Quinone-based antibody labeling reagent for enzyme-free chemiluminescent immunoassays. Application to avidin and biotinylated anti-rabbit IgG labeling.

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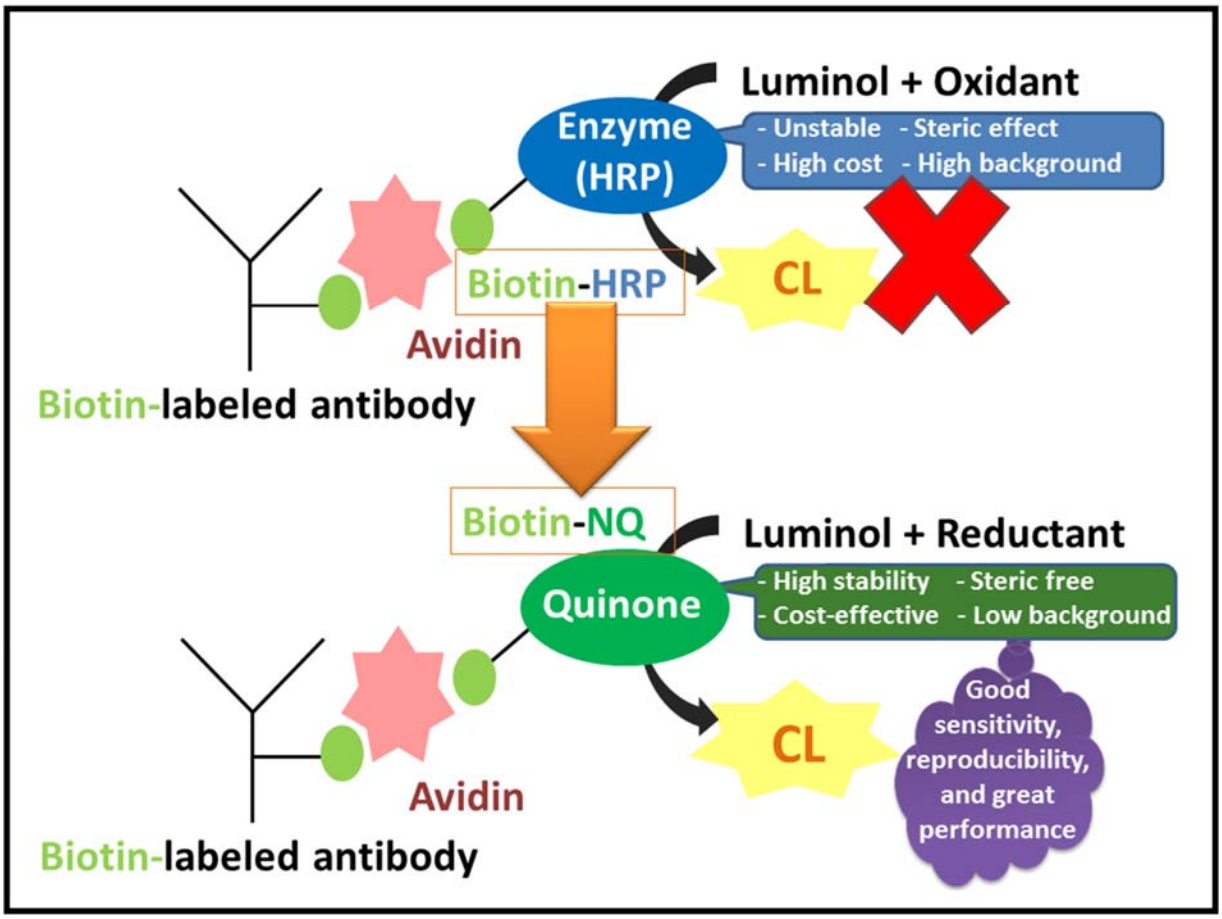
Graphical abstract



Abstract:

Chemiluminescence-enzyme immunoassays make it possible to measure trace components with high sensitivity and selectivity due to the high specificity of the antigen-antibody reaction and high sensitivity of chemiluminescence assays. However, using an enzyme-labeled antibody suffers from many problems such as low reproducibility due to the instability of the enzyme and inhibition of antigen-antibody reaction due to its steric effect. Therefore, herein we report an innovative non-enzymatic chemiluminescence immunoassays labeling reagent through using quinone as a signal-generating tag coupled with biotin as a binder, to overcome enzymatic labeling problems. Biotinylated-1,4-naphthoquinone (biotin-NQ) was synthesized and characterized and it could produce long-lasting chemiluminescence upon mixing with dithiothreitol and luminol based on the redox cycle of quinone. Biotin-NQ showed exceptional stability towards different stress factors that may be encountered during performing the immunoassay such as high temperatures, highly acidic and alkaline conditions, and repeated freeze-thaw cycles. On the other hand, all these conditions lead to decreased labeling enzyme reactivity due to possible denaturation of its protein structure. Finally, the measurement of the biotin-labeled antibody was successfully performed using biotin-NQ and avidin. As a result, the antibody could be detected down to 25.7 nM which is 2.5 times sensitive than biotin-HRP chemiluminescence-enzyme immunoassays. Moreover, our method was applied successfully for determination of avidin using immobilized biotinylated antibody and biotin-NQ, which simulates immunoassays. Avidin could be detected down to 23.4 nM with excellent linearity (r=0.996). Accordingly, our developed reagent, biotin-NQ, could be used as a universal highly stable, cost-effective, and steric free non-enzymatic label for immunoassays.

Keywords: non-enzymatic sensor; biotin; quinone; chemiluminescence; immunoassay, stability

1. Introduction:

Immunoassays make it possible to measure trace components with high sensitivity and selectivity due to the high specificity of the antigen-antibody reaction (Gan and Patel, 2013; Marquette and Blum, 2009). Hence, it is used in a wide range of areas from basic to clinical research including, biological (Shim et al., 2017; Zong et al., 2012), environmental (Ahn et al., 2011), pharmaceutical (Han et al., 2013), and toxicological assays (Moody, 2006). Immunoassays can be categorized into several types depending on the way of analyte detection and differences in antibodies or antigens labeling (Marquette and Blum, 2009). One of the most used immunoassays is the enzyme immunoassay that uses an enzyme as a labeling substance for antibodies for the signal generation (color development, fluorescence or chemiluminescence (CL)). The targeted antigen can be quantified by measuring the enzyme activity of the antigen-antibody immune-conjugate (Gan and Patel, 2013). This method is widely used at present as it is superior in sensitivity, safety, and applicability as compared with radioimmunoassay that uses radioactive isotope label (Banga-Mboko et al., 2003; Wang et al., 2007). Among enzyme immunoassay, a method utilizing an enzyme to catalyze CL reaction is called CL-enzyme immunoassays (Marquette and Blum, 2009; Nishizono et al., 1991). CL detection has many advantages such as excellent sensitivity and rapidity, wide dynamic range. Besides, CL can be measured with a relatively simple and inexpensive device because it does not require an excitation light source. Thus, CL-enzyme immunoassays are the widest used type of immunoassays (Bi et al., 2009; Chen et al., 2012; Zhao et al., 2009). In CL-enzyme immunoassays, different enzymes are often used for signal generation such as horseradish peroxidase (HRP) and alkaline phosphatase. HRP generates the CL signal through catalyzing the luminol/hydrogen peroxide CL reaction (Katsuragi et al., 2000), while alkaline phosphatase catalyzes the CL reactions of dioxetane phenyl phosphate

derivative, such as Lumigen APS-5 (Xie et al., 2009). However, using an enzyme-labeled antibody suffer from many problems such as low reproducibility due to the instability of the enzyme and inhibition of antigen-antibody reaction due to steric hindrance caused by the molecular size of the enzyme. Additionally, high background CL is caused by the direct reaction of luminol and oxidizing agents such as hydrogen peroxide (He et al., 2013; Lu et al., 2001; Mercadal et al., 2018; Shibata et al., 2019).

Recently non-enzymatic detection methods were developed to overcome these problems and to further expand the usefulness of the immunoassay (Halawa et al., 2018; Mercadal et al., 2018; Nsabimana et al., 2019; Qin et al., 2018; Shim et al., 2017; Shu et al., 2019). The previously reported non-enzymatic methods either depended on using fluorescence microspheres (Shim et al., 2017), gold nanoparticles (Halawa et al., 2018) that could be combined with functionalized TiO2 nanoluminophores (Shu et al., 2019), biotinylated silver nanoparticles (Mercadal et al., 2018), functionalized metal−organic framework combined with ruthenium-based electrochemiluminescence (Qin et al., 2018), or electrochemical sensors based on nanomaterials (Nsabimana et al., 2019). However, these methods also suffered from some drawbacks such as inhibition of the signal by avidin or the non-selective adsorption of the used labels, fluorescence microspheres, on the well (Shim et al., 2017), or its low reproducibility, as it depends on intensity depletion mechanism, and high vulnerability to any change in the surrounding environment of the assay (Halawa et al., 2018; Mercadal et al., 2018; Nsabimana et al., 2019; Qin et al., 2018; Shu et al., 2019), or the need of highly experienced analyst (Nsabimana et al., 2019; Qin et al., 2018). Thus, non-enzymatic labeling for immunoassay with improved stability, small molecular weight, facile procedure, and lower cost has become urgently required.

Our research group is focusing on studying the interesting CL properties of the chemically stable and small molecular weight compounds, quinones. We found that adding a reductant such as dithiothreitol (DTT) with luminol to a compound having a quinone structure produces a long-lasting intense emission. This CL is produced from a series of oxidation/reduction cycles of quinones with reductant and luminol (Fig. S1) and it could be used for the quantitative determination of quinones sensitively and selectively (Fukuda et al., 2017; Kishikawa et al., 2009). In this research, we aim at using quinones as a signal-generating tag for the development of a new detection method for immunoassays to overcome the problems of enzymatic assays and existing non-enzymatic ones. Quinones are chemically stable compared to enzymes, and it is expected to have good reproducibility even under comparatively severe conditions or after long-term preservation. Besides, quinones, which are small molecules, will have a little influence on antigen-antibody reaction, and reduction of nonspecific adsorption to antibody, microplate, etc. can be expected. Furthermore, since the addition of an oxidizing agent is not needed for the CL reaction of quinones, high sensitivity is attained by reduction of the background emission.

As well known, avidin has extremely high affinity and specificity for biotin (Mercadal et al., 2018; Schmidt and Healy, 2013). Taking into account the wide range of applications and ease of preparation, the avidin-biotin system is used as a quinone labeling method for antibodies. The commercially available quinone compound with a free carboxylic group, 2-(9-carboxynonyl)-1,4-naphthoquinone, is coupled with biotin hydrazide to form biotin-1,4-naphthoquinone conjugate (biotin-NQ). Labeling of the detection antibody will be conducted through binding the synthesized biotin-NQ and biotin-labeled detection antibody via avidin (Fig. 1). The biotin-NQ bonded to the antibody could be detected quickly by the addition of a reductant and luminol which produce CL stably lasts for a long time as discussed before. Since the labeling method using the avidin-biotin

system is widely used in commercially available immunoassay kits and currently there are many available biotin-labeled antibodies for various antigens (Mercadal et al., 2018; Schmidt and Healy, 2013), we can easily prepare labeled antibodies against various antigens merely by changing the type of biotin-labeled antibody. Thus, we synthesized the novel compound biotin-NQ in which a biotin moiety is introduced to the 1,4-naphthoquinone structure as a CL labeling reagent for immunoassay, and its CL property investigation and stability evaluation were carried out and compared with the biotin-conjugated enzyme, biotin-HRP. Then, we applied biotin-NQ for the measurement of biotinylated antibody based on the avidin-biotin system as a fundamental study for the development of immunoassay using biotin-NQ and examined the practicality of this assay method.

2. Experimental

The used reagents and chemicals, instrumentation and materials, and procedures for CL measurement of biotin-NQ with a luminometer and CL measurement of biotin-HRP were described in the Supplementary Material (Sections S1-S4).

2.1 Synthesis of biotin-NQ

Biotin-NQ was synthesized through the amide formation reaction of a carboxylic derivative of 1,4-naphthoquinone with biotin hydrazide (Fig. S2) using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride as a catalyst for amide bond formation (Montalbetti and Falque, 2005). 2-(9-Carboxynonyl)-1,4-naphthoquinone (15 mg, 46 μmol) was dissolved in 0.6 mL DMF, then, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (13 mg, 47 μmol) was added and the mixture was stirred at room temperature. Subsequently, 1.5 mL of a DMF solution

of biotin hydrazide (9.8 mg, 38 μmol) was added dropwise and the mixture was stirred at 50 °C for 17 hours. Thereafter, the precipitated crystals were suction filtered to obtain 15 mg (70 % yield) of pale yellow crystals of biotin-NQ.

2.2. CL measurement of biotin-NQ with a microplate reader

Aliquots of 50 μL of biotin-NQ and 50 μL of a NaOH (30 mM) solution of luminol (50 μM) were sequentially added to wells of a 96-well microplate and set in the microplate reader. The CL generated after automatic injection of a 50 μL of a DTT (700 μM) acetonitrile solution was measured for 3 minutes. The value obtained by integrating the produced CL for 3 minutes after the addition of DTT was taken as the CL intensity.

2.3. CL measurement of avidin using biotin-NQ assisted with ultrafiltration

Aliquots of 750 μL of avidin (0-25 μM) were transferred to different Eppendorf tubes followed by addition of 250 μL of 4 times excess NQ-biotin. Then, avidin was incubated with biotin-NQ for 1 hr at 37 °C. Afterward, biotin-NQ tagged avidin was separated from free biotin-NQ and purified through centrifugal ultrafiltration (cut off, 10 K). In brief, 0.5 mL of the incubated reaction mixture was transferred to Amicon Ultra (0.5 mL, 10 K) followed by centrifugation at 9,000 rpm for 15 minutes to purify and concentrate the high molecular weight avidin. Then, the inner tube that contains the avidin concentrate was reversed inside the outer tube and centrifuged for 2 min at 1,000 rpm, for recovering the concentrated solution containing the biotin-NQ tagged avidin. Then avidin-biotin-NQ was determined with the CL microplate method mentioned previously through adding luminol and DTT.

2.4. CL measurement of microplate immobilized avidin using biotin-NQ

Immobilization of avidin into the microplate wells was carried out through transferring 100 µL of avidin (0 - 0.8 µM) dissolved in carbonate-bicarbonate buffer (0.05 M, pH 9.6) to the wells and incubated overnight at 4°C. Then, washing was carried out with 300 µL of PBS-T buffer three times through repetitive adding of the buffer and removing it after 1-2 minutes. Next, 300 µL of 1% bovine serum albumin (BSA) was added and incubated at room temperature for 2 hours for blocking of the free sites at the wells. Afterward, another three times washing with PBS-T was performed followed by the addition of 100 µL of 12.8 µM biotin-NQ solution and the microplate was then incubated at 37 °C for 2 hours to label the **immobilized** avidin. Finally, after washing three times with PBS-T, the microplate was set in the microplate reader and luminol and DTT were added to each well, then the generated CL was measured for 3 minutes as mentioned previously.

2.5. CL measurement of microplate immobilized biotin-labeled antibody using biotin-NQ

Aliquots of 100 µL of the biotin-labeled antibody (0 - 0.8 µM) dissolved in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was transferred to the wells of the microplate and incubated overnight at 4°C for immobilization of the antibody on the wells. Then, washing was carried out with 300 µL of PBS-T buffer three times. After the washing step, 300 µL of 1% BSA was added and incubated at room temperature for 2 hours. After washing three times with PBS-T, 100 µL of avidin (0.8 µM) aqueous solution was added and incubated at room temperature for 1 hour to form a biotin-labeled antibody-avidin complex. After washing three times with PBS-T, 100 µL of 12.8 µM biotin-NQ solution (16 times excess) was added and incubated at 37 °C for 2 hours to label the biotinylated antibody-avidin complex. Finally, after washing three times with PBS-T, the microplate was set in the microplate reader and luminol and DTT were added to each well, then the generated CL was measured for 3 minutes as mentioned previously.

2.6. CL measurement of avidin using microplate immobilized biotin-labeled antibody and biotin-NQ

Aliquots of 100 μL of 0.8 μM biotin-labeled antibody dissolved in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was transferred to the wells of the microplate and incubated overnight at 4°C for immobilization of the antibody on the wells. Then, washing was carried out with 300 μL of PBS-T buffer three times. After removal of the washing solution, 300 μL of 1% BSA was added and incubated at room temperature for 2 hours. After washing three times with PBS-T, 100 μL of avidin (0.0- 0.8 μM) aqueous solution was added and incubated at room temperature for 1 hour. After washing three times with PBS-T, 100 μL of 12.8 μM biotin-NQ solution (16 times excess) was added and incubated at 37 °C for 2 hours. Finally, after washing three times with PBS-T, the microplate was set in the microplate reader and luminol and DTT were added to each well, then the generated CL was measured for 3 minutes as mentioned previously.

3. Results and Discussions

3.1. Structure confirmation of the synthesized biotin-NQ and CL properties characterization

2-(9-Carboxynonyl)-1,4-naphthoquinone and biotin hydrazide were condensed together to form biotin-NQ (Fig. S2). Fast atom bombardment mass spectrometry analysis was performed for the obtained crystals and as shown in Fig. 2a, the molecular ion peak is at 569.4 which corresponds to $[M+H]^+$ of biotin-NQ. Electrospray ionization mass spectrometry was also performed giving a clear molecular ion peak at 591.2 corresponding to $[M+Na]^+$ of biotin-NQ (Fig. S3). Besides, when biotin-NQ was reacted with 4-dimethylaminocinnamaldehyde for confirmation of the biotin

moiety presence, it exhibited red color indicating that biotin-NQ had a biotin structure (Livaniou et al., 2000; McCormick and Roth, 1970; Szychowski et al., 2010). Furthermore, the UV spectrum of biotin-NQ and the substrate, 2-(9-carboxynonyl)-1,4-naphthoquinone were measured. 1,4-Naphthoquinone moiety was reported to have a characteristic absorbance maximum at about 330 nm (Cenas et al., 2004; Elgawish et al., 2015a; Rodrigues et al., 2006). As shown in Fig. S4, the absorption maxima of both biotin-NQ and 2-(9-carboxynonyl)-1,4-naphthoquinone are observed around 330 nm demonstrating the presence of NQ moiety in the product. From these results, we confirmed that the intended biotin-NQ containing intact biotin and quinone moieties was obtained.

After confirming the structure of biotin-NQ, its CL properties were evaluated and compared with the substrate 2-(9-carboxynonyl)-1,4-naphthoquinone using the redox cycle reaction of quinone with DTT and luminol (Fig. S1). Procedure for biotin-NQ luminometer based CL measurement was described in the Supplementary Material (Section S3). As shown in Fig. 2b, upon the reaction of biotin-NQ (10 μM) with luminol and DTT, intense (signal to blank (S/B) ratio of 24) and long-lasting CL (persist for at least 20 min) was produced which is similar to that obtained with 2-(9-carboxynonyl)-1,4-naphthoquinone. Regarding the used solvents in the assay, we tested water instead of acetonitrile as a solvent for DTT as water is more compatible with immunoassays and the results are shown in Fig. S5. However, using acetonitrile as a solvent for DTT provided a long-lasting intense glow-type CL comparing to water, which showed a semi flash type CL (Fig. S5a). The glow-type CL shows a nearly constant emission that not fade rapidly with time, which greatly increases analytical sensitivity and accuracy. On the other hand, semi flash-type emission, that possesses a fast kinetic curve, would lead to poor analytical accuracy (Liu et al., 2017; Roda et al., 2003). Besides, as shown in Fig. S5b, the 3 min integrated CL of both

solvents is the same, while the 10 integrated CL is much more in case of using acetonitrile, another proof for its appropriateness. Thus, we preferred to use acetonitrile as a solvent for DTT.

3.2. Microplate based CL assay of biotin-NQ and its comparison with biotin-HRP

Considering the simultaneous analysis of a large number of samples and integration of the designed reagent with immunoassay, a microplate reader-based CL measurement method for biotin-NQ was developed and optimized. As mentioned in the experimental part, biotin-NQ was transferred to the wells of the microplate followed by the addition of an alkaline solution of luminol and consecutive injection of DTT. The concentrations of the used reagents were carefully studied and optimized. Luminol concentration was studied in the range of 30-90 μM and NaOH concentration of luminol solution was studied in the range of 10-50 mM, while DTT concentration was studied in the range of 0.2-1.0 mM. As shown in Fig. S6, the optimum concentrations of luminol, NaOH, and DTT giving the highest relative S/B ratio were found to be 50 μM, 30 mM, and 0.7 mM, respectively. Using the optimum conditions, a calibration curve of biotin-NQ was constructed and a linear relationship with a correlation coefficient of 0.991 was obtained between biotin-NQ in the concentration range of 0.0-2.0 μM according to the following regression equation:

$$Y = 5.44 \times 10^5 X + 6.8 \times 10^4$$

Where, Y is the integrated CL intensity after 3 min and X is the corresponding biotin-NQ μM concentration.

The detection limit is defined as $3\sigma/K$, where σ is the standard deviation of blank CL and K is the slope of the calibration regression equation (Liu et al., 2016; Yang et al., 2020). The detection limit for biotin-NQ was found to be 50 nM.

3.3. Comparison of the stability and merits of biotin-NQ vs. biotin-HRP

To clarify the usefulness of biotin-NQ, its stability against various stress conditions affecting immunoassay was investigated and compared with the stability of biotin-HRP. The investigated stress conditions included heating, acidic and alkaline pH, repeated freeze and thaw, and effect of preservatives, such as sodium azide, and proteolytic enzymes, such as pepsin. After subjecting them to different stress conditions, biotin-NQ was quantified as mentioned previously in the experimental section where, biotin-HRP was measured according to the literature method (Katsuragi et al., 2000) as described briefly in the Supplementary Material (Section S4).

At first, the stability of biotin-NQ and biotin-HRP against temperature change was evaluated. After heating each biotin derivative at 50 °C and 100 °C for 15 minutes, CL was measured. As shown in Fig. 3a, the biotin-HRP activity was decreased to about 80% at 50 °C and about 10% at 100 °C, whereas the biotin-NQ reactivity was not affected at all under the same conditions. From these results, it can be considered that CL measurement can be performed for biotin-NQ after its transportation or storage under extremely high temperatures without lowering its activity. In the same context, the stability of biotin-NQ and biotin-HRP against pH change were evaluated. Each biotin derivative was prepared at pH 4.0, 8.0 and 12.0 buffer and stored at 4 °C. After 48 hours, biotin-NQ was diluted by 10 times with 75% acetonitrile and biotin-HRP was diluted with pH 8 buffer, then, their reactivity was measured. As a result, the biotin-HRP reactivity was decreased to about 70% at pH 4 and about 85% at pH 12 (Fig. 3b). On the other hand, the biotin-NQ response was not affected at all under the studied pH range (Fig. 3b). Thus, biotin-NQ could be used without any problem even after its storage under extreme acidic and alkaline conditions. Afterward, the effect of the freeze-thaw cycles on the reactivity of biotin-NQ and biotin-HRP was investigated. For each biotin derivative, CL was measured after repeated freeze

at -80 °C and thawing at room temperature three times. From the results shown in (Fig. 3c), it can be seen that biotin-HRP reactivity decreased down to about 80% by three freeze-thaw operations, whereas the biotin-NQ showed no significant change. Owing to this fact, it is written in all HRP using CL-enzyme immunoassays KITs that repeated freeze and thaw of the reagents should be avoided. Therefore, the sample labeled with biotin-HRP could suffer from low reproducibility of the measurement due to the repetition of freeze and thaw. On the other hand, biotin-NQ showed excellent durability under these conditions giving it excellent merits when compared to biotin-HRP. The poor stability of biotin-HRP upon heating, exposure to highly acidic and highly alkaline conditions, and/or repeated freeze-thaw procedures could be attributed to denaturation of its protein conformation under these conditions (Lee et al., 2015; Machado and Saraiva, 2005; Malomo et al., 2011).

After demonstrating the excellent stability of biotin-NQ against different stress conditions, we evaluated its response towards some commonly used chemicals in the immunoassays, such as preservatives, and enzymes that might be present in the sample matrix. Sodium azide is commonly used as a preservative for antibody products. Therefore, the influence of the addition of sodium azide on the CL of biotin-NQ and biotin-HRP was investigated. 1 M sodium azide aqueous solution or water as control were added to each biotin derivative and CL was measured after 24 hours. As a result, as shown in Fig. 3d, for biotin-HRP, the CL intensity decreased to about 60%, whereas biotin-NQ showed no significant change in the CL intensity after the addition of sodium azide. The reason behind that could be attributed to the inhibitory effect of azidyl radical on the prosthetic heme group of HRP (Ortiz de Montellano et al., 1988; Van Deurzen et al., 1997). Thus, when applying biotin-HRP to CL-enzyme immunoassays, pretreatment for antibody products such as dialysis may be necessary to remove sodium azide, whereas biotin-NQ could be used directly

without special pretreatment for the used antibodies. Furthermore, some analyzed samples may contain proteolytic enzymes, such as gastric juice and intestinal fluids samples. Therefore, the stability of biotin-HRP and biotin-NQ were evaluated towards pepsin, a proteolytic enzyme present in gastric juice. A solution of 0.4% pepsin, or water as a control, were added to each biotin derivative. After 1 hour, biotin-NQ was diluted 10 times with 75% acetonitrile and biotin-HRP was diluted 10 times with pH 8.0 buffer, then, their CL performances were measured. As a result, as shown in Fig. 3e, the biotin-HRP reactivity was decreased to about 20% by the addition of pepsin. This could be attributed to the hydrolytic activity of pepsin on the peptide bonds of the protein structure of HRP (Thomas et al., 2004). On the other hand, the biotin-NQ showed no change in CL intensity despite the presence of the amide group in its structure. This is because biotin-NQ is not recognized by the active site of pepsin, thus, it cannot be hydrolyzed. Accordingly, biotin-NQ can stably perform CL measurement without being influenced by proteolytic enzymes that could be co-existed in the samples.

From the results of the stability studies, we can conclude that biotin-NQ has exceptional stability towards all stress factors and chemicals that may affect immunoassay and it could be used as a signal generator tag without the need for special treatment procedures or specific precautions.

In addition to the previous privileges of biotin-NQ over biotin-HRP, cost-effectiveness is also great merit for biotin-NQ. The use of HRP makes the analytical procedure expensive (Sahu et al., 2019), while on the other hand, quinones are known to be inexpensive and widely available (Huskinson et al., 2014). Moreover, extraction of HRP from natural sources and synthesize of its products such as biotin-HRP is expensive (Spadiut and Herwig, 2013) and complicated (Rao et al., 1999), while, synthesizes of biotin-NQ was very simple through simple one-pot reaction followed by filtration and washing of the produced precipitate.

3.4. Selectivity study of the sensor vs. immunoassay components.

As our sensor is aimed for immunoassays labeling, the selectivity of the CL reaction towards biotin-NQ vs. commonly used immunoassay component (10 equivalents each) were tested. As shown in Fig. S7, there was no CL produced from any immunoassay component including avidin, BSA, biotinylated antibody, and some antigens such as ovalbumin, β-casein, and β-lactoglobulin. Besides, our previous study about proteins and their quinones adducts showed that many natural proteins and enzymes, that could be candidate targeted analytes for immunoassay, such as cytochrome c, alcohol dehydrogenase, β-lactoglobulin, lysozyme, and HSA, does not show any CL response to the DTT luminol CL system in the absence of quinones (Elgawish et al., 2015a, 2015b). All this evidence the selectivity of our CL reaction towards the developed sensor, biotin-NQ, and demonstrate the suitability of the use of biotin-NQ as immunoassays labeling reagent.

3.5. CL measurement of Avidin *via* biotin-NQ labeling

We investigated the ability of biotin-NQ to efficiently label avidin and maintain its redox-cycle ability, thus, avidin could be measurable by quinone redox-cycle based CL assay for the attached biotin-NQ. Avidin was measured either with the aid of ultrafiltration or through immobilization in the microplate wells as mentioned in the experimental section. We first incubated avidin with 4 times excess biotin-NQ, as avidin could bind to four biotins (Mercadal et al., 2018; Schmidt and Healy, 2013), then separated the labeled avidin through ultrafiltration and determined the potentially produced avidin-biotin-NQ though CL assay after addition of luminol and DTT. As shown in Fig. S8a, the CL signal increase linearly with the concentration of avidin in the range of 0-25 μM and it showed excellent linearity (Fig. S8b) in the range of 0-10 μM ($r = 0.9992$). These results indicate the ability of biotin-NQ to bind with avidin efficiently and quantitatively while retaining its CL properties.

Secondly, avidin was immobilized in the microplate wells, then biotin-NQ was added sequentially and excess biotin-NQ was washed away. Next, the generated CL after adding luminol and DTT was measured, thereby quantifying avidin. Avidin showed a linear relationship with a correlation coefficient of 0.995 in the range of 0.1 to 0.8 μM as shown in Table 1. Besides, when the detection limit defined as $3\sigma/K$ was calculated (Liu et al., 2016; Yang et al., 2020), it was found to be 18.3 nM. For comparison purposes, we carried out the same experiment using biotin-HRP instead of biotin-NQ while using the CL measurement conditions suited to biotin-HRP measurement (Katsuragi et al., 2000) (Supplementary Material, Section S4). The sensitivity of biotin-NQ was about 3 times higher than that of biotin-HRP. These results demonstrate the superiority of biotin-NQ for labeling avidin vs. enzymatic labeling.

The results obtained from the analysis of avidin either assisted with ultrafiltration or well immobilization indicate the ability of biotin-NQ to bind with avidin efficiently and quantitatively while retaining its CL properties.

3.6. CL measurement of biotin-labeled antibody using biotin-NQ

To demonstrate that our designed reagent, biotin-NQ, could be efficiently used for labeling antibodies in the immunoassay, we investigated its ability to label biotin-labeled IgG. First, the biotin-labeled antibody to be measured was immobilized in the microplate well. Then avidin and biotin-NQ were added sequentially and labeling through avidin-biotin interaction occurred. Afterward, the CL generated by adding luminol and DTT was measured, thereby quantifying the biotin-labeled antibody (Fig. 1). The full analytical procedure was optimized to obtain the highest sensitivity.

At first, we studied and optimized the interaction of avidin with the fixed biotinylated antibody in terms of molar ratio and incubation time using 0.4 μM biotin-labeled antibody. Avidin solution concentration was studied in the range of 0.2 to 2.0 μM corresponding to a molar ratio of 0.5 to 5 times that of the 0.4 μM biotin-labeled antibody. As seen in Fig. 4a, the CL intensity increased with increasing avidin concentration, however, the blank CL increased similarly and the relative S/B ratio decreased. When the avidin: biotinylated antibody molar ratio was 1:1, the highest S/B ratio was obtained, and thus, it was considered as the optimum condition. Next, the influence of the incubation time after the addition of avidin to the biotin-labeled antibody on the CL intensity and the S/B ratio was investigated. As shown in Fig. 4b, the best incubation time was 1 hr giving the highest relative S/B ratio. Therefore, avidin was added to the fixed antibody in a 1:1 ratio and incubated for 1.0 hr in the measurement procedure.

More importantly, we investigated the interaction of avidin with biotin-NQ in terms of molar ratio and incubation time. The effect of the biotin-NQ concentration was studied in the range of 1.6-8.0 μM corresponding to a molar ratio of 4-20 times to 0.4 μM avidin-biotinylated antibody complex. As a result, 6.4 μM biotin-NQ, corresponding to a molar ratio of 16:1 avidin, gave the largest S/B ratio (Fig. 4c). Next, the influence of incubation time after the addition of biotin-NQ to avidin was investigated. The incubation time was varied from 0.5 to 4.0 hours, and it was found that 1 hr incubation yielded the highest S/B ratio (Fig. 4d). Therefore, biotin-NQ was added in an excess molar ratio of 16:1 and incubated for 1 hr at the measurement procedure. Finally, the optimized measurement procedure described in the experimental section was followed.

When a calibration curve of the biotin-labeled antibody was prepared under optimal conditions, a linear relationship with a correlation coefficient of 0.971 was obtained between the biotin-labeled antibody concentration and the CL intensity in the range of 0.2 to 0.8 μM as shown

in Table 1. Besides, when the detection limit defined as 3σ/K was calculated (Liu et al., 2016; Yang et al., 2020), it was found to be 25.7 nM. For comparison purposes, we carried out the same experiment using biotin-HRP instead of biotin-NQ while using the CL measurement conditions suited to biotin-HRP measurement (Katsuragi et al., 2000) (Supplementary Material, Section S4). The sensitivity of biotin-NQ was about 2.5 times higher than that of biotin-HRP. The higher sensitivity of biotin-NQ vs. biotin-HRP either in measuring immobilized biotinylated antibody (2.5 times) or immobilized avidin (3 times) could be due to the less steric hindrance influence of biotin-NQ compared with biotin-HRP. Thus, avidin binding with multiple biotin-NQ is more likely to occur than with biotin-HRP, as well, biotin-NQ is less likely to cause nonspecific binding with the well surface. In addition, no oxidant is used with luminol during CL measurement, thus, no significant blank signal was recorded. These results demonstrate the reliability of biotin-NQ for labeling antibodies and its potential merits vs. the enzymatic labeling.

3.7. CL measurement of avidin using immobilized biotin-labeled antibody and biotin-NQ

To test the reliability of our designed reagent, biotin-NQ, for labeling an analyte in a design very similar to the immunoassay, we investigated its ability to label avidin bounded to immobilized biotin-labeled IgG. First, the biotin-labeled antibody was immobilized in the microplate well as a capture entity for avidin. Then, different concentrations of avidin (0.2-0.8 μM) were added. Afterward, biotin-NQ was added to label avidin bound to the immobilized biotin-labeled IgG. After that, the CL generated by adding luminol and DTT was measured, thereby quantifying the avidin. When a calibration curve of the avidin was prepared, a linear relationship with a correlation coefficient of 0.996 was obtained between the avidin concentration and the CL intensity in the range of 0.2 to 0.8 μM as shown in Table 1. Besides, when the detection limit defined as 3σ/K was calculated (Liu et al., 2016; Yang et al., 2020), it was found to be 23.4 nM which, as expected, is

very similar to that of biotin-labeled antibody (25.7 nM) that was determined in the previous section. All these results indicate the reliability of biotin-NQ for labeling avidin as a model analyte and demonstrate its convenience for labeling immunoassays.

4. Conclusion

Biotin-NQ was designed and synthesized and fully investigated as a new CL signal generator tag for immunoassay instead of the commonly used enzymatic tagging. Biotin-NQ can form intense and long-lasting CL signal upon its redox reaction with DTT and luminol based on the redox cycle of quinones. Moreover, biotin-NQ was found to possess exceptional stability towards different stress factors that may be encountered during the immunoassay performance. Biotin-NQ was found to be not affected by heating or storing at highly acidic and alkaline condition and interestingly it was not affected by repeated freeze-thaw cycles. In addition, biotin-NQ was not affected by chemicals, such as sodium azide, and proteolytic enzymes, such as pepsin that could be present as preservatives for the antibodies or in the analyzed sample, respectively. On the other hand, all these conditions lead to decreased reactivity of the enzymes due to possible denaturation of its protein conformation or deactivation of its prosthetic group. All these show the outstanding merits of biotin-NQ when compared with the enzymatic label biotin-HRP. Furthermore, CL measurement of biotin-labeled antibody using biotin-NQ and avidin was carried out as a final confirmation. We could successfully detect biotinylated antibody with good linearity and good sensitivity in the nanomolar level which was about 2.5 times the sensitivity of the biotin-HRP method.

In conclusion, the designed biotin-NQ could be used as a highly stable and efficient CL signal generator tag for immunoassay overcoming all the demerits of enzyme labels. As our reagent depended on the biotin-avidin system, thus, it could be used for rapid quantification and

monitoring of various analytes through changing the type of biotinylated antibody. As well, due to its excellent stability, it could be applied in the immunoassay at different fields ranging from disease biomarkers analysis to environmental toxic materials and food allergens monitoring. Currently, we are trying to improve the sensitivity of the designed reagent through varying the type of the used quinone and increasing the number of bound quinones to biotin through polymerization approach.

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Table 1: Calibration curves and detection limits of avidin biotin-labeled antibody using biotin-NQ and biotin-HRP CL assay methods.

Analyte	Method	Range (μM)	Equation [a]	Linearity (*r*)	Detection limit (nM)[b]
Immobilized Avidin	biotin-NQ	0.1-0.8	$Y = 12.21 \times 10^5 X + 2.6 \times 10^5$	0.995	18.3
	biotin-HRP	0.1-0.8	$Y = 14.72 \times 10^5 X + 3.9 \times 10^5$	0.953	53.9
Immobilized Biotinylated anti-rabbit IgG	Avidin + biotin-NQ	0.2-0.8	$Y = 9.09 \times 10^5 X + 3.4 \times 10^5$	0.987	25.7
	Avidin + biotin-HRP	0.2-0.8	$Y = 11.98 \times 10^5 X + 2.5 \times 10^5$	0.917	63.0
Avidin	Immobilized biotinylated anti-rabbit IgG + biotin-NQ	0.2-0.8	$Y = 9.09 \times 10^5 X + 2.0 \times 10^5$	0.996	23.4

[a] Y = CL intensity; X = Concentration of biotin conjugated antibody

[b] 3SD/slope

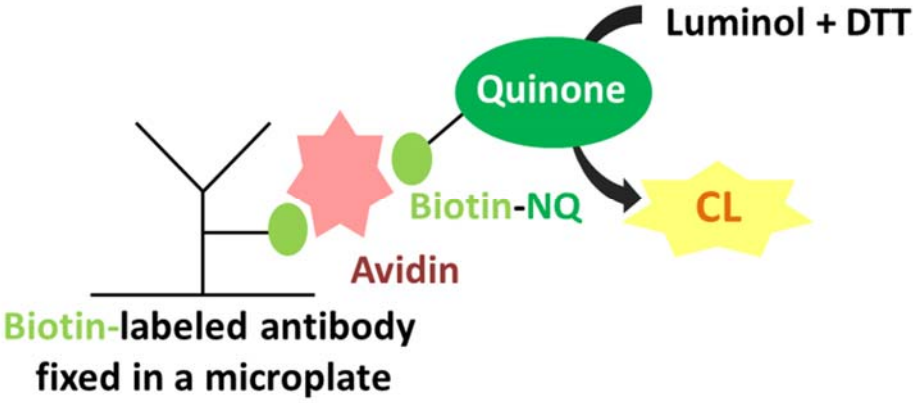

Fig. 1: Illustration for labeling of the biotin-labeled antibody with biotin-NQ using avidin as a binder followed by their determination with luminol and DTT.

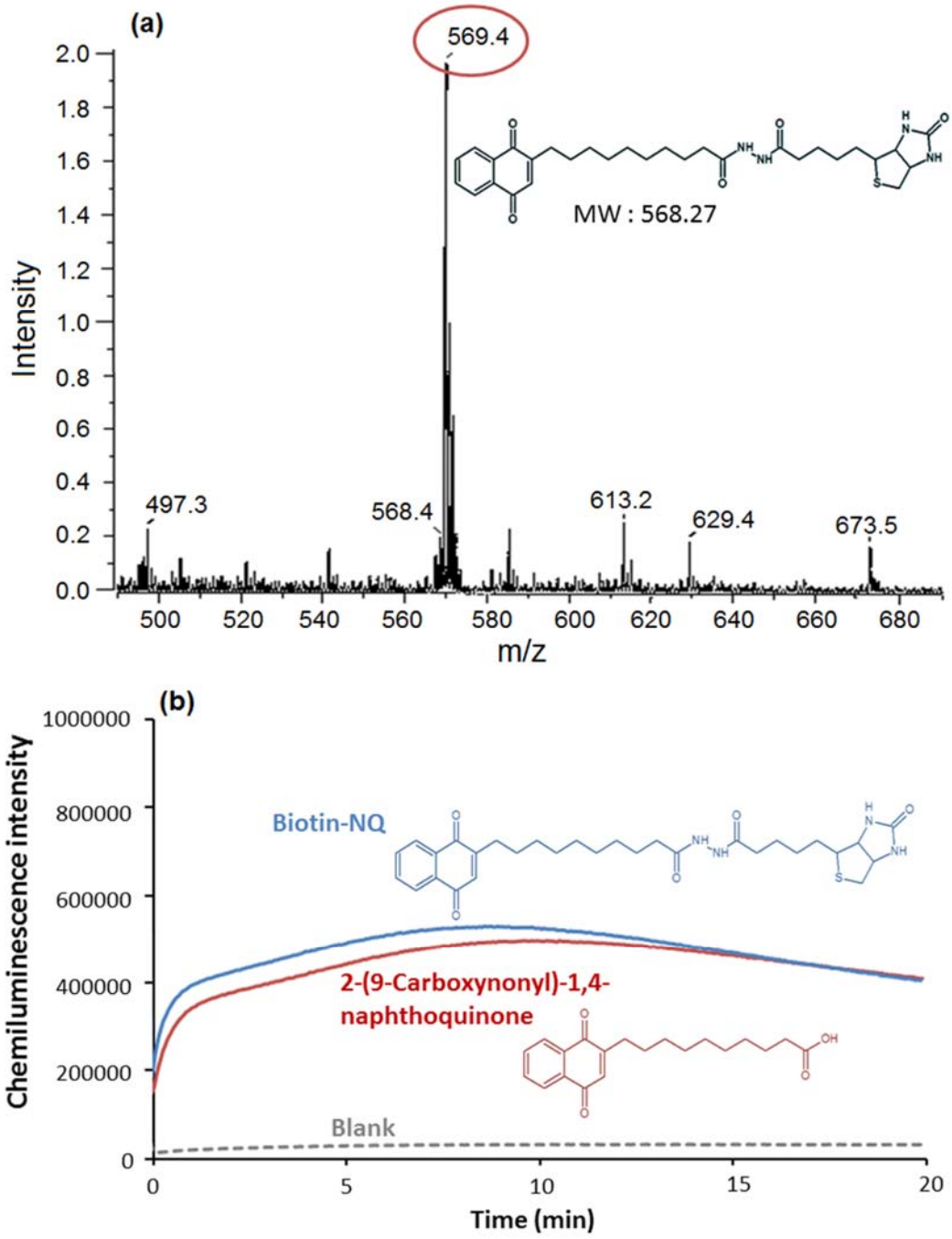

Fig. 2: Characterization of biotin-NQ, where (a) Fast atom bombardment mass spectrometry spectrum of biotin-NQ and (b) is the time profile CL spectra of 2-(9-carboxynonyl)-1,4-naphthoquinone (10 µM) and biotin-NQ (10 µM) showing nearly identical CL time profile.

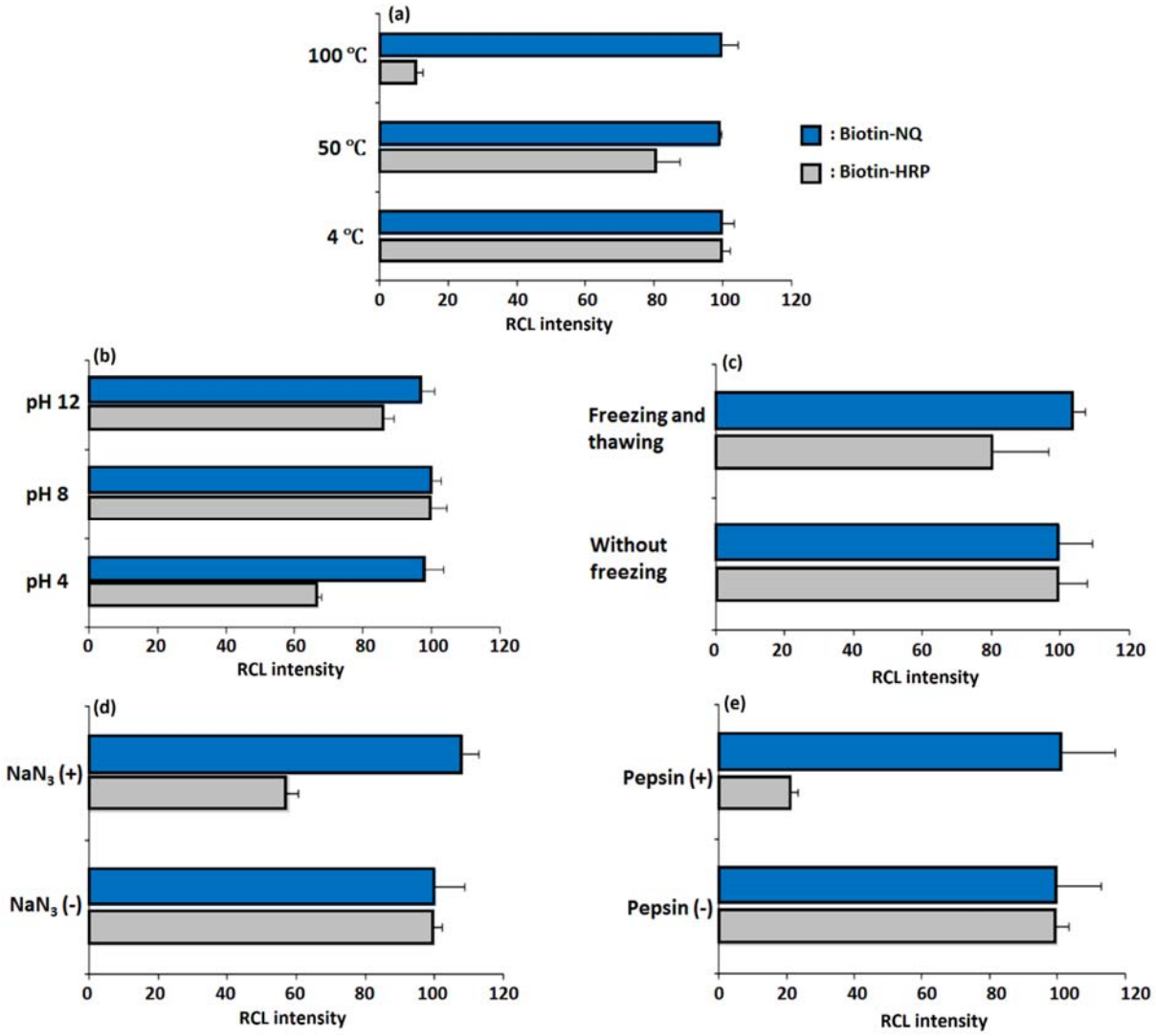

Fig. 3: Effect of different stress factors on the reactivity and stability of biotin-NQ and biotin-HRP; where (a) is the effect of temperature, (b) is the effect of pH, (c) is the effect of freeze and thaw cycle, (d) is the effect of the antibody preservative, sodium azide, and (e) is the effect of the proteolytic enzyme, pepsin. RCL signals are expressed as mean + SD (n=3). The blue columns represent the CL response of biotin-NQ, while the grey columns represent the CL response of biotin-HRP.

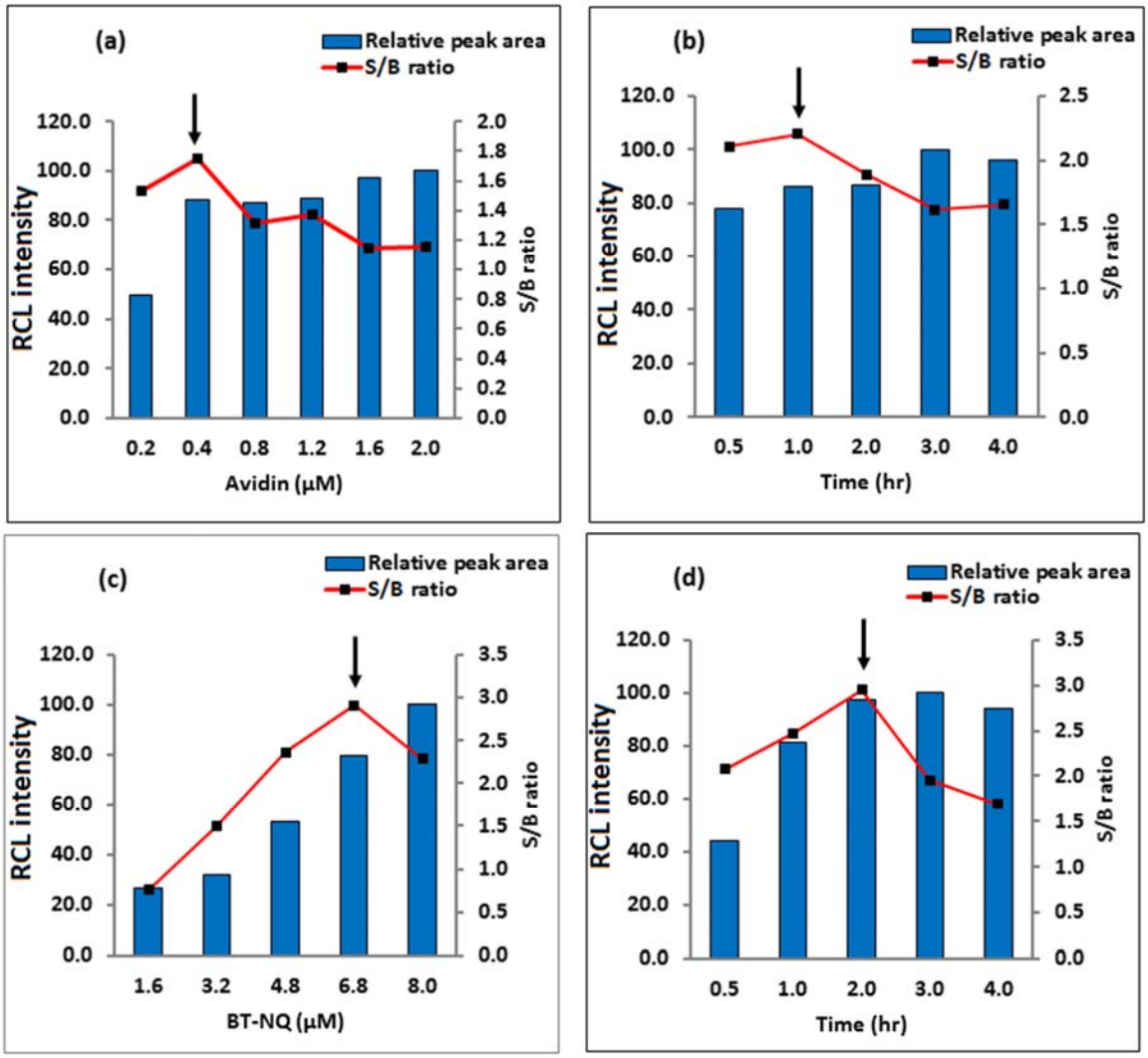

Fig. 4: Effect of measurement conditions on the RCL and S/B ratio of biotin-labeled antibody determination using biotin-NQ CL method where (a) is the effect of avidin concentration, (B) is the effect of incubation time of avidin with the antibody, (c) is the effect of biotin-NQ concentration, and (d) is the effect of incubation time of biotin-NQ with avidin.

Supplementary Materials

Section S1: Reagents and chemicals

Anti-rabbit IgG-biotin conjugate and BT-HRP was sourced from ImmunoReagents, Inc. (NC, USA) and ROCKLAND, INC (PA, U.S.A), respectively, while avidin was from CALZYME Laboratories, INC. (CA, U.S.A). Biotin hydrazide and 2-(9-carboxynonyl)-1,4-naphthoquinone were from Sigma-Aldrich (MO, USA). Luminol, DTT, bovine serum albumin (BSA), sodium chloride, sodium bicarbonate, sodium carbonate, sulfuric acid, hydrochloric acid, sodium acetate, disodium hydrogen phosphate, acetic acid, and DMF were obtained from Nacalai Tesque (Kyoto, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), and 4-dimethylaminocinnamaldehyde (DCAC) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile (HPLC grade) and sodium hydroxide were from Kanto Chemical Co., Ltd (Tokyo) and Merck (Darmstadt, Germany), respectively. 2-Amino-2-hydroxymethyl-1,3-propanediol, pepsin 1: 100 (derived from swine gastric mucosa), potassium chloride, sodium azide, trisodium phosphate, tween 20, hydrogen peroxide, and potassium dihydrogenphosphate were sourced from Wako Pure Chemical Industries (Osaka, Japan). Purified water was used throughout the experiments and it was produced using Autostill WG 203 (Yamato Scientific Co., Ltd., Tokyo).

Tris-HCl buffer (0.1 M, pH 8.4), was prepared from of 2-amino-2-hydroxymethyl-1,3-propanediol and HCl, and acetate buffer (0.1 M, pH 4) was prepared from mixing sodium acetate and acetic acid, while phosphate buffer solution (0.1 M, pH 12) was prepared from trisodium phosphate and disodium hydrogenphosphate. Phosphate buffered saline (PBS) (pH 7.4) was prepared from potassium dihydrogen phosphate (0.1 g), potassium chloride (0.1 g), disodium

hydrogen phosphate (0.576 g), and sodium chloride (4.0 g) dissolved in 500 mL water. The immobilizing buffer, carbonate-bicarbonate buffer (0.05 M, pH 9.6) (Al-amri and Hashem, 2020; Ma et al., 2013; Zeng et al., 2020; Zhang et al., 2006), was prepared from sodium carbonate and sodium bicarbonate and the pH was adjusted with few drops of 1 M sodium hydroxide aqueous solution (Dawson et al., 1986). BSA (1%) was prepared in PBS, while PBS-T buffer was prepared by dissolving tween 20 (0.05%) in PBS. The solution for biotin dyeing was prepared by mixing 0.2% DACA ethanol solution with 2% sulfuric acid ethanol at a ratio of 1:1.

Section S2: Instrumentation and materials

A Lumat LB-9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) was used for the preliminary CL study. A microplate (cell culture microplate, 96 well, PS, F-bottom (CHIMNEY WELL), white, CELLSTAR®, TC, LID with condensation rings, sterile, Greiner Bio-One Co. Ltd., Tokyo, Japan), microplate reader (Spectra Max M5, Molecular Devices, CA, USA), and data processing software (Molecular Device, Softmax® Pro 5 software) were used in the microplate-based assay. F22 pH-meter (Horiba, Kyoto) was used for the pH measurement. Ultrafiltration was carried out using Amicon Ultra-0.5 mL, 10 K (Merck).

Section S3: CL measurement of BT-NQ with a luminometer

Fifty µL of a 75% acetonitrile solution of BT-NQ and 50 µL of a NaOH (7 mM) solution of luminol (35 µM) are sequentially added to a small test tube and mixed, after setting in a luminometer, a 50 µL of a DTT (500 µM) acetonitrile solution is automatically injected. CL was measured for 20 minutes.

Section S4: CL measurement of BT-HRP

The procedure reported previously (Katsuragi et al., 2000) was used with a small modification, where a 50 µL BT-HRP was transferred to the wells of the microplate, followed by addition of 50 µL of a 35 µM luminol aqueous solution and automatic injection of 25 µL of a 0.6 mM H_2O_2 aqueous solution. The generated flash CL was measured for 5 seconds.

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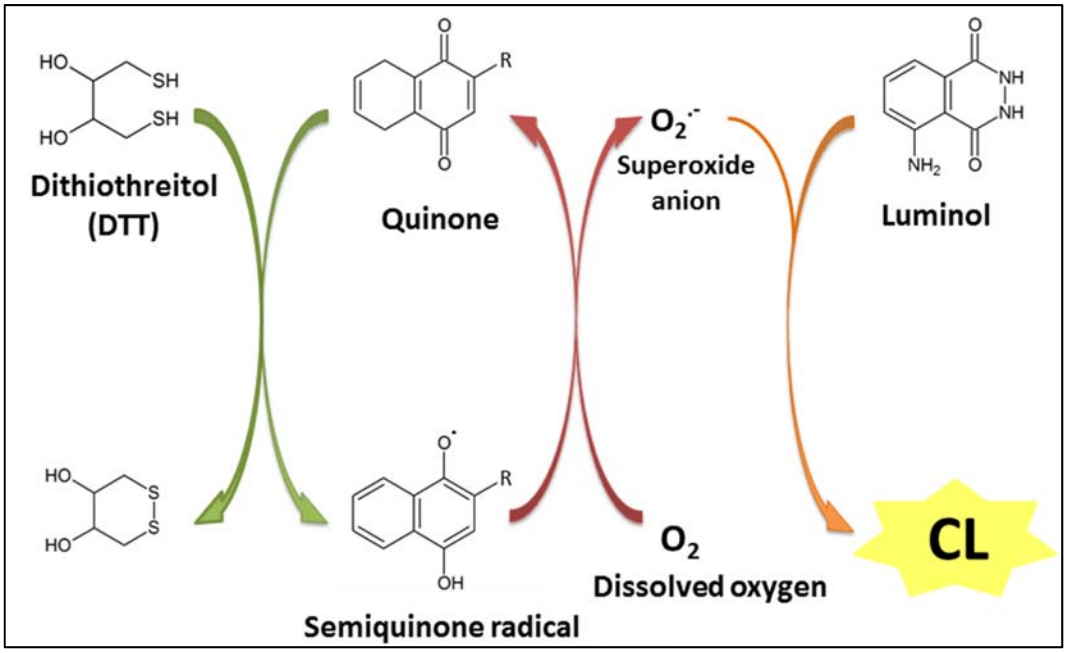

Fig. S1: Luminol chemiluminescence mechanism of quinone based on their redox cycle.

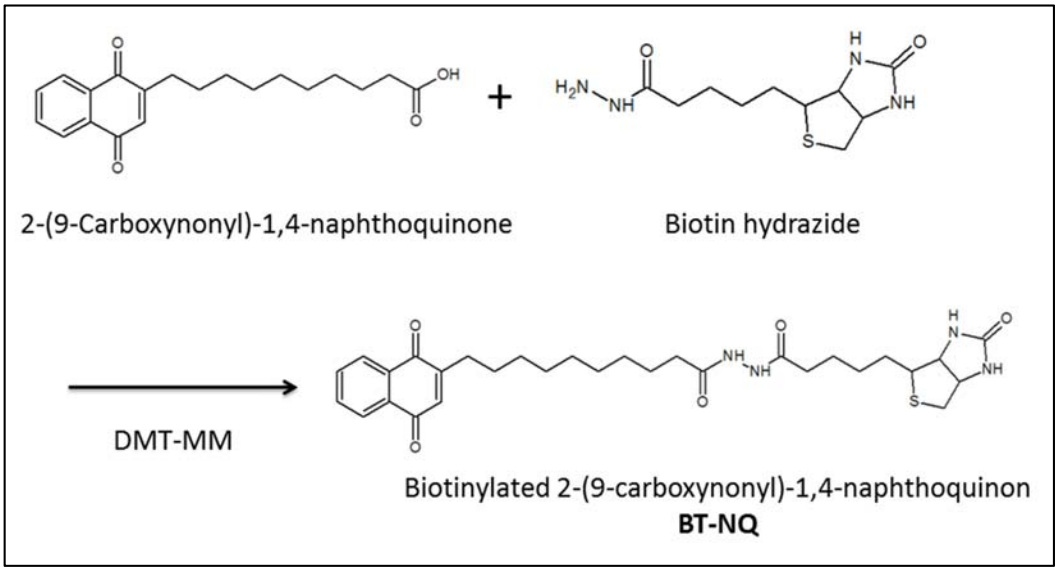

Fig. S2: Reaction schcmes used for the synthesis of BT-NQ.

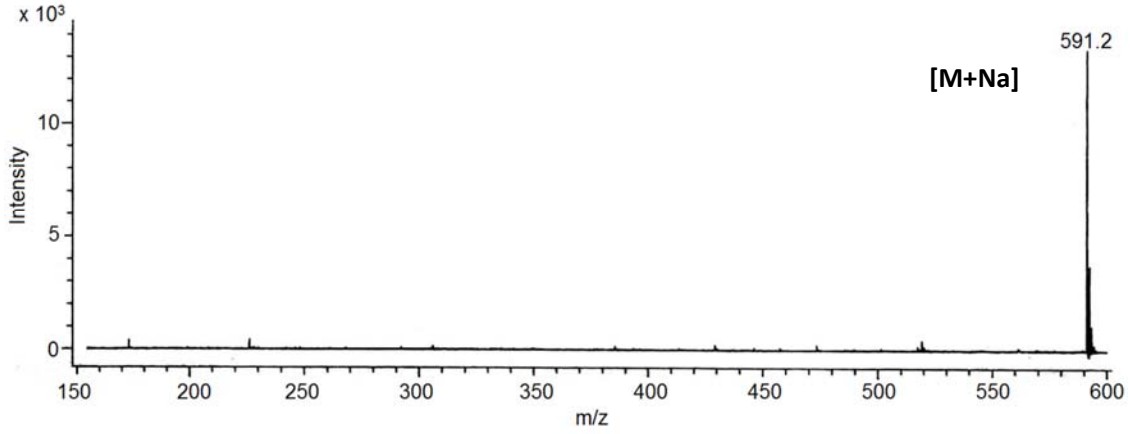

Fig. S3: Electrospray ionization-mass spectrometry spectrum of biotin-NQ.

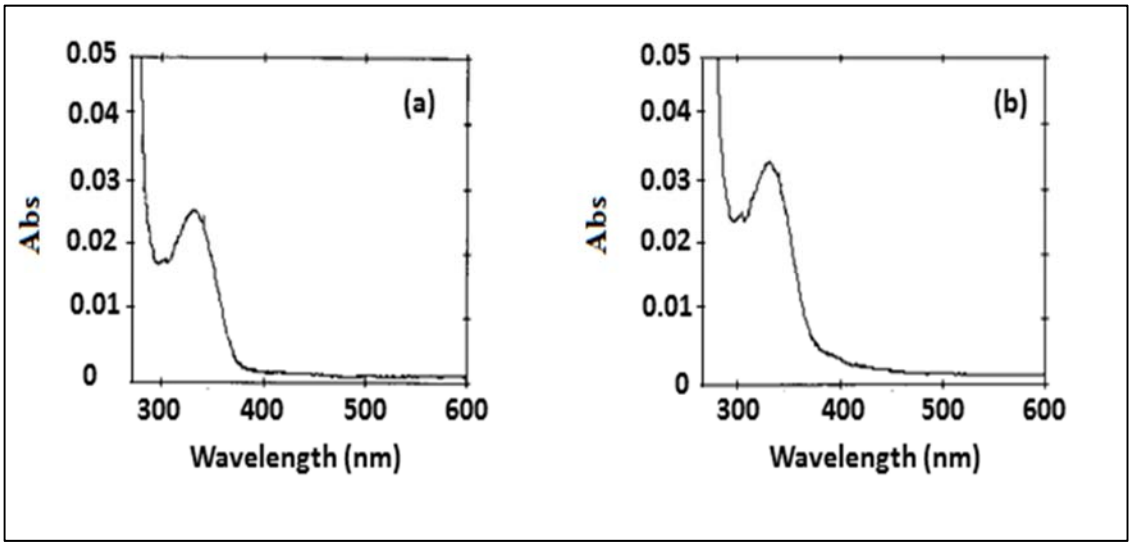

Fig. S4: UV spectra of (a) 2-(9-carboxynonyl)-1,4-naphthoquinone and (b) BT-NQ showing the same *λmax* at 330 which is characteristic for 1,4-naphthoquinione.

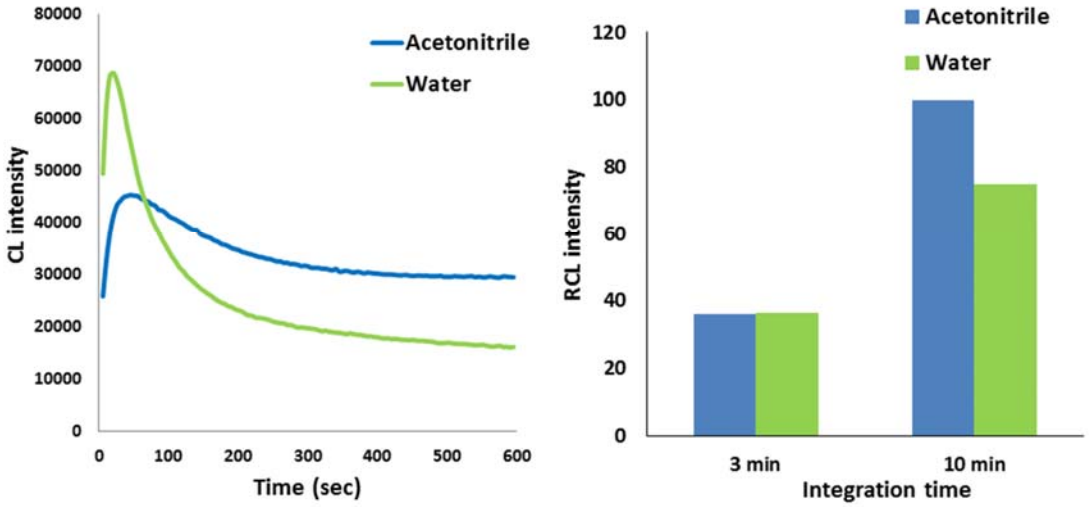

Fig. S5: Effect of DTT solvent on the quione redox cycle CL reaction with DTT and luminol where (a) is the CL time cure and (b) shwos the integrtated RCL intensity.

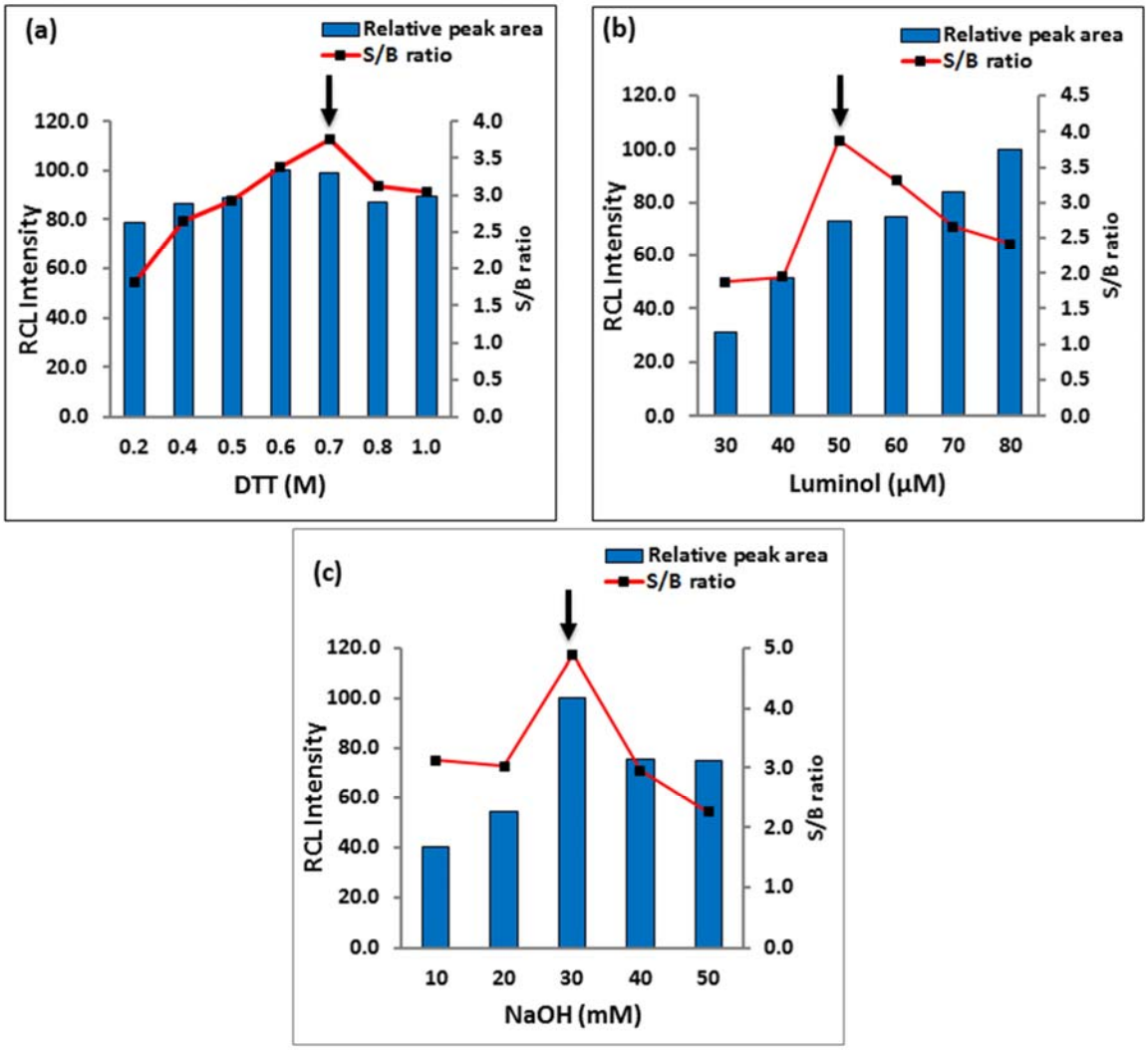

Fig. S6: Effect of reagents concentrations on the RCL and S/B ratio of BT-NQ CL reaction with luminol and DTT where (a) is the effect of DTT concentration, (B) is the effect of luminol concentration, and (c) is the effect of NaOH concentration.

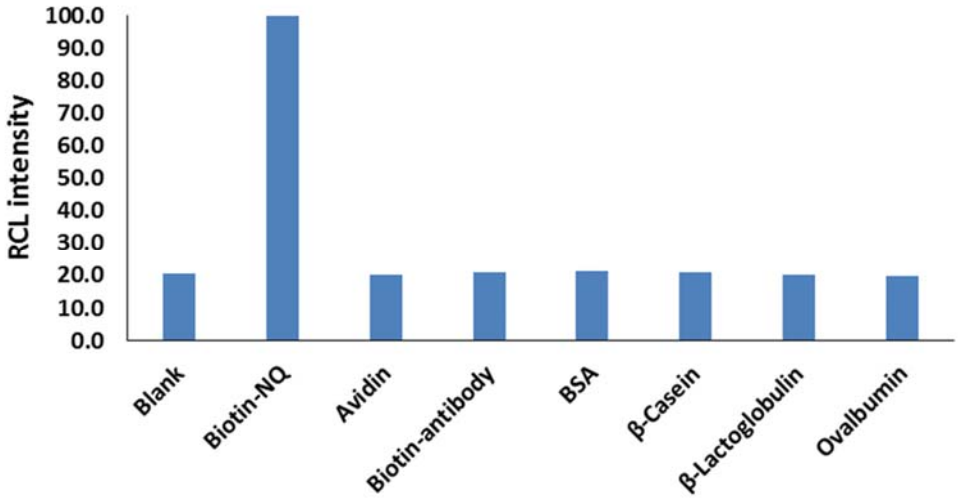

Fig. S7. Selectivity study of the luminol-DTT CL system towards the developed sensors, biotin-NQ (2 vs the commonly used component of the immunoassay (10 equivalent) where the RCL intensity of biotin-NQ was considered as 100%.

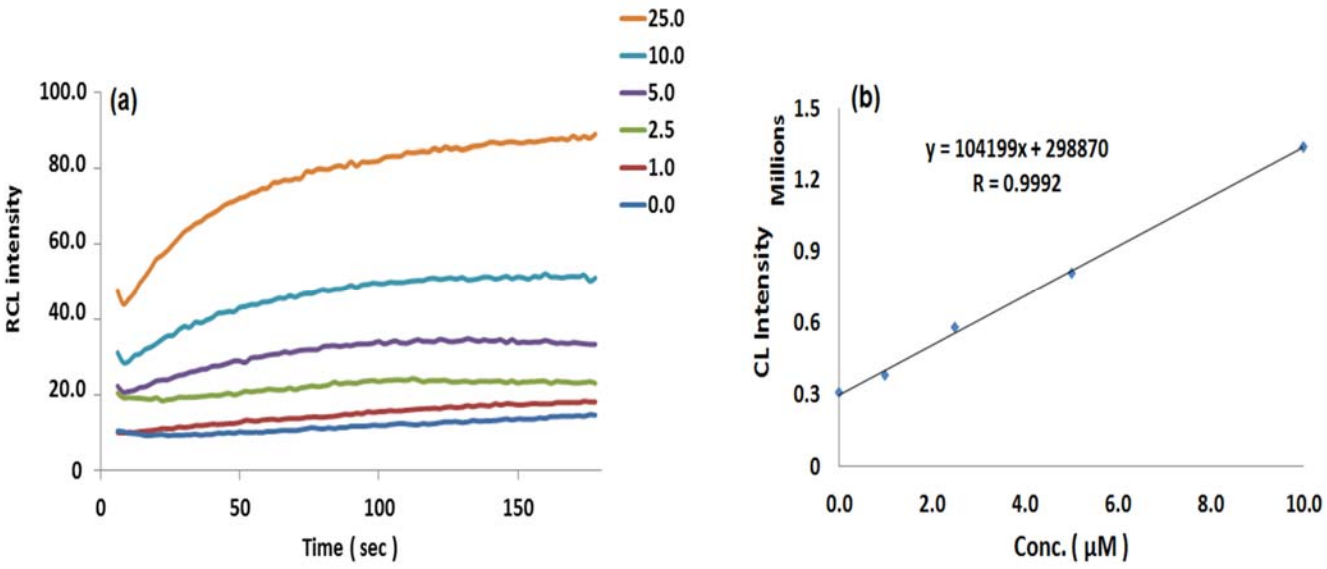

Fig. S8: Results of avidin analysis *via* labeling with BT-NQ followed by ultrafiltration purification and CL determination with luminol and DTT, where (a) time profile CL spectra of avidin at different concentrations (0-25 µM) and (b) is the linear calibration curve of avidin vs. the integrated CL intensity.