substrate solution (100 µl) containing Amplex Red and H_2O_2 was dispensed into the strip-well.

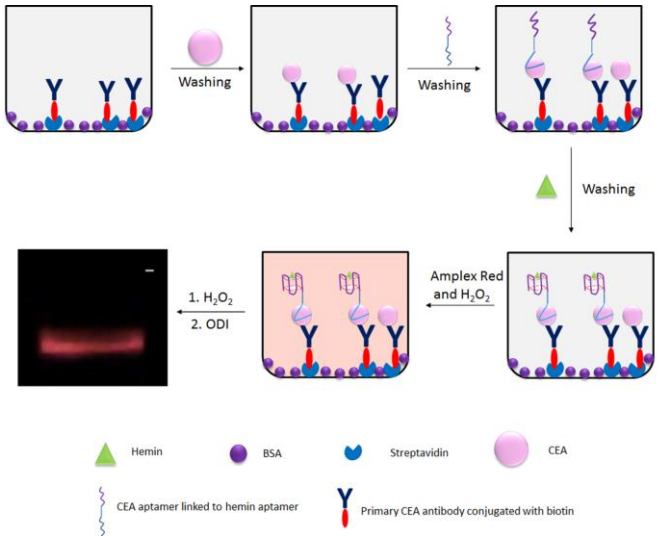


Fig 1. Quantification of CEA using hybrid biosensor with ODI-CL detection.

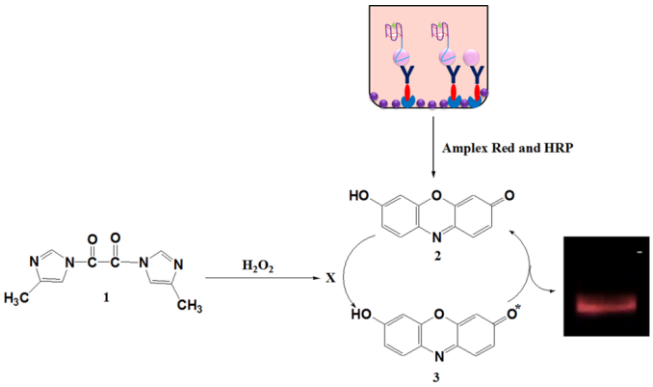


Fig.2 ODI-CL reaction mechanism for the quantification of CEA using hybrid biosensor. **1** ODI, **2** resorufin under the ground state, **3** resorufin under the excited state.

After a 10-minute incubation, CEA in the strip-well was quantified with the measurement of CL intensity with the addition of ODI-CL reagents based on the mechanism shown in Fig. 2.

Results and Discussion

Quantification of CEA under various environmental conditions

Based on the diagram shown in Fig. 1, CEA added in the strip-well was incubated for 20 minutes at 37 °C. Then, CEA aptamer linked to hemin aptamer (500 nM, 100 l) added in the strip-well was incubated for 30 minutes at 37 °C. After the incubation and washing of the strip-well, 500 nM hemin was added in the strip-well. After a 15-minute incubation at room temperature and washing of the strip-

well, substrate solution (100 µl) added in the strip-well was incubated for 10 minutes at room temperature. Finally, relative CL intensity of each sample was measured with the luminometer.

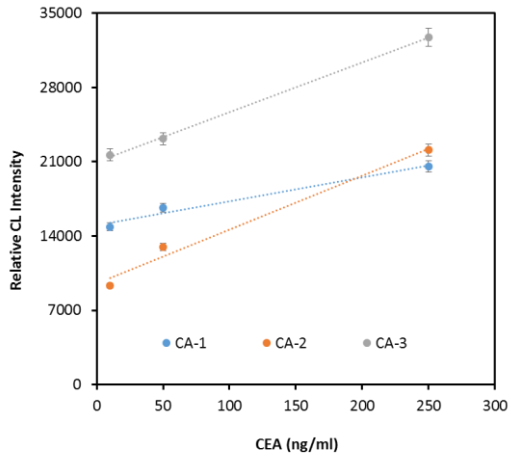


Fig. 3 Selection of CEA aptamer linked to hemin aptamer for the quantification of CEA

As shown in Fig. 3, the linearity of CA-3 for the quantification of CEA was better than those of CA-1 and CA-2. However, the slope of the linear calibration curve (y = 46.21 +21045, R^2 = 0.998) obtained with CA-3 was so small that accuracy and precision of the hybrid biosensor weren't good. In addition, the background noise of hybrid biosensor was relatively. Thus, the limit of detection (LOD = 3 /slope, 8.5 ng/ml) of hybrid biosensor was too high to diagnose breast cancer in patients (cut off value = 5 ng/ml). σ is standard deviation of background noise (n = 5) measured in the absence of CEA.

In order to enhance the accuracy and precision of hybrid biosensor, the incubation time after adding CEA aptamer linked to hemin aptamer was extended up to 1 hour. The rest of the experimental conditions were the same as those used to obtain the results shown in Fig. 3. As shown in Fig. 9, the slope of linear calibration curve obtained with the 1-hour incubation was higher than that determined with the 30-minute incubation (See Fig. 3) even though the background noise in the former condition was similar to that in the latter because the incubation time after adding free hemin was invariable. In addition, the dynamic range of linear calibration curve in the former was narrower than that in the latter as shown in Fig. 3 and the inset of Fig. 4. LOD (2.11 ng/ml) computed under the condition of Fig. 4 was about 4-fold lower than that calculated under the condition of Fig. 3. However, the limit of quantification (LOQ = 10 /slope, 7.03 ng/ml) is still higher than the cut-off value to determine whether patient has breast cancer.

We modified the experimental condition again based on the results observed in Figs 3 and 4. The incubation time for the binding interaction between CEA and CEA aptamer linked to hemin aptamer was 1 hr. Also, the incubation time

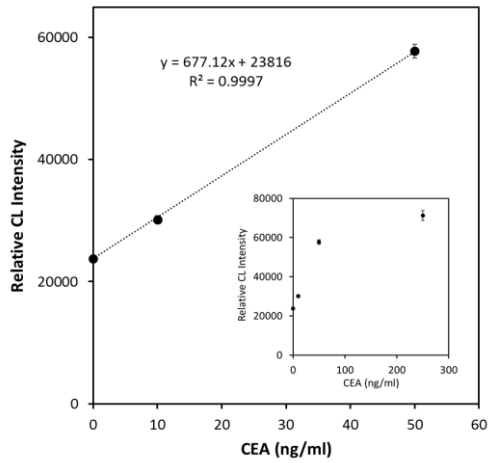


Fig. 4 Calibration curve obtained under the condition to enhance slope of linear calibration curve.

between hemin and hemin-aptamer linked to CEA aptamer-bound CEA in the strip-well was 5 minutes because most of hemin molecules was able to bind with hemin aptamer linked to CEA aptamer.

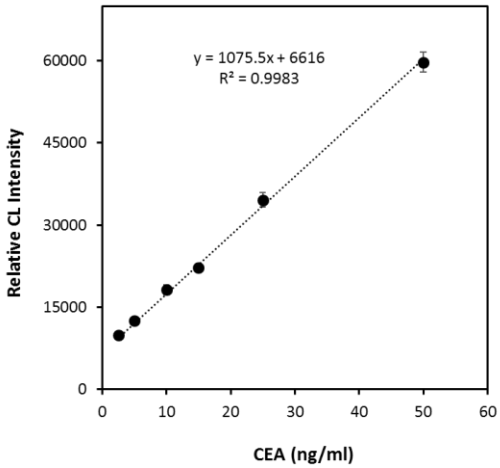


Fig. 5 Calibration curve capable of quantifying trace levels of CEA.

As shown in Fig. 5, the background of hybrid biosensor under the modified condition was about 3.5 fold lower than those under the conditions of Figure 3 even though the former was still about 3-fold higher than that in the absence of hemin. Also, the slope of linear calibration curve was bigger than those obtained in Figs 3 and 4. Thus, LOD and LOQ of hybrid biosensor computed under the condition were as low as 0.55 and 1.84 ng/ml. These results indicate that hybrid biosensor can quantify lower concentration of CEA than 5 ng/ml for the diagnosis of breast cancer. However, LOD of hybrid biosensor using a primary antibody, CEA aptamer, and HRP-mimicking DNAzyme is still higher than those of conventional enzyme immunoassays using two antibodies and HRP.(*4*) This is because the background of hybrid biosensor, generated by the adsorption of free hemin on the surface of strip-well, is

higher than those of HRP enzyme immunoassay. Fig. 10 and Table 1 indicate that accuracy, precision, and recovery of hybrid biosensor with ODI-CL detection were good within acceptable error range.

Table 1 Recovery of hybrid biosensor for the quantification of CEA. (N=5)

Sample A[1]	Sample B[1]	Expected[1,2]	Measured[1]	Recovery (%)
10	5	7.5	6.9	92
20	10	15	16.3	108.6
20	30	25	23.8	95.2

[1] Unit of CEA: ng/ml
[2] Expected value = (sample A + sample B)/2

Table 2 Correlation between hybrid biosensor and conventional enzyme immunoassay with ODI-CL detection (N =3).

Sample	Hybrid Biosensor (ng/ml)	Enzyme Immunoassay (ng/ml)
1	3.7 ± 0.4	3.5 ± 0.2
2	6.7 ± 0.3	6.3 ± 0.4
3	15.4 ± 0.6	15.7 ± 0.4
4	43.2 ± 1.6	45.7 ± 1.3

As shown in Table 2, the good correlation between hybrid biosensor and conventional enzyme immunoassay[10] for the quantification of CEA in four samples indicates that hybrid biosensor can be applied as a new analytical method capable of early diagnosing and rapid monitoring breast cancer.

Conclusions

A hybrid biosensor capable of quantifying trace levels of CEA in a sample was developed for the first time. I confirmed that the hybrid biosensor with ODI-CL detection can be applied as a new tool for the diagnosis of breast cancer. Thus, We expect that the method reported in this manuscript can be applied to develop hybrid biosensors capable of diagnosing various human diseases such as cancer and infectious diseases as well as monitoring toxic materials in food and drink.

However, the hybrid biosensor was not highly sensitive because of the high background occurring with the rapid adsorption of free hemin on the surface of BSA pre-coated strip-well. In order to reduce the background noise of the hybrid biosensor, We propose that CEA aptamer conjugated HRP needs to be used instead of CEA aptamer linked to hemin aptamer and hemin. This is because HRP isn't adsorbed on the surface of BSA pre-coated strip-well, whereas hemin is rapidly adsorbed on the surface of BSA pre-coated strip-well in the absence of a target marker. Thus, using CEA aptamer conjugated with HRP, we plan to develop a more sensitive hybrid biosensor as future work.

REFERENCES

[1] M. Y. Liu,, C. P. Jia, Y. Y. Huang, X. H. Lou, S. H. Yao, Q. H. Jin, J. L. Zhao, J. Q. Xiang, *Analyst 135*, 327, 2010.

[2] M. Y. Liu, C. P. Jia, Q. H. Jin, X. H. Lou, S. H. Yao, J. Q. Xiang, J. L. Zhao, *Talanta 81*, 1625, 2010.

[3] A.. Pakdel, F. Naghibalhossaini, P. Mokarram, M. Jaberipou, A. Hosseini, *Mol. Biol. Rep. 39*, 3695, 2012.

[4] J. Kim, J. Kim, T. H. D. Rho, J. H. Lee, *Talanta 129*, 106, 2014.

[5] J. Jiang, S. Zhao, Y. Huang, G. X. Qin, F. G. Ye *J. Chromatogr. A 1282*, 161, 2013.

[6] R. Findeisen, S. Albrecht, B. Richter, K. Deutschmann, T. Zimmermann, W. Distler, *Luminescence 15*, 283, 2010.

[7] J. H. Lee, J. E. R. Rho, T. H. D. Rho, J. G. Newby, *Biosens. Bioelectron. 26*, 377, 2010.

[8] R. Chong, J. E. R. Rho, H. J. Yoon, T. H. D. Rho, P. S. Park, Y. H. Kim, J. H. Lee, *Biosens. Bioelectron. 32*, 19, 2012.

[9] H. Shu, W. Wen, H. Y. Wen, H. Y. Xiong, X. H. Zhang, S. F. Wang, *Electrochem. Commun. 37*, 15, 2013.

[10] N. Savory, K. Abe, K., K. Sode, K. Ikebukuro, *Biosens. Bioelectron. 26*, 1386, 2010.

[11] T. Cha, S. Cho, Y. T. Kim, J. H. Lee, *Biosens. Bioelectron. 62*, 31, 2014.

[12] J. Jun, J. S. Lee, D. H. Shin, J. Jang, *ACS Appl. Mater. Interfaces 6*, 13859, 2014.

[13] L. Kashefi-Kheyrabadi, M. A. Mehrgardi, E. Wiechec, A. P. F. Turner, A. Tiwari, A. *Anal. Chem. 86*, 4956, 2014.

[14] E. Golub, R. Freeman, I. Willner, *Anal. Chem. 85*, 12126, 2013.

[15] J. Lee, C. H. Jeon, S. J. Ahn, T. H. Ha, *analyst 139*, 1622, 2014.

[16] A. L. Sun, Q. C. Qi, X. N. Wang, P. Bie, *Biosens. Bioelectron. 57*, 16, 2014.