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A cost-effective chemiluminescent biosensor capable of early diagnosing cancer using a combination of magnetic beads and platinum nanoparticles

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Abstract

Using thyroid stimulant hormone (TSH) detection antibody-conjugated horseradish peroxidase (HRP) immobilized on platinum (Pt) nanoparticle, a highly sensitive biosensor designed based on the principle of 1,1'-oxalyldiimidazole chemiluminescent enzyme immune assay (ODI CLEIA) was developed for the early diagnosis of thyroid cancer. Trace levels of TSH in human serum was rapidly quantified with the highly sensitive biosensor. The time necessary for the quantification of TSH using the biosensor with ODI CL detection was at least 3 times more rapid than commercially available enzyme immunoassay with colorimetric detection. The linear calibration curve (0.013 ~ 12 mU L^{-1}) of biosensor obtained with 3 ~ 6 % coefficient of variation was wider than that of the commercial product. Also, the limit of detection (LOD = background + 3σ, 0.005 mU L^{-1}) of biosensor was about 100-fold lower than that of commercial enzyme immunoassay. We expect that the newly developed biosensor, which has excellent accuracy, precision, and reproducibility, can be applied as a new method for the early diagnosis of thyroid cancer.

Keywords: Chemiluminescent enzyme immunoassay (CLEIA); Tumor marker; Stimulant hormone (TSH); Nanoparticle; 1,1'-Oxalyldiimidazole (ODI) chemiluminescence; Biosensor

[1] Gina Choi, Emory Kim, and Edward Park contributed equally in this research

1. Introduction

The thyroid, a gland at the base of the throat near the windpipe, creates hormones to support body temperature and blood pressure as well as to control the heart rate, the amount of calcium in the blood, and weight. Thyroid cancer occurring in people of all ages starts in the thyroid gland located inside the front of lower neck [1]. National Institute of Health (NIH) reported that anaplastic thyroid cancer is hard to cure with currently available treatment because this cancer is rare and spreads quickly. However, other thyroid cancers such as papillary, follicular, and medullary cancer can usually be cured if it is diagnosed early (http://www.cancer.gov/types/thyroid).

Thyroid stimulant hormone (TSH) in blood is a biomarker widely applied to early diagnose thyroid cancer [2, 3]. Thus, various analytical methods to quantify trace levels of TSH in human serum were developed [4-6]. Sandwich enzyme immunoassays (EIAs), using a capture antibody immobilized on polystyrene strip-well and a detection antibody conjugated with enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (ALP), are widely used for quantifying TSH in human serum because EIAs is more sensitive than other methods [7, 8]. However, the cut-off value (3 mU L^{-1}) of TSH in human serum is too low to rapidly quantify TSH using conventional EIAs with colorimetric detection [9].

In order to enhance the sensitivity of EIAs, fluorescence [10, 11] and chemiluminescence [12, 13] were applied as a detection method of EIAs. For example, it was reported that 1,1'-oxalyldiimidazole chemiluminescent enzyme immunoassay (ODI CLEIA) is more sensitive than conventional EIAs with colorimetric, fluorescence, luminol and 1,2-dioxetane detections for the quantification of cancer biomarkers such as prostate specific antigen, alpha-fetoprotein, carcinoembryonic antigen [13-15]. Thus, the analytical times of biosensors with ODI CLEIA

were more rapid than those with other optical detections [13-15].

Protein such as antibody and enzyme can be immobilized on nanoparticles such as gold (Au), platinum (Pt), and silver (Ag) due to the electrostatic interaction between NH_2 of protein and surface of nanoparticles [16, 17]. Using this phenomena, it was possible to enhance the biosensor fabricated based on the EIA methods. In order to develop the EIA biosensor using nanoparticles, in general, detection antibodies, capable of rapidly binding with a specific biomarker, and enzymes (e.g., HRP, ALP) are simultaneously added in an aqueous solution containing nanoparticles. In other words, detection antibody and enzyme competitively interact with nanoparticles. The function of detection antibody immobilized on a nanoparticle is to bind with the biomarker-capture antibody, whereas the objective of enzyme immobilized on the nanoparticle is to enhance the sensitivity of the biosensor. If detection antibodies are predominantly immobilized on the nanoparticle, it is difficult to enhance the sensitivity of biosensor. Also, if enzymes are predominantly immobilized on the nanoparticle, it is difficult to develop a rapid biosensor due to the delay of the interaction between the detection antibody and the biomarker-capture antibody.

Antibody and enzyme have two function groups such as NH_2 and COOH. Using the chemical reaction of two function groups, thus, antibody can strongly bind with enzyme. After the reaction, the complex still have NH_2 capable of electrostatically interacting with nanoparticles. If NH_2 of antibody reacts with COOH of enzyme for the conjugation the enzyme will still have multiple NH_2. Thus, it is possible that enzymes conjugated with antibody will be immobilized on nanoparticles. If the hypothesis is correct, the detection antibody of nanoparticle complex can rapidly bind with a specific biomarker-capture antibody. In other words, it is possible to develop a highly sensitive biosensor using the nanoparticle complex without any critical problems

observed when antibodies and enzymes are simultaneously and consecutively added in an aqueous solution containing nanoparticles. Based on the hypothesis, we studied to develop a highly sensitive biosensor designed based on ODI CLEIA method. Details are described in detail in this manuscript.

2. Experimental

2.1. Chemicals and materials

TSH enzyme immunoassay kit containing capture antibody coated on magnetic bead and detection antibody conjugated with ALP, TSH capture antibody (MIX.11 AB), and TSH detection antibody (BIS.6 AB) were purchased from Immunometrics (UK), Ltd. Horseradish peroxidase (HRP, 5 KU) and 4-iodophenol were purchased from Sigma-Aldrich. SureLink HRP conjugation kit was purchased from KPL, Inc. Bis (2, 4, 6-trichlorophenyl) oxalate (TCPO) and 4-methylimidazole (4MImH) were purchased from TCI America. Gold (Au) nanospheres (10 nm) in citrate buffer were purchased from NanoHybrids. Platinum nanoparticles (30 and 70 nm) in citrate buffer were purchased from NanpComposix. Deioninzed water (HPLC grade), ethyl acetate (HPLC grade), isopropyl alcohol (HPLC grade), 10 × PBS (pH 7.4), and Tris-HCl (pH 8.5, 1 mol L^{-1}) were purchased from VWR. Streptavidin and streptavidin conjugated with HRP were purchased from Thermo Fisher Scientific, Inc.

2.2. Optimization of the reaction between substrate and HRP

Scheme 1

In order to develop a highly sensitive ODI CLEIA using HRP for the early diagnosis of

thyroid cancer, we studied to optimize the reaction of substrate (e.g., Amplex Red, Amplex Ultrared which is a derivative of Amplex Red) and H_2O_2 in the presence of H_2O_2 using ODI CL detection. Scheme 1 shows the brief reaction mechanism of ODI CL reaction generated from the reaction between substrate and HRP. We optimized various variable factors – e.g., the type of substrate, the concentration of substrate, the type of buffer solution, the concentration of buffer solution, the concentration of enhancer (4-iodophenol, 4-IP), incubation time to form resorufin from the reaction between substrate and HRP – to emit bright light in ODI CL reaction. Resorufin (10 µl) formed under the optimization condition was added in a borosilicate test tube (12 × 75 mm). The test tube was inserted into the detection area of luminometer (LB 9507, Berthold, Inc). Then, H_2O_2, (25 µl) was injected into the test tube using a syringe pump of LB 9507. Finally, ODI (25 µl) formed form the reaction between TCPO (5 µM) and 4MImH (20 µM) was injected into the test tube using the other syringe pump. Then, the luminometer immediately measured the strength of light emitted in the test tube for 1 sec.

2.3. Determination of nanoparticle for ODI CLEIA

Fig. 1

In order to study the physical property of nanoparticle capable of binding with HRP, 10 µl of HRP (e.g., 0.5, 1, and 2 mg/ml) was added in the PBS solution (pH 7.4, 990 µl) containing 0.05 mg/ml of gold nanospheres (10 nm) or platinum nanospheres (30 or 70 nm). The solution was incubated for 1 hr at room temperature. After the incubation, HRP was immobilized on nanoparticles through the electrostatic interactions between nanoparticle and NH_2 of HRP. In order to obtain the pure nanoparticle bound with HRP, the solution containing nanoparticle bound with HRP and free HRP was centrifuged at 14,000 rpm for 10 min. After the

centrifugation, the solution containing free HRP was removed from the centrifuge tube. In order to prepare a stock solution, pure nanoparticle bound with HRP was stored in Tris-HCl (pH 8.5) containing 1 % BSA.

The stock solution was 100-fold diluted with Tris-HCl (pH 8.5) to prepare the working solution. The working solution (100 µl) was mixed with substrate (e.g., the mixture of Amplex Red, H_2O_2, and 4-IP). Resorufin (10 µl) formed form the first chemical reaction of Fig, 1 was inserted into a borosilicate test tube. Using LB 9507 with syringe pumps, light emitted from the test tube was measured.

2.4. Competition of streptavidin and HRP to bind with Pt nanoparticles

In order to investigate the chemical and physical differences of streptavidin and HRP capable of binding with Pt nanoparticles, they were immobilized on Pt nanoparticles using two different pathways. First, streptavidin (10 µl of 1 mg/ml) was added in 1 ml of PBS containing Pt nanoparticles (0.05 mg/ml) for 30 min at room temperature. Then, HRP (10 µl of 1 mg/ml) was spiked in the solution. The final mixture was incubated for 30 min more at room temperature. Second, HRP (10 µl of 1 mg/ml) was added in 1 ml of PBS containing Pt nanoparticles (0.05 mg/ml) for 30 min at room temperature. Then, streptavidin (10 µl of 1 mg/ml) was spiked in the solution. The final mixture was incubated for an additional 30 min at room temperature.

In addition, streptavidin conjugated with HRP (10 µl of 1 mg/ml) was added in 1 ml of PBS containing Pt nanoparticles (0.05 mg/ml). Then, the mixture was incubated for 1 hr at room temperature.

100 µl of each streptavidin immobilized on Pt nanoparticle prepared using three different methods was added in a biotin coated strip-well. The strip-well was incubated for 30 min at room temperature. After the incubation, the strip-well was washed using PBST washing solution. Then,

100 µl of Amplex Red was added in the strip-well and left for 2 min at room temperature to produce resorufin. After the incubation, resorufin (10 µl) was transferred to a borosilicate test tube. Then, the strength of light emitted in the test tube was measured with LB 9507 with two syringe pumps.

2.5. Preparation of TSH detection antibody-conjugated HRP immobilized on Pt nanoparticle

TSH detection antibody was conjugated with HRP using SureLink HRP conjugation kit (KPL, Inc). NH_2 of TSH detection antibody (100 µg) was bound with the activated HRP. 100 µl of TSH detection antibody conjugated with HRP (0.1 mg/ml) was added in 900 µl of PBS containing Pt nanoparticles (0.05 mg/ml). Then, the mixture was incubated for 1 hr at room temperature. TSH detection antibody-conjugated HRP immobilized on Pt nanoparticles obtained after the centrifuge (1,4000 rpm) for 10 min were stored in Tris-HCl (pH 8.5) containing 1 % BSA. The stock solution was diluted 40 times in Tris-HCl to prepare the working solution.

2.6. Quantification of TSH using capture antibodies coated on magnetic beads and TSH detection antibody-conjugated HRP immobilized on Pt nanoparticles

TSH (50 µl) in human serum mixed with TSH capture antibody coated on magnetic bead (100 µl) in a centrifuge tube was incubated for 15 min at 37 °C. After the incubation, the TSH-bound TSH capture antibody coated on magnetic bead was separated from the mixture using a centrifuge tube holder containing magnetic bar. The TSH-bound TSH capture antibody coated on magnetic bead was washed 3 times using PBST washing buffer. After the washing, TSH detection antibody-conjugated HRP immobilized on Pt nanoparticles (100 µl) was added in the centrifuge tube containing the TSH-bound TSH capture antibody coated on magnetic bead. The mixture was incubated for 20 min at 37 °C. After the incubation, the final complexes were

separated with the centrifuge holder and washed 4 times using the PBST washing solution. After the washing, Amplex Red (100 µl) for ODI CL detection was added in the centrifuge tube containing the particles. The strength of light emitted from resorufin formed after a 1-min incubation was measured with LB 9507 with two syringe pumps as described in detail in the other procedures of this research.

3. Results and discussion

3.1. Optimization of reaction between substrate and HRP

Fig. 2

One of the main variable factors to quantify trace levels of TSH with ODI CLEIA is the optimization of substrate (e.g., Amplex Red, Amplex Ultrared) and HRP.

First, we studied the efficiency of two similar substrates (e.g., Amplex Ultrared, Amplex Red, 5 µM) capable of producing resorufin in the presence of H_2O_2 (0.4 mM) and HRP (10 µU ml^{-1}). As shown in Fig. 2(a), the relative CL intensity in the presence of Amplex Ultrared was about 2-fold higher than that in the presence of Amplex Red. Thus, we selected Amplex Ultrared for the development of a highly sensitive biosensor capable of early diagnosing thyroid cancer.

Second, we investigated effects of various buffer solutions in enzyme assay with ODI CL detection. Based on the experimental results shown in Fig. 2(b), we selected sodium phosphate buffer (pH 7) for the reaction between Amplex Ultrared and HRP.

Third, Fig. 2(c) shows that 20 mM sodium phosphate (pH 7) is the best to enhance the sensitivity of ODI CLEIA.

Forth, we investigated the best concentration of Amplex Ultrared in sodium phosphate (pH 7,

20 mM) to enhance the relative CL intensity. As shown in Fig. 2(d), light emitted in the presence 4.1 μM Amplex Ultrared was brighter than those in the presence of Amplex Ultrared lower or higher than 4.1 μM. In other words, relative CL intensity in the presence of Amplex Ultrared higher than 4.1 μM was lower than that in the presence of 4.1 μM. This is because the concentration of resorufin from the reaction of excess Amplex Ultrared and H_2O_2 in the presence of HRP is too high to emit bright light due to the effect of self-quenching.

Fifth, we determined the best concentration of 4-iodophenol capable of enhancing the relative CL intensity with the rapid reaction between substrate and HRP. As shown in Fig. 2(e), relative CL intensity in the presence of 5.4 mM 4-iodophenol was higher than those in the presence of lower than 5.4 mM 4-iodophenol. Also, we conducted more experiments with higher 4-iodophenol than 5.4 mM. Due to self-quenching, the light emitted in the presence of higher 4-iodophenol than 5.4 mM was not as bright as that in the presence of 5.4 mM 4-iodophenol (data not shown).

Finally, we confirmed that relative CL intensity was enhanced with the extension of incubation time. As shown in Fig. 2(f), trace levels of HRP (10 μU ml^{-1}) can be detected after the 1-min incubation because the relative CL intensity (signal) in the presence of 10 μU ml^{-1} HRP was about 10-fold higher than the background in the absence of HRP

With the optimization of reaction between substrate and HRP as shown in Fig. 2, we developed a highly sensitive biosensor, capable of rapidly quantifying TSH, with detection antibody-conjugated HRP, Pt nanoparticles, primary antibody immobilized on magnetic beads.

3.2. Determination of nanoparticle for ODI CLEIA

Fig. 3

Fig. 3 shows the activity of HRP was dependent on the property of nanoparticles. The concentration of HRP immobilized on platinum (Pt) nanoparticles was higher than that fixed on gold (Au) nanoparticles even though the total number of Pt nanoparticles were fewer than that of Au nanoparticles. The results indicate that the electrostatic interaction between platinum nanoparticle and protein (e.g., HRP, antibody) is stronger than that between gold nanoparticle and protein. Also, the relative CL intensity in the presence of HRP immobilized on Pt nanoparticle (30 nm) was higher than that in the presence of HRP immobilized on Pt platinum nanoparticle (30 nm). The results indicate that HRP activity is dependent on the number of platinum nanoparticles. As shown in Fig 3, relative CL intensity is dependent on the HRP concentration to produce resorufin from the reaction of Amplex Ultrared and HRP immobilized on nanoparticles. With the increase of HRP concentration added to produce HRP immobilized on nanoparticles, relative CL intensity was enhanced as shown in Fig. 3.

3.3. Competition of streptavidin and HRP to bind with Pt nanoparticles

Fig. 4

Fig. 5

Fig. 4 shows that the electrostatic interaction between HRP and Pt nanoparticle was more rapid than that between streptavidin and Pt nanoparticle. The results indicate that the number of HRP immobilized on Pt nanoparticle was more than that of streptavidin on Pt nanoparticle shown in Fig. 5(a). In addition, Fig. 4 shows that light emitted in the presence of streptavidin-conjugated HRP immobilized on Pt nanoparticle was brighter than those in the other two conditions. This is because most of the HRP conjugated with streptavidin was rapidly

immobilized on Pt nanoparticle as shown in Fig. 5(b).

3.4. Quantification of TSH in human serum

In order to develop highly sensitive and rapid ODI CLEIA based on the results of Figs. 2 - 4, NH_2 of TSH antibody was conjugated with activated HRP using SureLink HRP conjugation kit. Thus, TSH antibody conjugated with HRP cannot be directly immobilized on Pt nanoparticle because TSH antibody doesn't have free NH_2 capable of binding with Pt nanoparticle. In other words, only NH_2 of HRP conjugated with TSH antibody can directly bind with Pt nanoparticle based on the electrostatic interaction between NH_2 of HRP and Pt nanoparticle. Thus, it is possible that TSH antibody-conjugated HRP immobilized on Pt nanoparticle will be able to rapidly bind with TSH. Also, it is possible that the sensitivity of the biosensor operated with ODI CLEIA will be enhanced.

Fig. 6

Using the TSH capture antibody coated on magnetic bead and TSH detection antibody-conjugated HRP immobilized on Pt nanoparticle, a highly sensitive biosensor capable of early diagnosing TSH was developed based on the reaction scheme as shown in Fig. 6. The time necessary for quantifying TSH using the new biosensor was about 3 times more rapid than that using the commercial TSH enzyme immunoassay kit purchased from Immunometrics, Ltd. This is because the biosensor operated with TSH detection antibody-conjugated HRP immobilized on Pt nanoparticle and ODI CL detection is at least 100-fold more sensitive than that operated with TSH detection conjugated with ALP and colorimetric detection.

Fig. 7

Fig. 8

Fig. 7 indicates that the new biosensor operated with ODI CLEIA shown in Fig. 6 is more rapid and more sensitive than the conventional ODI CLEIA shown in Fig. 8. The limit of detection (LOD = background + 3σ) determined using the biosensor was as low as 0.004 mU L^{-1}. σ is the standard deviation of background measured in the absence of TSH. LOD of the rapid biosensor was about 4-fold lower than that (0.017 mU L^{-1}) of conventional ODI CLEIA. The limit of quantification (LOQ = background + 10σ) of new biosensor was as low as 0.013 mU L^{-1}. The wide dynamic range (0.013 ~ 12 mU L^{-1}) of linear calibration curve shown in Fig. 7 indicates that the biosensor operated with ODI CLEIA can rapidly quantify TSH within the lower and higher concentration of TSH than the cut-off value (3 mU L^{-1} in human serum) for the early diagnosis of thyroid cancer.

Table 1

Table 2

Table 1 shows that the sensitivity of the biosensor is better than those of other biosensors recently reported by other research groups [4-6]. The recovery of the biosensor with excellent accuracy and precision, as shown in Table 2, was good within the acceptable statistical error range. Thus, we confirmed that the newly developed biosensor with ODI CLEIA can accurately, precisely, and rapidly quantify TSH in human serum for the early diagnosis of thyroid cancer.

4. Conclusions

With the understanding of electrostatic interaction between protein (e.g., antibody, HRP) and nanoparticle (e.g., Au, Pt), a highly sensitive biosensor based on ODI CLEIA was developed for the early diagnosis of thyroid cancer. The time necessary for analyzing trace levels of TSH in human serum using the biosensor was at least 3 times more rapid than that using the commercially available enzyme immunoassay. Also, the LOD of the former was about 100-fold lower than that of the latter. Thus, the new biosensor with excellent accuracy, precision, and reproducibility can be a new method capable of early diagnosis of thyroid cancer. In other words, we expect that a portable biosensor based on the concept of point of care testing can be devised for the early diagnosis of thyroid cancer. Also, the new technology used in developing the biosensor can be applied to fabricate highly sensitive biosensors for rapidly quantification of various cancer biomarkers as well as a number of biomarkers used for the early diagnoses of human diseases.

In general, Au and Pt nanoparticles are widely applied in developing lateral flow assay kits designed based on the concept of immunoassay. Using the method developed with Pt nanoparticles in this research, we expect that a new lateral flow assay operated with ODI CL detection can be fabricated for public health.

In conclusion, we expect that the new technology used in the highly sensitive biosensor will be applied in various research and industrial fields to rapidly quantify biomarkers for the early diagnoses of human diseases and monitor toxic materials in food, drink, and natural environment.

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Scheme 1. ODI enzyme (HRP) assay. **1**: TCPO, **2**: 4MImH, **3**: ODI, **4**: resorufin under the ground state, **5**: resorufin under the excited state, **X**: high-energy intermediate.

Fig. 1. ODI CL reaction in the presence of HRP immobilized on Au or Pt nanoparticles.

Fig. 2. (a) comparison between Amplex Red and Amplex Ultrared, (b) Selection of buffer solution, (c) Effect of sodium phosphate (pH 7), (d) Effect of Amplex Ultrared concentration, (e) Effect of 4-iodophenol concentration, (f) Effect of incubation time.

Fig. 3. HRP activity immobilized on nanoparticles. The error range of each value is 3 ~ 6 %.

Fig. 4. Competition of streptavidin and HRP to bin with Pt nanoparticles.

Fig. 5. (a) Immobilization of streptavidin and HRP on Pt nanoparticle, (b) Immobilization of streptavidin conjugated with HRP on Pt nanoparticle.

Fig. 6. Biosensor operated with ODI CLEIA using TSH detection antibody-conjugated HRP immobilized on Pt nanoparticle.

Fig. 7. Linear calibration curves using the new biosensor operated with ODI CLEIA (blue dotted line) and conventional ODI CLEIA (red dotted line) for the quantification of TSH in human serum.

Fig. 8. Conventional ODI CLEIA for the early diagnosis of thyroid cancer.

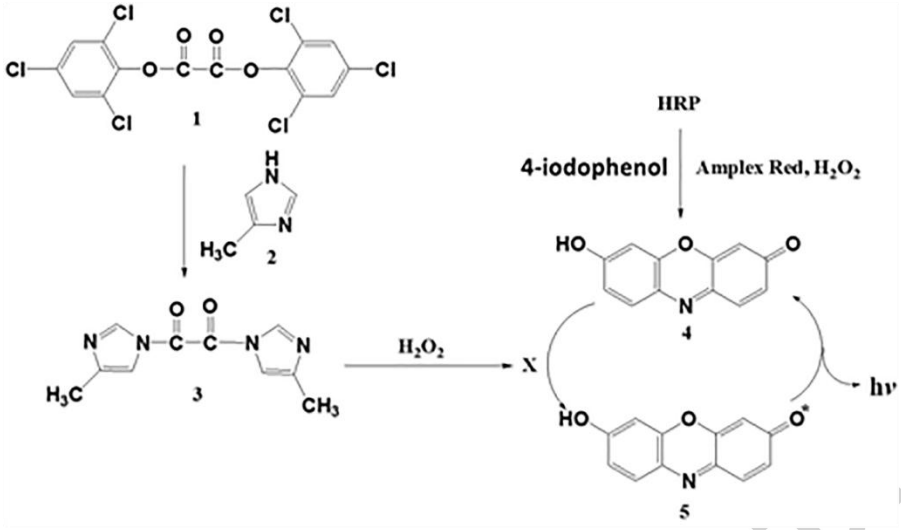
Table 1 Comparison between the new biosensor and other methods

Method	Dynamic range (mU L^{-1})	LOD (mU L^{-1})	Ref.
Microchip operated with enzyme immunoassay using electrochemical detection	1 - 8	2.4	[4]
Nanogold-functionalized magnetic beads with redox activity for sensitive electrochemical immunoassay	0.01 - 20	0.005	[5]
An electrochemiluminescence immunosensor based on polyamidoamine-norfloxacin functionalized Pd-Au core-shell hexoctahedrons as signal enhancers	0.05 - 20	0.02	[6]
Biosensor with ODI CLEIA	0.013 - 12	0.004	this research

Table 2 Recovery test (N = 5) of biosensor operated with ODI CLIEA.

1^{st} sample [a]	2^{nd} sample [a]	Calculated [a,b]	Measured [a]	Recovery [c]
0.5	1.0	0.75	0.69 ± 0.03	92.0
1.2	3.2	2.2	2.39 ± 0.12	108.6
2.4	3.2	2.8	2.59 ± 0.16	92.5
6.0	9.0	7.5	7.9 ± 0.29	105.3

[a] mU L^{-1},
[b] Calculated = (1^{st} sample + 2^{nd} sample)/2, [c] %



Scheme 1

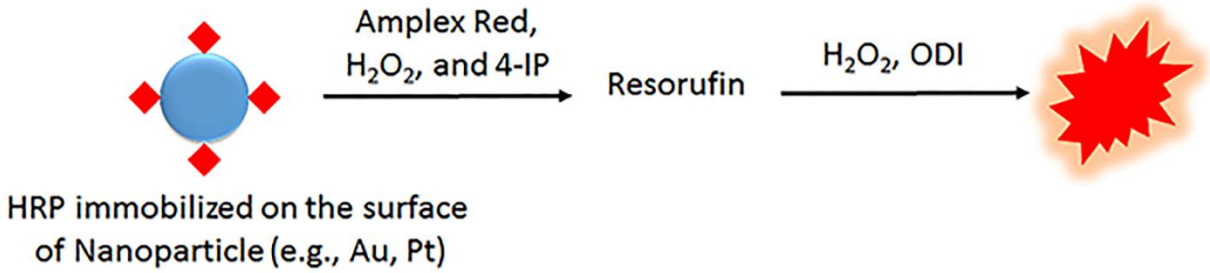


Fig. 1

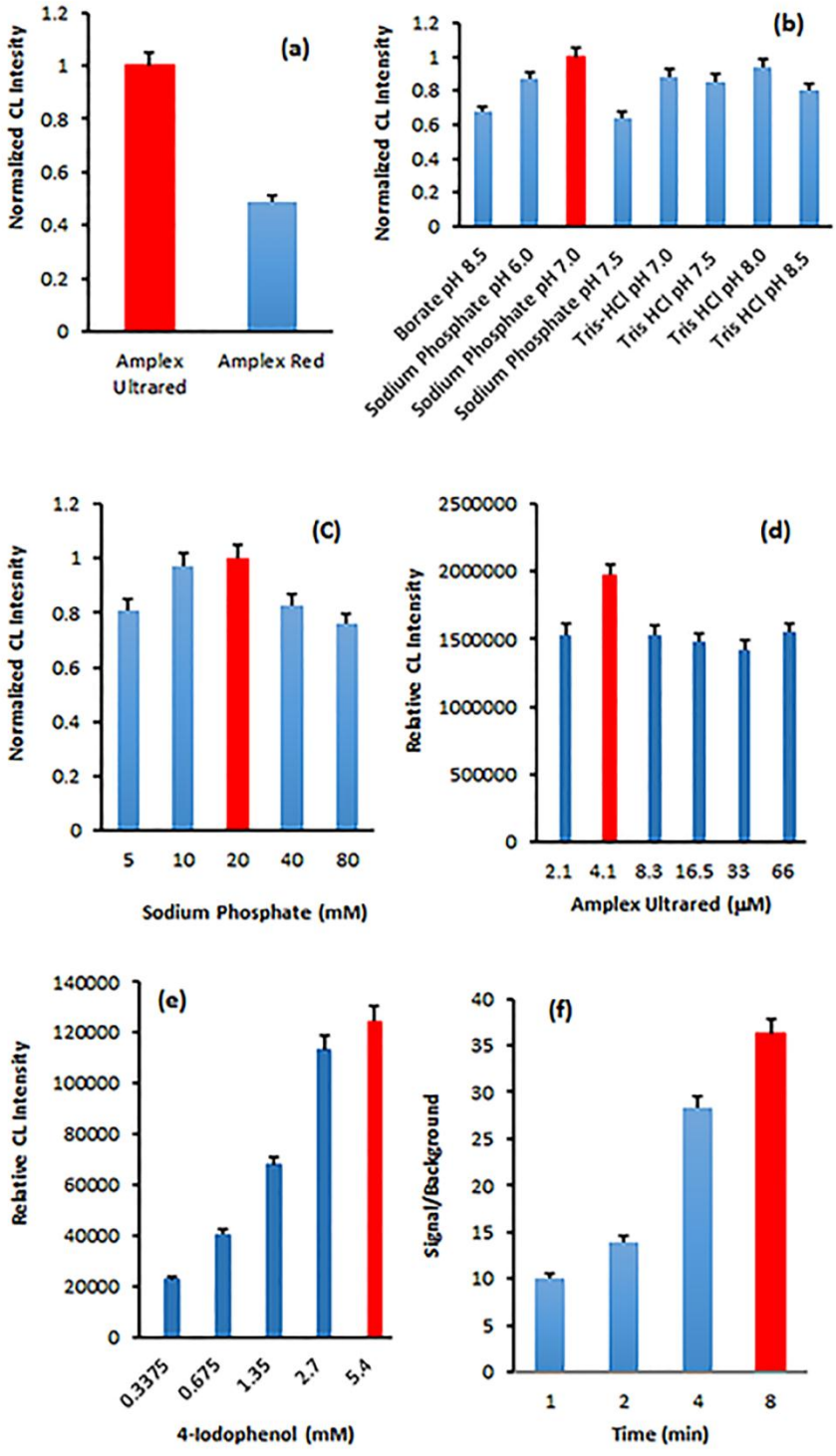


Fig. 2

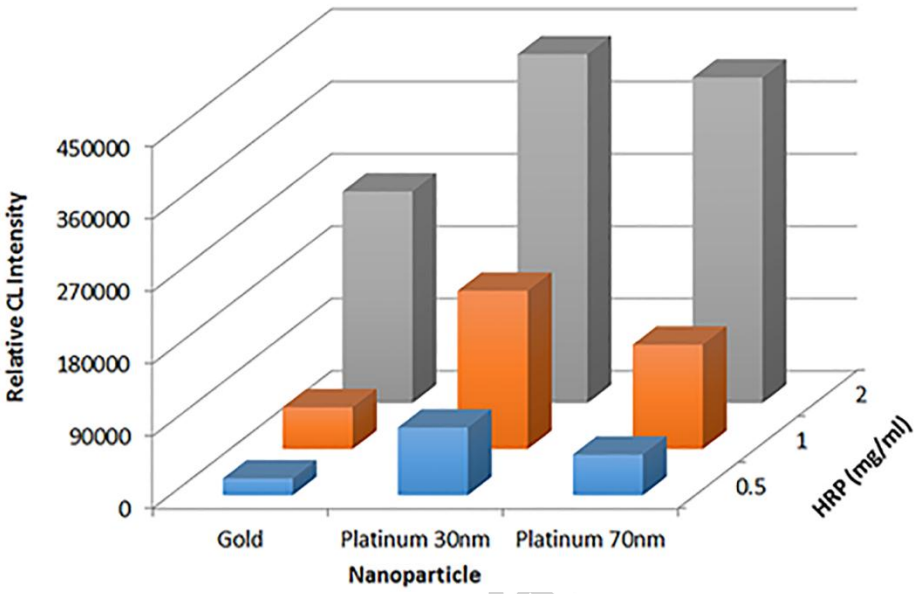


Fig. 3

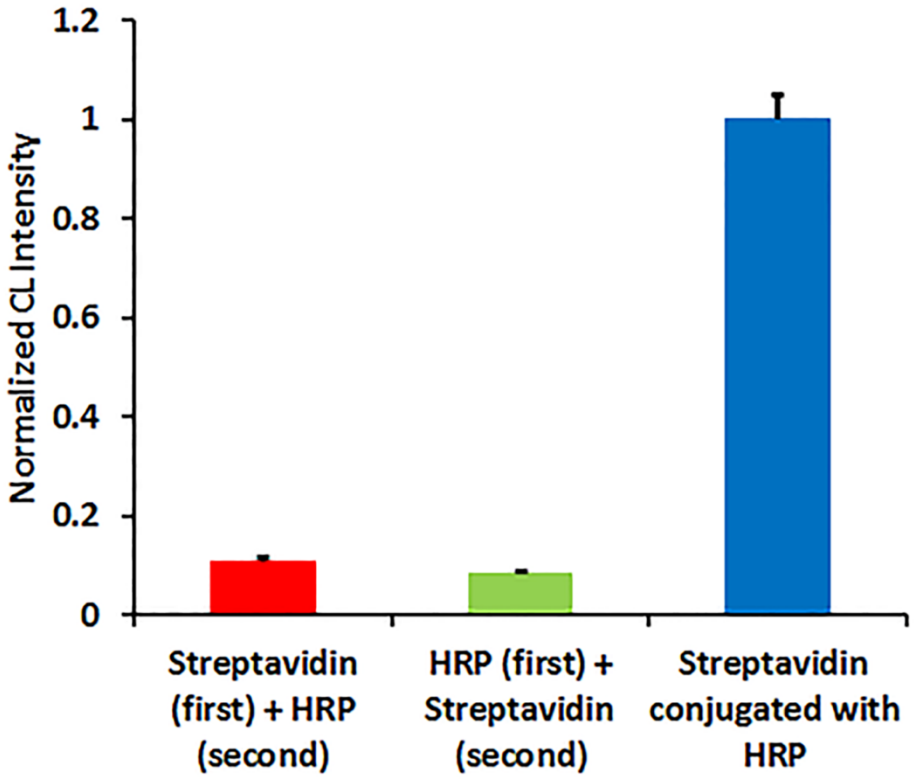


Fig. 4

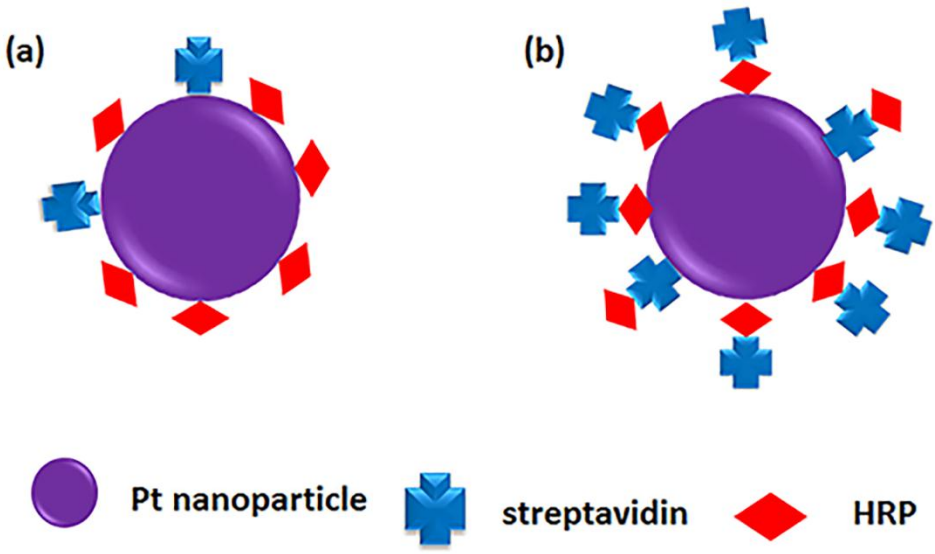


Fig. 5

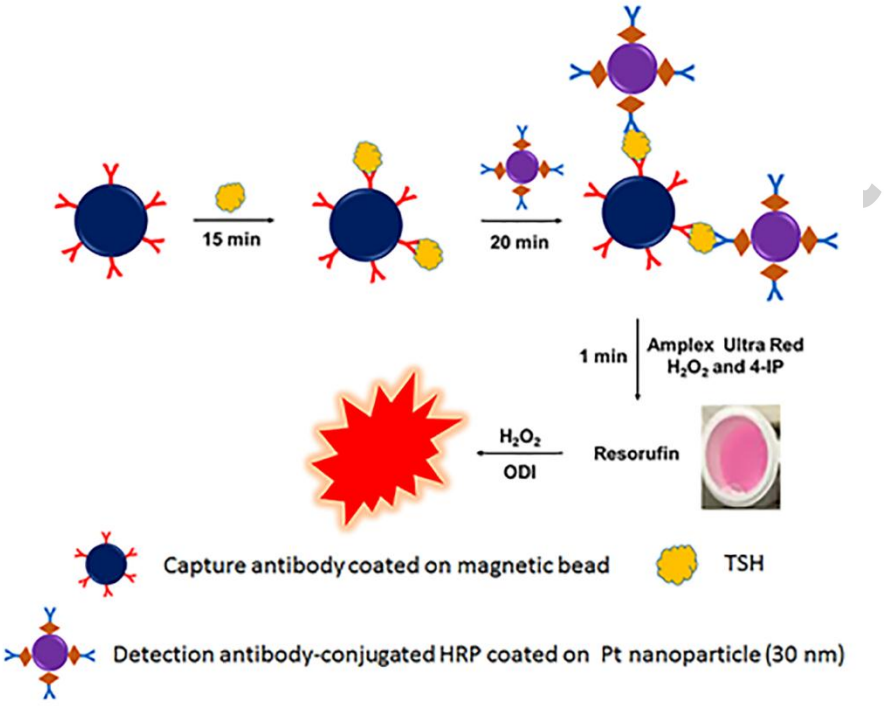


Fig. 6

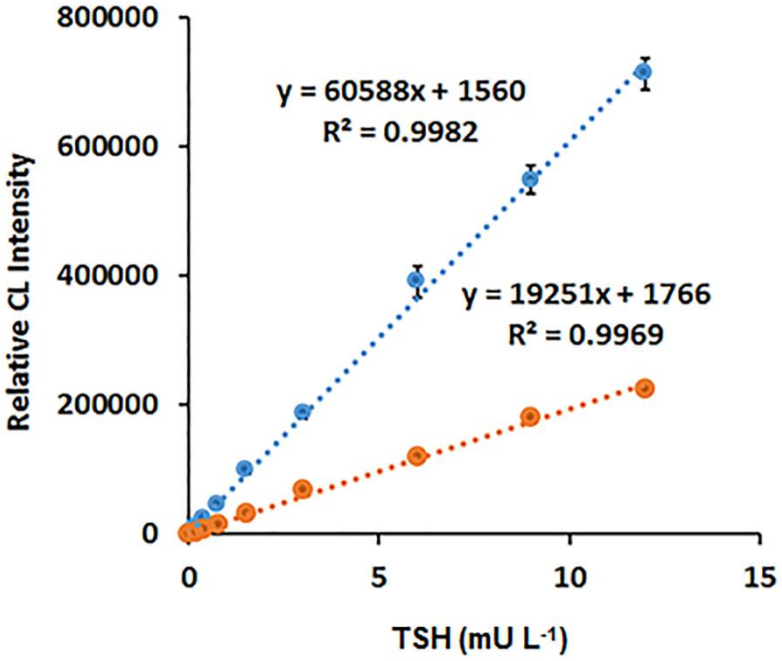


Fig. 7

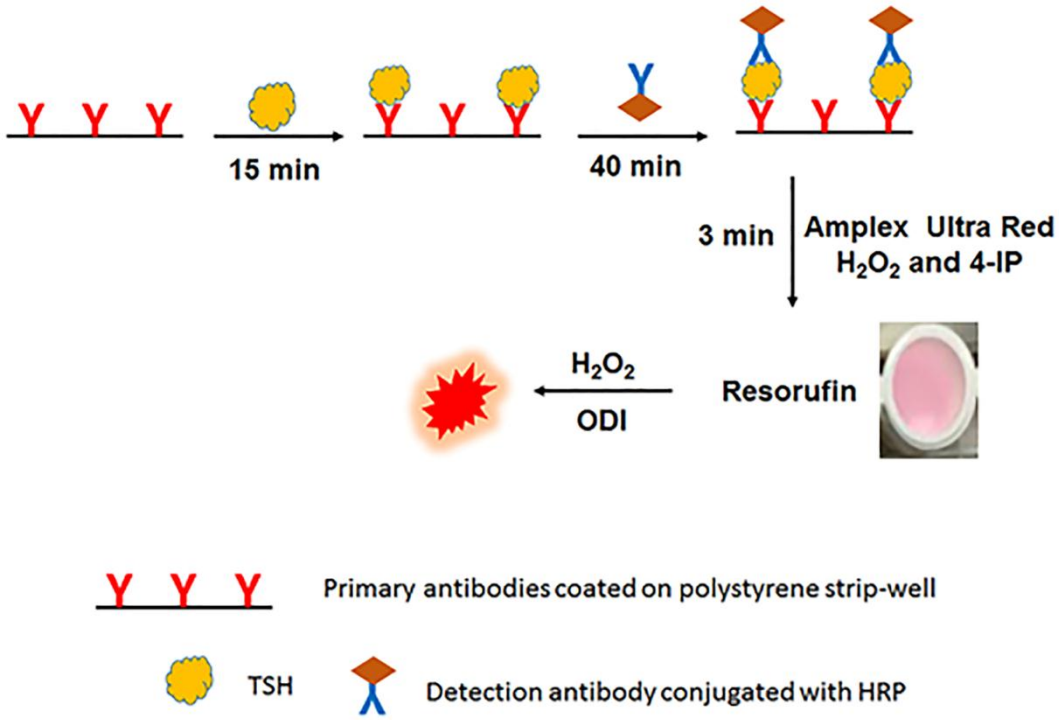


Fig. 8

Highlights

- Understanding of physical interaction between biomolecule and Pt nanoparticle
- Development of chemiluminescent biosensor for the early diagnosis of cancer
- Cost-effective and rapid biosensor with excellent accuracy and precision