Design of a dual functionalized chemiluminescence ultrasensitive probe for quinones based on their redox cycle. Application to the determination of doxorubicin in lyophilized powder and human serum.

Mahmoud El-Maghrabey[a,b,1,2], Naoya Kishikawa[a,1,*], Shuhei Kamimura[a], Kaname Ohyama[c], Naotaka Kuroda[a]

[a] Department of Analytical Chemistry for Pharmaceuticals, Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[b] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

[c] Department of Pharmacy Practice, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto-machi, Nagasaki 852-8588, Japan

[1] These authors have equal contributions to the manuscript.

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*Corresponding author: Naoya Kishikawa

Phone : +81-95-819-2445, Fax: +81-95-819-2446, E-mail: kishika@nagasaki-u.ac.jp

Abstract

Quinones are ubiquitous compounds that have two-sided nature; a beneficial one, especially in the electron transport chain, and a toxic one through the generation of reactive oxygen species *in vivo*. Herein we developed a new dual-function chemiluminescence probe for their determination namely N-(4-aminobutyl)-N-ethylisoluminol-lipoic acid (ABEI-LA). This probe has two moieties, a reductant one, lipoic acid (LA), which upon its redox cycle reaction with quinones, generate superoxide anion, and the other moiety is the luminophore, ABEI, which generates intense chemiluminescence upon reaction with the generated superoxide anion. ABEI-LA is the first single reagent that could generate CL with quinones in a one-step operation. A simple rapid chemiluminescence assay for the anticancer drug, doxorubicin, was developed using ABEI-LA as a probe. The method showed good linearity towards doxorubicin in the range of 1-200 nM and excellent sensitivity with a detection limit of 0.17 nM (25 pg/assay). The proposed probe, ABEI-LA, was successfully applied for the quantification of doxorubicin, in lyophilized powder for injections and human serum samples and it showed very good recoveries of 84-91% and 98-101%, respectively. These results demonstrate the ability of ABEI-LA for the determination of quinones in different matrices enabling the in-depth study of quinones two-sided nature.

Keywords: quinones; lipoic acid; aminobutyl-ethyl-isoluminol; chemiluminescence probe, pharmaceuticals, serum

1. Introduction

Quinones are dioxo aromatic compounds derived from mono, di, and polycyclic aromatic hydrocarbons. They are categorized based on the ring system into benzoquinones, naphthoquinones, anthraquinones, etc. [1]. Quinones are distributed naturally in many plants′ and animals′ tissues and they possess versatile important roles, especially in the electron transport chain. For instance, ubiquinone acts as an electron carrier in the electron transport chain of mitochondria that is essential for cellular respiration and production of energy in animals [2]. In the same context, pyrroloquinoline quinone (PQQ), a bacterial dehydrogenases redox cofactor, was categorized as a member of the vitamin B family and it was found to act as a radical scavenger and has the ability to protect against internal organs injuries [3]. Besides, the vitamin K family, including phylloquinone (vitamin K1) and menaquinone (vitamin K2) are structurally based on the 1,4-naphthoquinone compound, menadione, and they possess a valuable role in the coagulation of blood and in metabolism and growth of bone [4,5]. Other quinones, such as plastoquinone, are essential for the plants' photosynthesis reactions [6]. Furthermore, quinones have various applications in the industry as bleaching reagent. Other quinones are used as an herbicide, in cosmetics, or even as medicinal drugs [2,7,8]. For instance, the anthracycline quinones, daunorubicin and doxorubicin, are used as antineoplastic agents [9]. On the other hand, in addition to the beneficial and important activities of quinones in biological systems, they have another toxic face. For instance, 9,10-phenanthrenequinone (PQ), an environmental existing quinone, have potentially harmful effects on human health. PQ could inhibit some biological functions such as the activities of important enzymes including nitric oxide synthase [10] and glyceraldehyde-3-phosphate dehydrogenase [11]. In addition, some quinones could cause oxidative stress in the human body owing to their ability to generate reactive oxygen species (ROS) upon interaction with $NADPH_2$ [12–14]. Hence, there is a strong need for a sensitive and selective analytical method for the determination of quinones in order to study and clarify their two-sided nature from a physiological and pharmacological point of view in several areas of research.

Various analytical techniques were developed for quinones determination including batch and chromatographic methods. Batch methods included colorimetric microplate assay [15], fluorometry (FL) [16–18], photometric flow-injection analysis [19], electron spin resonance (ESR) assay [20,21], and chromatographic methods

included gas-chromatography coupled with mass spectrometry (GC-MS) [22–24] and high-performance liquid chromatography (HPLC) coupled with either with UV [25–27], FL [28–34], chemiluminescence (CL) [35–38], electrochemical detection [39–42] or MS [43–45]. Although some of these methods are sensitive enough to give valuable data about the quinone concentrations in different matrices [15,29,32–38,45], however, many of these analytical platforms have many demerits including low sensitivity, time consumption, and sometimes complexity of the procedure. Besides, methods based on UV detection suffer from low sensitivity and non-selectiveness. In addition, despite the inherent high selectivity and sensitivity of fluorescence detection methods [46], quinones are non or weakly fluorescent compounds, thus FL methods prerequisite derivatization of quinones into a fluorescent product [16–18]. Besides, quinones are weakly ionizable compounds, hence their mass detection requires prior derivatization and/or atmospheric-pressure chemical ionization [43–45]. Regarding electrochemical detection methods, despite their sensitivity, they suffer from complexity and time consumption due to the requirement of beforehand reduction of quinones prior to their detection [39–42]. GC-MS methods [22–24] also suffer from complexity and time consumption due to the use of complicated instruments and the prerequisite of derivatization to convert quinones into volatile compounds. For the ESR analysis [20,21], a complicated special and costive instrument is required, limiting the use of this technique.

On the other hand, the analytical methods that use CL detection grabbed high interest due to their great sensitivity and the simplicity of the used device. The CL detection method excites molecules by a chemical reaction; thus, it does not require an excitation light source as compared with the FL method and is not affected by stray light or scattered light originating from the light source. The previously reported CL based assays for quinones, have great sensitivity, however, they required photoreactor for the photochemical generation of ROS from quinones [35–37] or suffer from the high background due to the use of oxidizing agents [38]. In our laboratory, we developed a CL assay for quinones based on their redox cycle (Fig. S1, Supplementary Material) through reacting them with a reductant, such as dithiothreitol (DTT), and luminol [3,8]. Quinones are reduced by DTT and converted into unstable semiquinone radicals, which are then oxidized by dissolved oxygen returning into quinone and producing superoxide anion radicals [8,47–49]. The produced superoxide anion radical

oxidatively decomposes luminol forming the excited intermediate (3-aminophthalic acid dianion) producing long-lasting strong CL [50]. This method can selectively measure quinones by utilizing their unique redox cycle. However, in this method, the reductant and the luminescent reagent had to be added separately, which might result in relative complications of the procedure and lowering its precision. Therefore, in this study, we aimed to design and synthesize a new CL analytical reagent that combines both the reductant and the luminol derivative to further simplify the measurement procedure and increase the efficiency of light emission by bringing the two reagents in close proximity. α-Lipoic acid (LA) was selected as the reductant [49,51] and the luminol derivative, *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI), was selected as a luminophore, as they possess reactive functional groups that can interact binding them to each other (Fig. 1). The CL characteristics of the reagent were investigated by measuring the CL generated by adding ABEI-LA to different kinds of quinones. The efficiency of the CL reagents, ABEI-LA, for CL determination of the model quinone anticancer drug, doxorubicin (DXR), was compared with that of a mixture of ABEI/LA as intact non-bonded system, DTT/luminol system, and LA derivative of isoluminol (IL), namely, IL-LA. ABEI-LA demonstrated its superiority to all of these compared systems. Also, the selectivity of ABEI-LA was tested and verified. Then we applied ABEI-LA for the CL quantification of DXR in lyophilized powder for injections and human serum samples.

2. Experimental

2.1. Chemicals, reagents, and materials

Doxorubicin hydrochloride (DXR), LA, methylparaben, potassium chloride, calcium chloride, silica gel (Wakosil® C-200, for column chromatography) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Luminol, DTT, phylloquinone, lactose monohydrate, ethanol, chloroform, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and sodium carbonate were obtained from Nacalai Tesque (Kyoto, Japan). Methanol and acetonitrile (HPLC grades) were purchased from Kanto Chemical (Tokyo, Japan). Magnesium chloride and pyridine were obtained from Kishida Chemical Co., Ltd. (Osaka). ABEI and pyrroloquinoline quinone (PQQ) were from Tokyo Chemical Industry (Tokyo) and Mitsubishi Gas

Chemical (Tokyo), respectively, while coenzyme Q10 (CoQ10, ubiquinone) and sodium hydroxide were from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. DXR injection formulation (Adriacin® 10 for injection) was obtained from Kyowa Hakko Kirin (Tokyo). Serum samples were bought from Kohjin Bio (Saitama, Japan). Purified water was obtained using the water purifier WG220 from Yamato Scientific Co. Ltd. (Tokyo). Other chemicals were of extra pure grade.

DXR stock solution was prepared in water, while PQQ, CoQ10, and phylloquinone were dissolved in ethanol. ABEI-LA, ABEI, LA, IL-LA, DTT, and luminol solutions were all prepared in aqueous solutions of sodium hydroxide. The tested additive in the selectivity study were all dissolved in water. The lyophilized formulation for injection was reconstituted in water and further dilute with the same solvent.

2.2. Synthesize of ABEI-LA

The synthesis of ABEI-LA was performed through amide formation between the amino group of ABEI and the carboxylic group of LA (Fig. 1). LA (0.15 mmol, 30 mg), ABEI (0.10 mmol, 27 mg), and the amide bond formation catalyst EDC (0.15 mmol, 27 mg) were dissolved in methanol, then, pyridine was added as a base catalyst and the mixture was stirred for 10 hours. The reaction mixture solution was dried under reduced pressure, then the residue was dissolved in chloroform: methanol (9:1, v/v) solution which was then purified by open column chromatography. After evaporation of the collected fraction, the residue was recrystallized using acetonitrile yielding 27 mg (58% yield) yellow crystals of ABEI-LA and its structure was confirmed by fast atom bombardment mass spectrometry (FAB-MS) using (JMS DX-303 mass spectrometer, Joel Ltd., Tokyo) and by elemental analysis using the PerkinElmer® 2400 Series II Elemental Analyzer (PerkinElmer, MA, USA. The measured values by elemental analysis for ABEI-LA with a chemical formula of $C_{22}H_{32}N_4O_3S_2 \bullet 1/2\ H_2O$ were C, 56.1%; H, 7.1%; N, 11.5%, which are in good agreement with the theoretically calculated values: C, 55.8%; H, 7.0%; N, 11.8%.

For a comparison purpose, the LA derivative of isoluminol (IL), namely, IL-LA was synthesized using the same procedure for ABEI-LA synthesis. Instead of using 0.1 mmol ABEI (27 mg), 0.1 mmol IL (26 mg) was used and then the same procedure was followed.

2.3. CL measurement for quinones

To a 50 µL of the quinones solution placed in a small test tube which was set in a luminometer, a 200 µL of ABEI-LA (400 µM) dissolved in 300 mM NaOH was injected automatically. The CL intensity was then measured for 180 seconds after the injection of ABEI-LA using the Lumat LB-9507 luminometer from Berthold Technologies (Bad Wildbad, Germany). This method was used for measuring different types of quinones including PQQ, DXR, CoQ10, and phylloquinone. Additionally, for comparison purpose, instead of using ABEI-LA as CL probe, the CL measurement of the model quinone, DXR, was performed after sequential addition of either 100 µL ABEI (800 µM) dissolved in 300 mM NaOH and 100 µL LA (800 µM) dissolved in 300 mM NaOH, 200 µL IL-LA (400 µM) dissolved in 300 mM NaOH, or 100 µL luminol (800 µM) dissolved in 300 mM NaOH and 100 µL DTT (800 µM) dissolved in 300 mM NaOH.

2.4. Validation of the CL method

For the validation of the developed CL assay, the calibration curve was constructed and limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision were evaluated [52–54]. A standard calibration curve was made using increasing concentrations of the model targeted quinone, DXR, in the range of 1.0-200 nM and the produced CL was measured each time using the procedure described previously. The LOD and LOQ were considered as the concentrations that could give integrated CL intensity corresponding to that of (Blank+3SD) and (Blank+5SD), respectively [49,53,54], and were calculated adopting the regression equation of the calibration curve. The accuracy and precision of the method were investigated by analysis of three quality control concentrations including 2.0, 10.0, and 200 nM. Five repeated measurements of each concentration were performed on the same day and for further four following days. The accuracy was calculated as the % found for each concentration, where the %RSD for each concentrations were measured for the same

day to calculate the intra-day precision, and on five successive days to calculate the inter-day precision [52].

2.5. Measurement of DXR in lyophilized powder for injection

The Adriamycin® 10 vial containing 10 mg DXR.HCl was reconstituted with 5 mL of water. Then the produced solution was diluted as needed with water followed by CL assay of DXR using ABEI-LA as a probe following the procedure mentioned previously. The CL generated by adding the ABEI-LA solution was measured and the DXR content was quantified.

2.6. Measurement of DXR in human serum

Serum samples, blank and pre-spiked with DXR, were extracted using liquid-liquid extraction adopting the method of Daeihamed et al. [55] with some modifications. In brief, 100 µL of serum samples were mixed with 1 mL of chloroform containing 20 % methanol. Then, the mixture was mixed and then centrifuged for 15 min at 4000 x *g* using Himac CR refrigerated centrifuge (Hitachi, Tokyo). Next, the lower organic phase was collected and evaporated in Eyela CVE-3100 solvent centrifugal evaporator (Tokyo Rikakikai, Tokyo). Afterward, the residues were reconstituted in water and analyzed by the CL assay mentioned previously using ABEI-LA as a probe.

3. Results and Discussions

3.1 Characterization of the synthesized ABEI-LA structure and its potential as a CL probe for quinones.

In this manuscript, we aimed at synthesizing the one-pot CL probe for quinones, ABEI-LA. ABEI-LA was synthesized through amide bond formation between the carboxylic group of LA and the amino group of ABEI. The amide bond formation catalysts EDC and pyridine [56] were used in the synthesis reaction. The synthesized ABEI-LA (M.P., 112-115 °C) structure was characterized and confirmed by FAB-MS and elemental analysis. As shown in the FAB-MS spectrum (Fig. 2), the molecular ion is peaking at m/z = 465.3, which corresponds to $[M+H]^+$. Other ion peaks including 434.0, 260.2, and 218.2 were also explained and assigned to different fragmented products resulted from ABEI-LA dissociation in FAB-MS analysis (Fig. 2). From the

above results and the previously mentioned elemental analysis results, the desired product structure of ABEI-LA was confirmed.

The ability of the designed probe, ABEI-LA, to produce CL upon its interaction with quinone in an alkaline medium was tested. The CL profiles of quinones such as PQQ, CoQ10, DXR, and phylloquinone after the addition of ABEI-LA are shown in Fig. 3. Strong long-term CL was observed for CoQ10 and DXR. A relatively weak CL was observed with PQQ and a very week CL was observed with phylloquinone. The performance of ABEI-LA was compared initially with that of the luminol/DTT method reported previously by our research group [8]. As shown in Fig. S3, the RCL of the studied quinones were obviously higher in the case of using ABEI-LA than using the luminol/DTT mixture which initially demonstrates the privileges of ABEI-LA as a new sensor for quinones.

From the electrochemical point of view, the redox potential of Lipoic-S-oxide/lipoic acid is about - 0.50 mv [57], where the studied quinones have redox potential ranged from -0.26 to + 0.09 [58–62]. From these values, the studied quinones underwent redox reactions with LA forming lipoic-S-oxide [57,63]. In order to elucidate the mechanism of the CL reaction of quinones with ABEI-LA, a reaction mixture of standard quinone, DXR, and ABEI-LA was injected into an ESI-MS system upon its neutralization with concentrated HCl and consequent dilution with methanol. The mass spectrum of reaction products is illustrated in Fig. 4a. As can be seen, in addition to the characteristic ion peak of ABEI-LA at m/z 465.19, there are two peaks at m/z 481.16 and 482.3 that correspond to the oxidized form of ABEI-LA, ABEI-LA thiosulfinate, and its 3-aminophthalate form, respectively. The formation of thiosulfinate derivative upon oxidation of lipoic acid is in good agreement with the previous study of Stary et al. [63] and another electrochemical study [57]. From this result, we can conclude that the reaction occurred as follow; at first, the reduction site of ABEI-LA induces a redox cycle in quinones, forming oxidized ABEI-LA (ABEI-LA thiosulfinate) and semiquinone radicals [49,63]. Then, the formed semiquinone radicals react with dissolved oxygens, producing superoxide anions and the radical itself is re-oxidized into quinone again [47,49]. Then, the formed superoxide anions decompose the luminol site of ABEI-LA into its 3-aminophthalate form generating CL [50,64]. To confirm that superoxide anion is the major reactive oxygen species involved in the chemiluminogenic reaction, an inhibitory assay was carried out using different

ROS scavenger. The used scavengers included sodium azide (singlet oxygen quencher) [65], mannitol (hydroxide radical scavenger), superoxide dismutase (SOD, superoxide anion radical scavenger [66], heat-denatured SOD (denaturated through heating at 95 °C for 90 min [67]), and catalase (hydrogen peroxide quencher) [66]. This assay was conducted using the standard quinone, DXR, alone as blank or after its mixing with the scavenger. The results of this study are shown in Fig. 4b. As can be clearly seen, among the tested scavenger, SOD can largely inhibit the CL reaction in a concentration-dependent manner due to its scavenging ability for superoxide anion radical. Additionally, denatured SOD does not affect the produced CL which confirms that the protein skeleton of SOD does not affect the CL reaction and that the original quenching of the SOD intact form was from its scavenging ability for superoxide anion radical which was lost by heat denaturation. On the other hand, other tested scavengers do not affect the produced CL which excludes other reactive oxygen species such as singlet oxygen, hydroxide radical, and hydrogen peroxide from having a role in the chemiluminogenic reaction. From all these results, the CL reaction of quinones with ABEI-LA is proposed to be in the sequence illustrated in Fig. 5.

3.2. Comparison of ABEI-LA performance with other alternative redox cycle-based probes

ABEI-LA showed good performance and high potential as a CL probe for quinones. For comparison purposes, we measured the CL of a model quinone, DXR, using ABEI and LA separately added. Besides, we synthesized IL-LA which is similar to ABEI-LA but it lacks the spacer attached to the amino group of luminol moiety. The structure of IL-LA was been demonstrated via FAB-MS analysis (Fig. S2, Supplementary Material). Furthermore, we measured the CL of DXR after adding the original protocol reported by our research group for quinones using DTT and luminol [8]. As shown in Fig. 6, ABEI-LA yielded the highest CL with DXR and also the highest signal to blank ratio (S/B) compared to IL-LA, luminol /DTT mixture, and ABEI/LA mixture, demonstrating its high efficiency for CL detection of quinones. The reason for the superiority of ABEI-LA over ABEI/LA added separately and luminol/DTT system could be due to the close proximity of the reductant and luminol moiety, thus the produced superoxide anion radical could interact more efficiently with the luminol

moiety producing more intense CL. Besides the high efficiency of ABEI-LA, it is noteworthy that using a single reagent is time and labor-saving. Additionally, we tried IL-LA, which lacks the spacer between the reductant and the luminol moiety, as a CL probe, however, the produced CL and S/B were surprisingly lower than those obtained with ABEI-LA. This could be due to the acylation of the amino group attached directly to the IL, producing low yield fluorescent species than 3-aminophthalate during the CL reaction as reported previously by Pantelia et al. [68].

3.3. Optimization and application of ABEI-LA for determination of DXR

DXR is one of the typical anti-cancer agents, produced from daunomycin-producing bacteria, and widely used for the treatment of malignant tumors including leukemia and breast cancer. DXR intervenes between DNA base pairs in tumor cells, inhibits DNA polymerase, RNA polymerase, and topoisomerase II reactions, and exerts an antitumor effect by suppressing the biosynthesis of both DNA and RNA [69]. It is also known to produce active oxygen by increasing the rate of physiological lipid peroxidation [70]. As mentioned in the previous sections, ABEI-LA produces a long-lasting strong luminescence just by mixing with DXR, thus, we thought that the CL-quantification of DXR could be performed with high sensitivity using ABEI-LA. Consequently, we optimized the CL measurement conditions for DXR using ABEI-LA to obtain the highest possible sensitivity. At first, the effect of the concentration of ABEI-LA was investigated in the range of 100 – 500 μM, and the highest RCL intensity and S/B ratio was obtained with 400 μM ABEI-LA (Fig. 7a). Thus, 400 μM was selected as the optimum ABEI-LA concentration. Then, the effect of concentration of the aqueous sodium hydroxide solution, used as the solvent for ABEI-LA, on the RCL intensity was studied. As shown in Fig. 7b, the RCL intensity amplified with the increase in sodium hydroxide concentration until reaching 300 mM and then kept nearly constant. However, the highest S/B ratio was obtained using 300 mM sodium hydroxide, thus, it was used in further studies. In addition, our target analyte, DXR, does not dissolve in organic solvents with low polarity but dissolves only in highly polar solvents such as methanol and water. Therefore, the effect of the solvent used to prepare the DXR solution on RCL intensity was studied. DXR was dissolved in methanol, water, and aqueous sodium hydroxide solution (300 μM), and the RCL intensity was measured

when mixed with ABEI-LA. The solvent greatly affected the CL behavior of DXR, where methanol made the reaction proceeds faster but lowered the RCL intensity, while sodium hydroxide changed the spectra from glow type into flash one. The highest integrated RCL intensity and S/B ratio and best CL behavior were obtained when water was used (Fig. 7c and d). Additionally, acetonitrile was tested as a solvent for DXR, however, its blank CL was 330 times higher than using water. Also, the final obtained S/B ratio was only 1.3 for 1 μM DXR dissolved in acetonitrile, compared to an S/B ratio of 20 for 1 μM DXR dissolved in water (Fig. S4). From all these results, water was selected as the solvent for DXR.

After the measurement conditions were optimized, different concentrations of DXR in the range of 1 – 1600 nM were measured using the ABEI-LA probe and the CL time profiles are shown in Fig. 8a. The saturation curve (Fig. 8b) indicates loss of linearity above 200 nM DXR, thus, a calibration curve was constructed using the DXR in the range of 1-200 nM (Fig. 8c). A good linear relationship with a correlation coefficient of 0.9998 was obtained between the DXR concentration and the 3 min integrated CL intensity in the range following the regression equation: $Y = 4616.3 X + 99439$, Where, Y is the 3 min integrated CL intensity and X is the DXR concentration in nM. The LOD (blank + 3SD) and LOQ (blank + 5SD) [53] were found to be 0.17 nM and 0.92 nM, respectively.

In addition, the accuracy and precision of the developed method were studied at four concentration points including the lower limit of quantification (LLOQ, 1 nM), the middle limit of quantifications (MLOQ, 2 and 20 nM), and the higher limit of quantification (HLOQ, 200 nM), and the results were abridged in Table 1. The accuracy was calculated as found% and it ranged from 95.4% to 101.1%. Regarding the precision, calculated as RSD%, the intra- and inter-day precision were ≤4.9% and ≤6.7%, respectively. All these results demonstrate the high accuracy and acceptable precision of the proposed method for the determination of DXR.

3.4. Application of ABEI-LA for CL determination of DXR in lyophilized powder for injection

In order to evaluate the practicality of the established CL analytical method for DXR, it was applied to the quantification of DXR in lyophilized powder for injection

(Adriacin® 10 for injection). This pharmaceutical formulation contains several additives including lactose (5 times than DXR) and paraben (0.1 times than DXR). Consequently, we tested the selectivity of our methods towards DXR in the presence of these additives and other common excipients. The CL intensity of DXR was measured while one hundred excess of the tested excipients were coexisted deliberately in the sample solution and the results are shown in Fig. 9. As a result, it was found that all the tested additives did not affect the CL intensity response of DXR even when they were present at a molar excess ratio of 100, demonstrating the high selectivity of the method towards DXR. Thus, DXR could be quantified in the pharmaceutical formulations without any pretreatment or separation method. Then, Adriacin® 10 lyophilized powder for injection were reconstituted simply with water and was further diluted with the same solvent until reaching the calibration range concentrations abridged in Table 2. Then, the accuracy and precision of the method and the nominal content of DXR in the formulation were evaluated (Table 1). The method showed good accuracy in the range of 97.6 – 101 % and very good precision of RSD≤6.9%. The nominal content of DXR in the formulation was 9.93 ±0.18 mg which when compared with the label claim (10mg) using a one-sample t-test [71] showed no significant difference ($p=0.28$). Moreover, for more demonstration of the reliability of the proposed CL method, a comparative fluorescence assay [72] for DXR in lyophilized powder for injection was carried out. The obtained results of the comparison method [72]were in good agreement with those obtained from the proposed CL assay. The accuracy and precision of the fluorometric and the developed CL method were compared using the student's t-test and the variance ratio F-test [71] and there was no significant difference between the two methods regarding accuracy and precision (Table 2). All these results demonstrate the ability of the proposed method to measure DXR concentrations accurately and precisely in its pharmaceutical formulations and prove the reliability and applicability of the developed assay.

3.5. Application of ABEI-LA for CL determination of DXR in human serum

As mentioned previously, DXR is an anthracycline cytotoxic antibiotic that is used for the treatment of many types of cancers. Though, it should be used clinically with high caution since it could cause irreversible chronic cardiomyopathy and

congestive heart failure that are cumulative dose-dependent. The DXR-induced cardiotoxic effects could be reduced through monitoring of its peak plasma concentration and adjusting the administration schedule to maintain this concentration with the therapeutic window [73]. The reported peak levels of DXR after intravenous administration is about 600 ng/mL (≈1μM) and it falls to 20-40 ng/mL (≈35-70 nM) within 10 h after administration [74,75]. These concentrations could be easily monitored by our proposed CL assay as our linear range is from 1-200 nM. Hence, we extended the applications of our method to the determination of DXR in human serum. As liquid-liquid extraction is among the most facile used extraction methods for biological fluids [76], we adopted it for the extraction of DXR. The serum samples were spiked with DXR, followed by extraction with a chloroform-methanol mixture (4:1, v/v) as reported previously by Daeihamed et al. [55]. Then, different concentrations of DXR, extracted from serum samples, were analyzed by the developed CL assay using ABEI-LA as a probe. The CL profiles of blank serum, blank water, and standard DXR and spiked serum samples are shown in Fig. S5. The blank serum showed no significant CL compared to the blank water demonstrating the specificity of the extraction method. The spiked serum CL profile was a little lower than that of the standard DXR, however, the recovery was acceptable. The recovery and repeatability of the analysis of DXR in serum were summarized in Table 2. The method showed acceptable recovery on average (87 %) with RSD% ≤4.3 %, demonstrating the suitability of the method for the determination of DXR in human serum.

All these results demonstrate the ability of the proposed method to measure DXR concentrations correctly in its pharmaceutical formulations (Table 1) as well as serum samples (Table 2) with very good accuracy and precision using simple equipment, a luminometer, and short measurement time, 3 min.

3.6. Comparison of the performance of the developed method with previously reported methods for DXR

Many analytical methods were reported for the determination of DXR in the literature and these methods included batch colorimetric [77], FL [72,78–80], CL [81,82], and electrochemical [83] assays, in addition to a capillary electrophoresis method with UV detection (CE-UV) [84], and several chromatographic methods with

UV [85,86], FL [55,74,75], electrochemical (ECD) [75], and CL [36] detections. The analytical performances of these methods were compared with the proposed CL assay probe, ABEI-LA, and the comparison is illustrated in Table 3. These methods suffered from many drawbacks such as very low sensitivity [77,85,86], high solvent consumption [36,55,74,75,84–86], dependence on quenching signal [79,80,82] decreasing their precision and reliability, need for relatively complicated instrumentation [36,81,84], tedious synthesis of the sensing material [81,83,84], and poor selectivity [81]. Comparing the sensitivity of our method with these previously reported ones, our developed CL assay was found to be 340-5100 times more sensitive than the batch colorimetric [77], HPLC-UV methods [85,86], native and CdTe/CdS quantum dots based FL methods [72,78,79]. Compared to HPLC-FL [55,74,75], HPLC-ECD [75], and molecular imprinted polymer permanganate CL methods [82], our assay was 47-100 times more sensitive than them. Furthermore, our method was about 4-6 times more sensitive than the CE-UV[84], HPLC-CL [36], luminol-ferricyanide CL [81], quantom dots based FL [80] and electrochemical [83] methods. In addition to the exceptional sensitivity of our method, it is also highly selective to quinones as it is based on their unique redox cycle. Furthermore, our probe synthesis was facile and performed through simple one-pot reaction. At last, our method did not use organic solvents in the assay and the used procedure and instrumentation are very simple rendering it an excellent environmental green approach for determination of quinones.

4. Conclusion

A unique novel CL probe for quinones, namely, ABEI-LA was successfully developed. When quinones are mixed with ABEI-LA, an intense glow type CL is generated proportional to the quinones concentration. ABEI-LA has a dual function in the CL reaction with quinones, a redox initiation function and reactive oxygen species detection ability. At first, LA moiety reacts with quinones through their redox cycle ability, in presence of dissolved oxygen, generating superoxide anion. Then, ABEI moiety, which is a luminol derivative, generates intense CL upon reaction with the firstly generated superoxide anion. Thus, ABEI-LA is the first probe with the ability to generate CL with quinones in a one-step procedure. The proposed CL probe is selective to quinones as it relies on their unique redox cycle ability. As a demonstration of the

applicability of the proposed probe, it was applied for the determination of the model anticancer quinone compound, DXR, in its lyophilized powder for injections and human serum samples. The proposed method showed ultrasensitivity (25 pg/assay, the most sensitive assay for DXR), good recovery (84-101), and acceptable precision (RSD≤6.9). In addition, ABEI-LA is the first single CL probe for quinones, which in addition to its exceptional sensitivity and selectivity, it is also time and labor-saving. In the end, we can conclude that ABEI-LA is an excellent CL probe for simple, rapid, and sensitive detection of quinones that could be of great value in studying quinones in different fields with various matrices.

Supplementary Material:

Mechanism of the redox cycle of the chemi-luminogenic reaction of quinones with luminol and reductants and the FAB-MS spectrum of isoluminol-lipoic acid (IL-LA).

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Table 1. The data of the accuracy and precision study for DXR CL analysis in the standard solution.

Validation point	DXR concentration (nM)	Accuracy (%)	Intra-day Precision (%RSD*)	Inter-day Precision (%RSD*)
LLOQ	1.0	95.4	3.1	5.4
MOQ	2.0	97.7	4.9	6.7
	20	101.1	2.0	4.3
HOQ	200	98.2	3.1	5.1

* Number of measurements (n=5)

Table 2. The data of the accuracy and precision study for DXR CL analysis in lyophilized powder for injection using the designed ABEI-LA probe and a comparison reference fluorometric method.

Proposed method					The comparison fluorometric method		
Validation point	DXR concentration (nM)	Accuracy (%)	Intra-day Precision (%RSD*)	Inter-day Precision (%RSD*)	DXR concentration (nM)	Accuracy (%)	Precision (%RSD*)
LLOQ	1.00	95.3	4.3	5.6	500	98.2	1.2
MOQ	2.0	97.6	2.7	6.9	1000	106	1.2
	20.0	101	4.2	5.3	2000	100	0.31
HOQ	200	99.2	3.5	5.7			
Labeled claim (10 mg)		Found 9.83±0.24			Found 10.14±0.4	t-test (p)** 0.2526	F-test (p)** 0.4153

* Number of measurements (n=5)

** t-test and F test were done on the found labeled claim using the developed method and the comparison fluorometric method and the p-value was higher than 0.05 indicating no significant difference.

Table 3. The data of the recovery and repeatability study for DXR CL analysis in human serum using the designed ABEI-LA probe.

Validation point	Concentration expected (nM)	Concentration found (nM)	Recovery* (%)	RSD* (%)
LLOQ	1.00	0.86	86.0	3.4
MOQ	20.0	18.2	91.0	2.8
	40.0	33.8	84.5	3.3
	100	88.7	88.7	4.3
HOQ	200	167.3	83.7	1.9

* Number of Measurements (n=5)

Table 3. Comparison of the proposed method with the previously reported ones for the measurement of DXR.

Method	Sensing compound	Reaction time or run time	LOD	Matrix	Remarks	Ref.
Colorimetric (four methods)	- Folin Ciocalteu - Fe(III) +1.10-ortho-phenanthroline - Folin-Ciocalteu - Periodate +3-methyl-2-benzothiazolinone hydrazone - Periodate +phenylhydrazine + ferricyanide.	15-37 min	34-420 ng/mL	Powder for injection	- Poor sensitivity - Some methods required boiling - Relatively long reaction time	[77]
FL assay	Native	none	(200 ng)	Aqueous matrices and Plasma	- Low sensitivity - Non validated	[72,78]
FL assay	CdTe/CdS quantum dots	none	90 ng/mL	Human serum and urine samples	- Based on FL quenching and DXR itself is FL which decreases their reliability. - Tedious probe synthesis - Sample pretreatment included 100 dilutions (low sensitivity)	[79]
FL assay	Carbon quantum dots	6 min	0.4 ng/mL	Human serum		[80]
HPLC-UV	None	10-15 min	500 & 510 ng/mL	Pharmaceuticals or rat plasma	- Use a high amount of solvent for chromatographic separation - Need a relatively long time for column conditioning and run time - HPLC-UV has very poor sensitivity - The HPLC-CL method Needs photosensitization, post-column reaction, and relatively complicated instrumentation	[85,86]
HPLC-FL	None	10-15 min	5 & 10 ng/mL	Human or rat plasma		[55,74]
HPLC-FL & HPLC-ECD	None	15 min	6.3 ng/mL	Human plasma		[75]
HPLC-CL	Photoirradiation + Bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl] oxalate	15 min	0.9 nM	Rat plasma		[36]

Method	Sensing compound	Reaction time or run time	LOD	Matrix	Remarks	Ref.
CE-UV	None	10 min	1 nM	Human plasma	- Need time for the separation of the analytes - relatively complicated instrumentation - Use a sweeping technique for sample preconcentration which increases the noise.	[84]
Electrochemical Cyclic-voltammetry	Multi-walled carbon nanotubes/poly-L-lysine	None	1 nM	Human plasma	- Expensive electrode material - Laborious electrode manufacture	[83]
CL assay (FIA)	Luminol + ferricyanide.	Few seconds	0.43 ng/mL	Rat plasma	- Non-selective assay - relatively complicated instrumentation	[81]
CL assay	Molecular imprinted polymer/ N-doped graphene quantum dots + permanganate	Few seconds	4.7 ng/mL	Human serum	- Expensive sensing material - Laborious probe synthesis - Use a high concentration of toxic acid (2.0 M Sulfuric acid) - Use a quenching signal for analysis, decreasing method reliability.	[82]
CL assay	ABEI-LA	3 min	0.17 nM ≈ 0.1 ng/mL (0.025 ng)	Powder for Injection and human serum	- Ultrasensitivity and high selectivity - One step simple probe synthesis. - Environmentally benign method (organic solvent-free and small amounts of reagent).	This work

*

Figures Captions

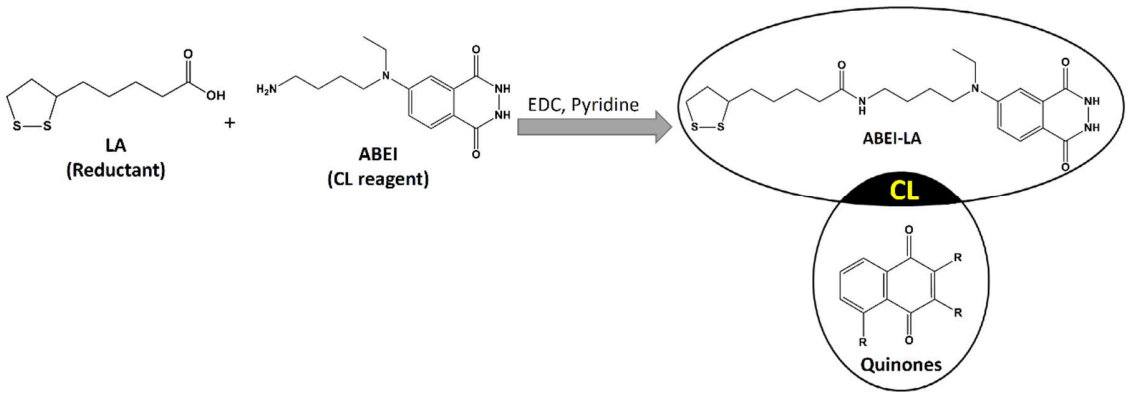


Fig. 1. The concept of the novel CL analytical reagent for quinones, ABEI-LA.

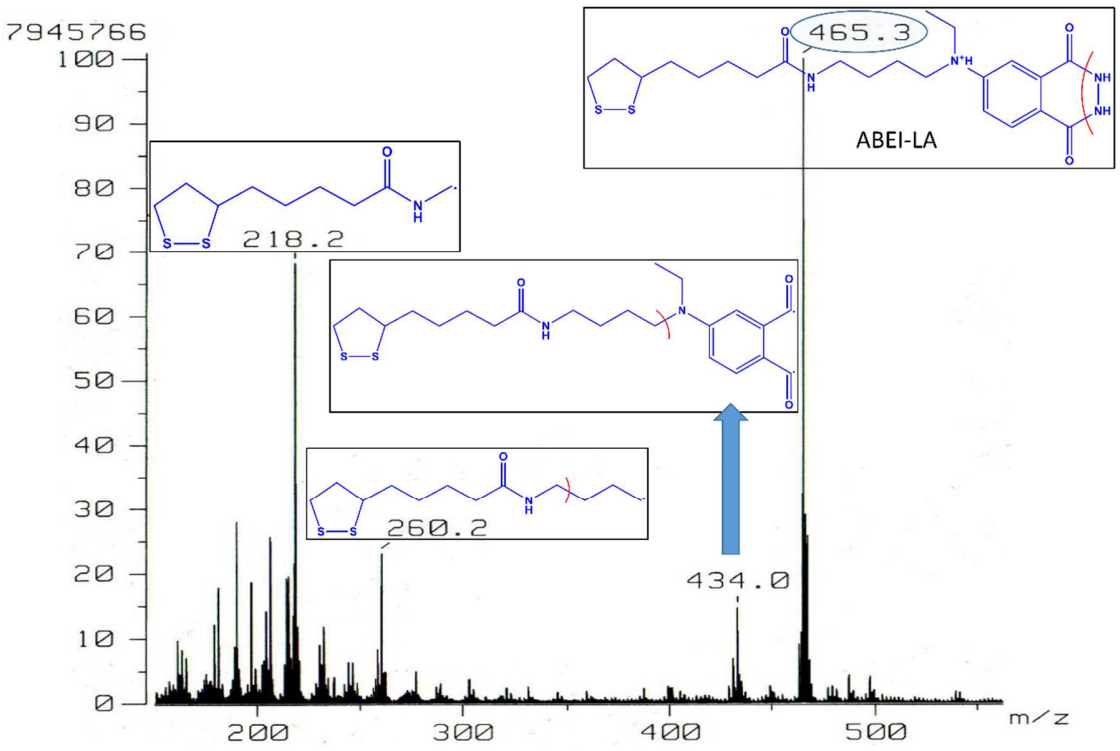


Fig. 2. FAB-MS spectrum of ABEI-LA

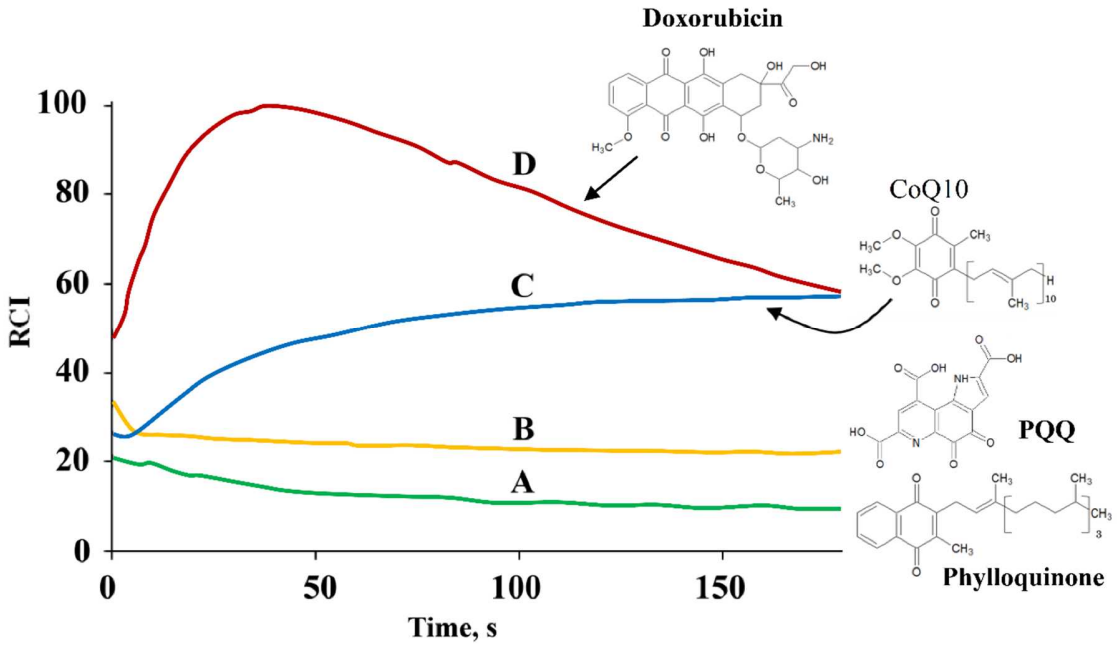


Fig. 3. Time profiles of CL spectra obtained from (A) phylloquinone, (B) PQQ, (C) CoQ10, and (D) doxorubicin. The solvent of phylloquinone, PQQ, and CoQ10 was ethanol, while doxorubicin was dissolved in water.

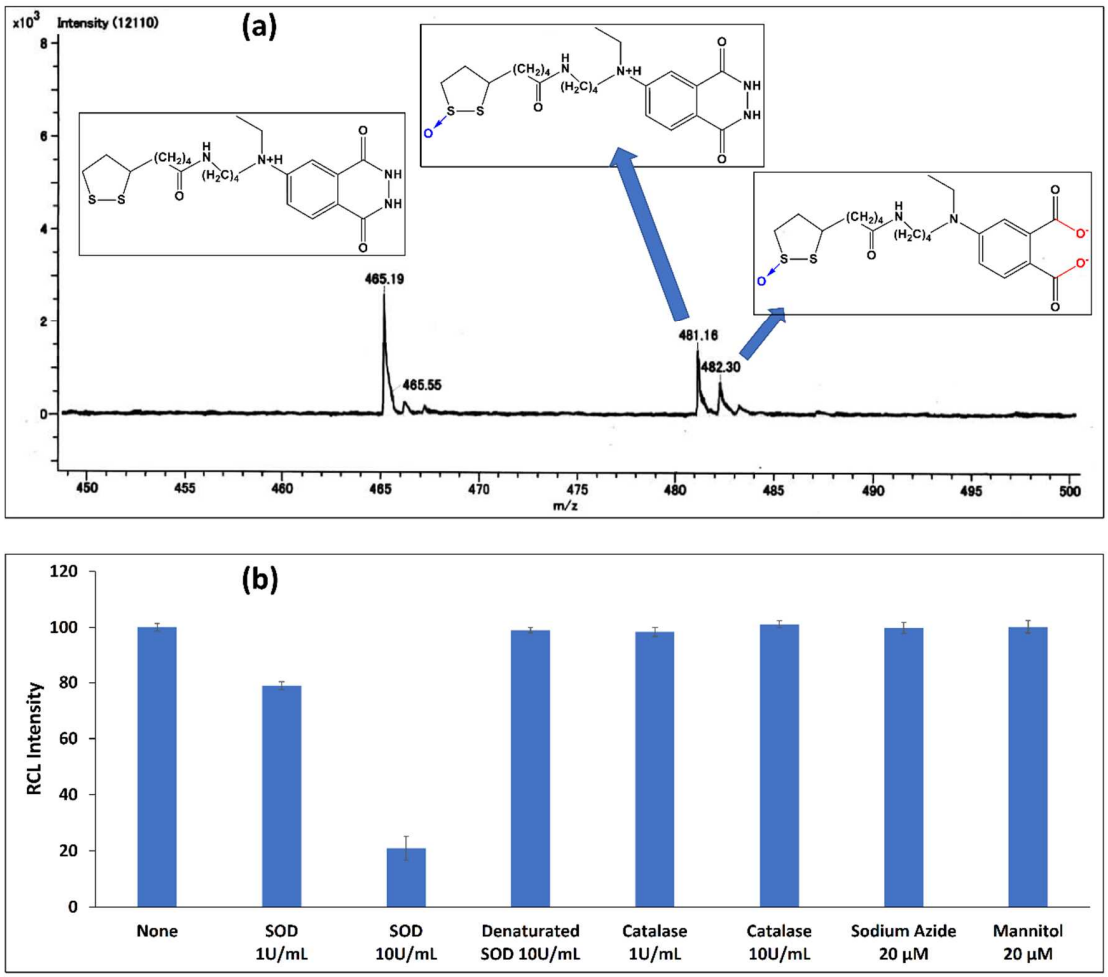


Fig. 4. Study of the chemiluminogenic reaction mechanism where (a) the ESI-mass spectra of the reaction mixture of DXR and ABEI-LA and (b) show the effect of different ROS scavengers on the CL reaction between DXR and ABEI-LA.

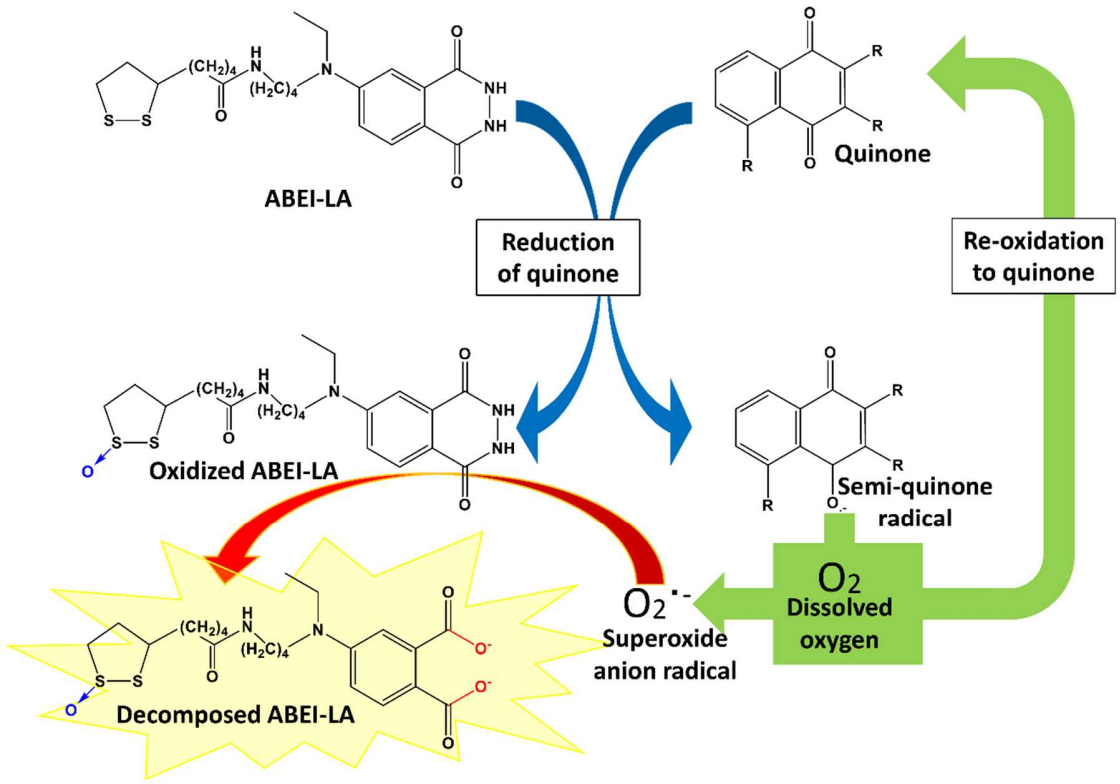


Fig. 5. Mechanism of the redox chemiluminogenic reaction between ABEI-LA and quinones.

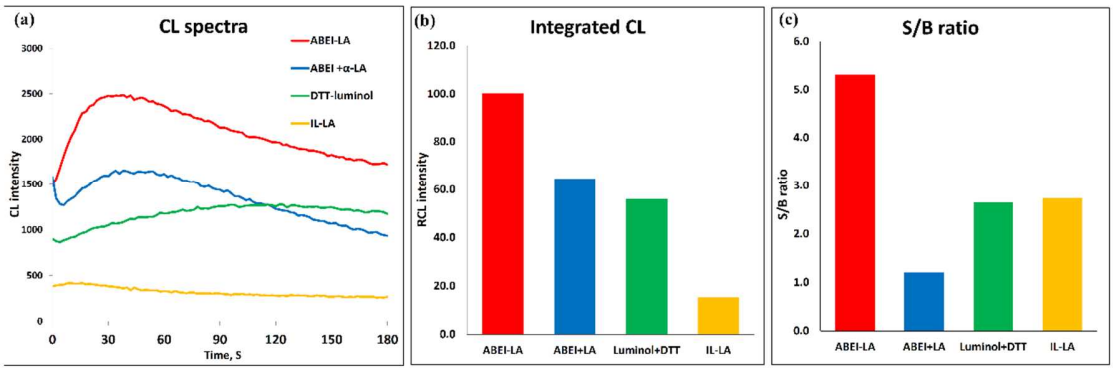


Fig. 6. The performance of ABEI-LA compared to other CL systems for quinones detection where (a) show the CL time profiles obtained using A; ABEI-LA, B; ABEI+LA, C; DTT+luminol, and D; IL-LA. While (b) show their integrated CL, and (c) show their signal to blank ratio (S/B). Concentrations of ABEI-LA and IL-LA are 400 µM 400, while concentrations of DTT, luminol, ABEI, LA are 400 µM.

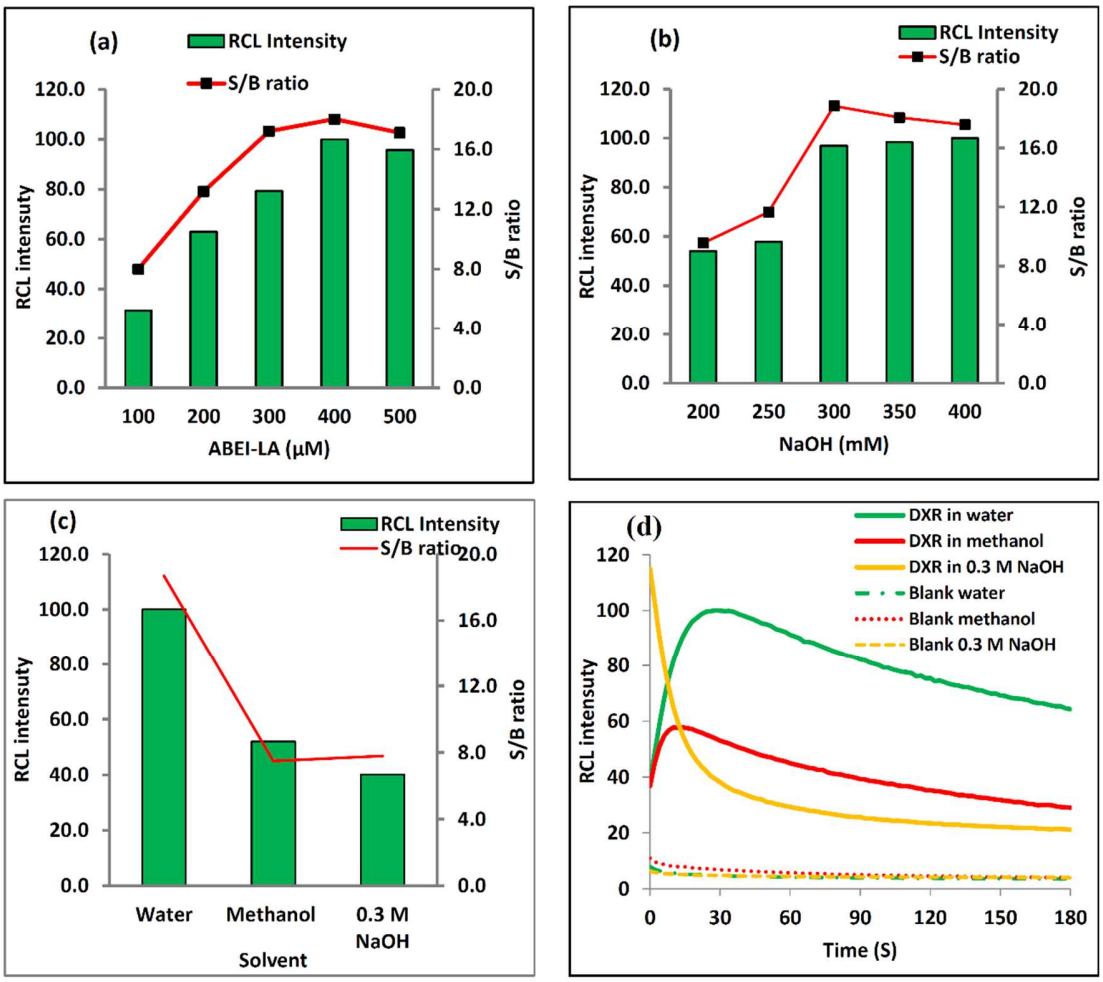
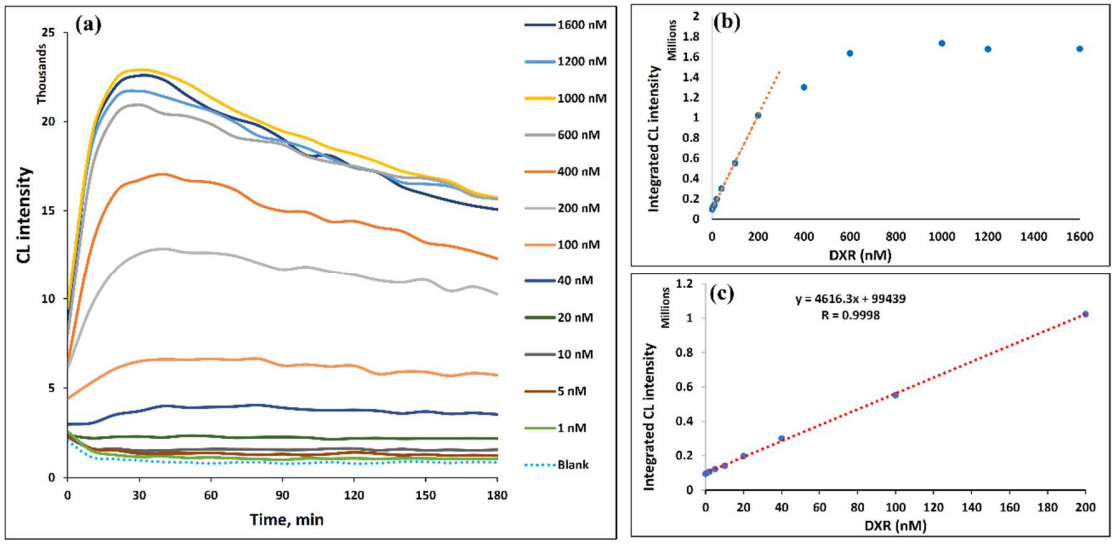


Fig. 7. Effects of reagents concentrations and solvents type on the CL reaction between ABEI-LA with DXR, where (a), (B) shows the effect of the concentrations of ABEI-LA and NaOH, respectively, on RCL intensity and S/B ratio, and (c), (d) shows the effect of DXR solvent of on RCL intensity and S/B ratio and CL spectra time profile, respectively.



Figs. 8. The CL spectra time profile of DXR (0-200 nM) (a) its saturation curve (b), and its calibration graph (c) using the ABEI-LA probe under the optimum measurement conditions.

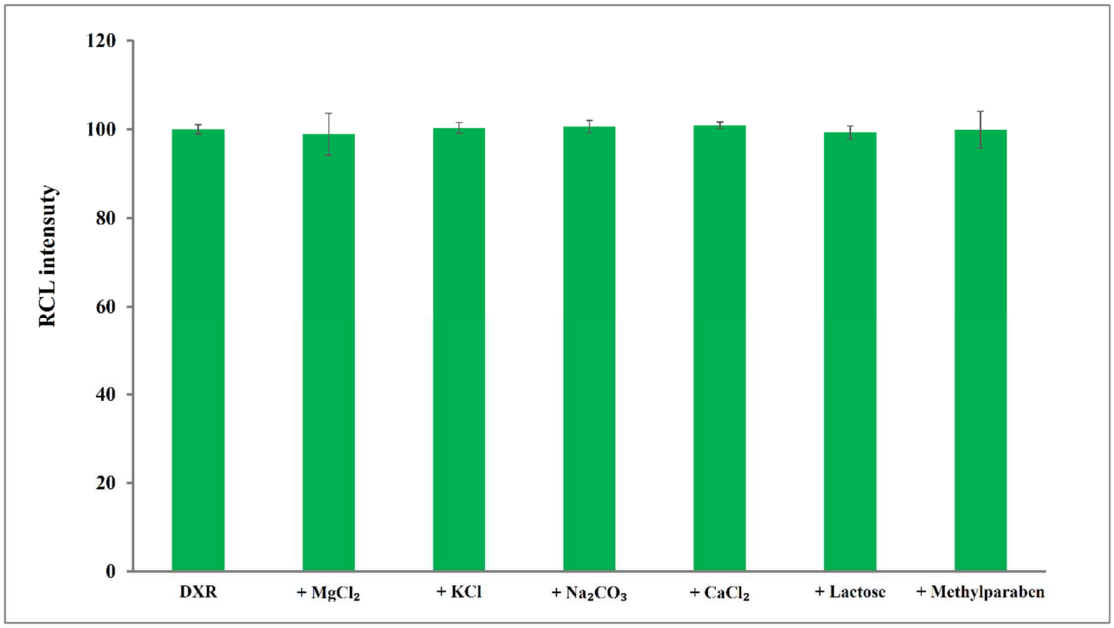


Fig. 9. Effects of possible additive on CL of doxorubicin.

Supplementary Material

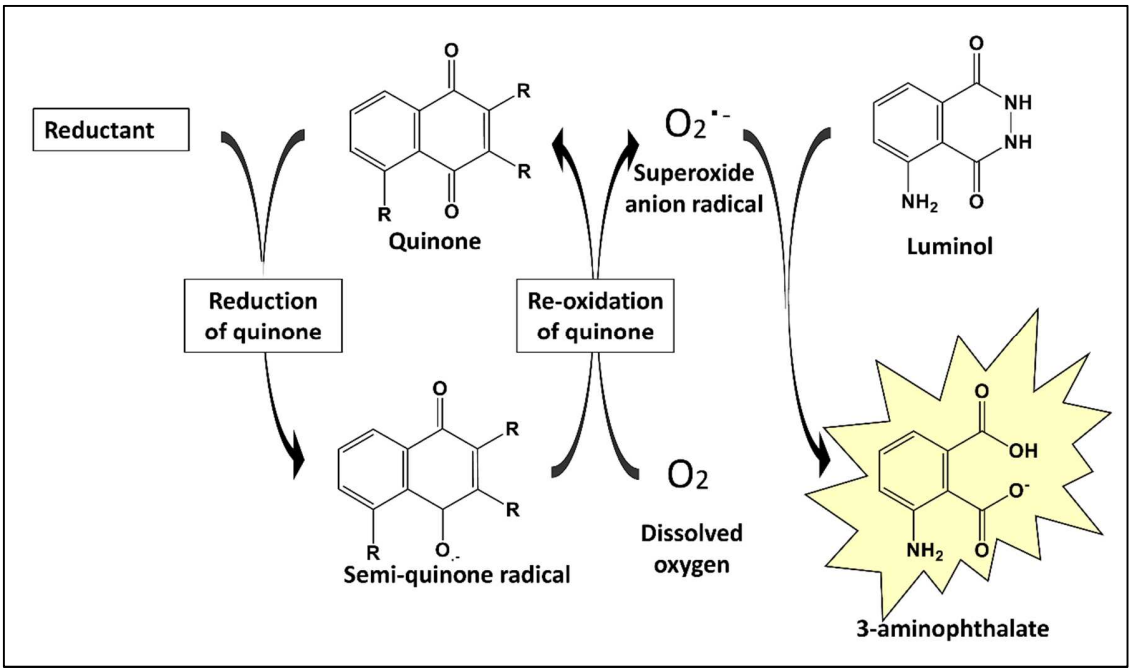


Fig. S1: Mechanism of the redox cycle of the chemi-luminogenic reaction of quinones with luminol and reductants.

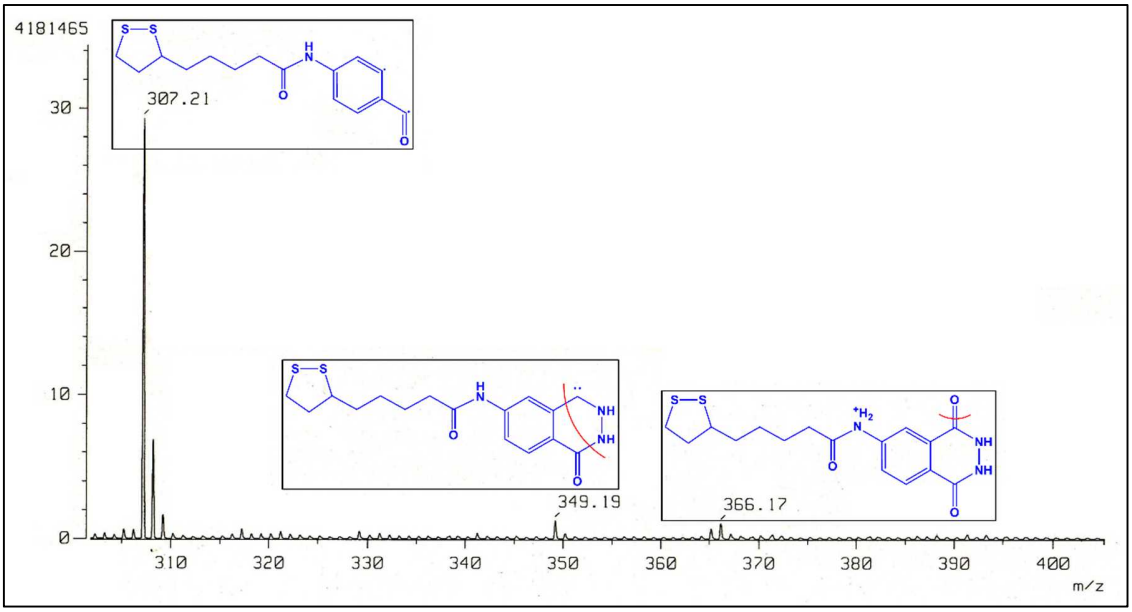


Fig. S2. FAB-MS spectrum of isoluminol-lipoic acid (IL-LA) showing molecular ion peak at 366.17 correspondings to $[M+H]^+$ of IL-LA.

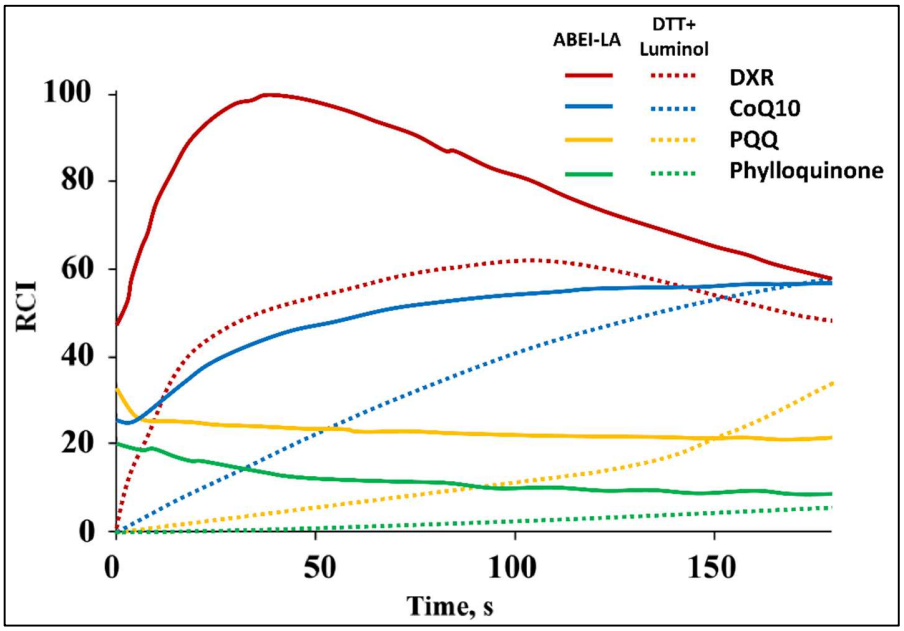


Fig. S3. The CL time profiles of the studied quinones upon their reaction with ABEI-LA and luminol/DTT mixture. The reaction of the studied quinones with luminol, and DTT was carried out following reference 8, where 100 µL of quinones (200 nM) and luminol (150 µM luminol dissolved in 6 mM NaOHaq) were mixed in a small test tube followed by injection of 100 µL of DTT (50 µM) then the CL time profile was measured.

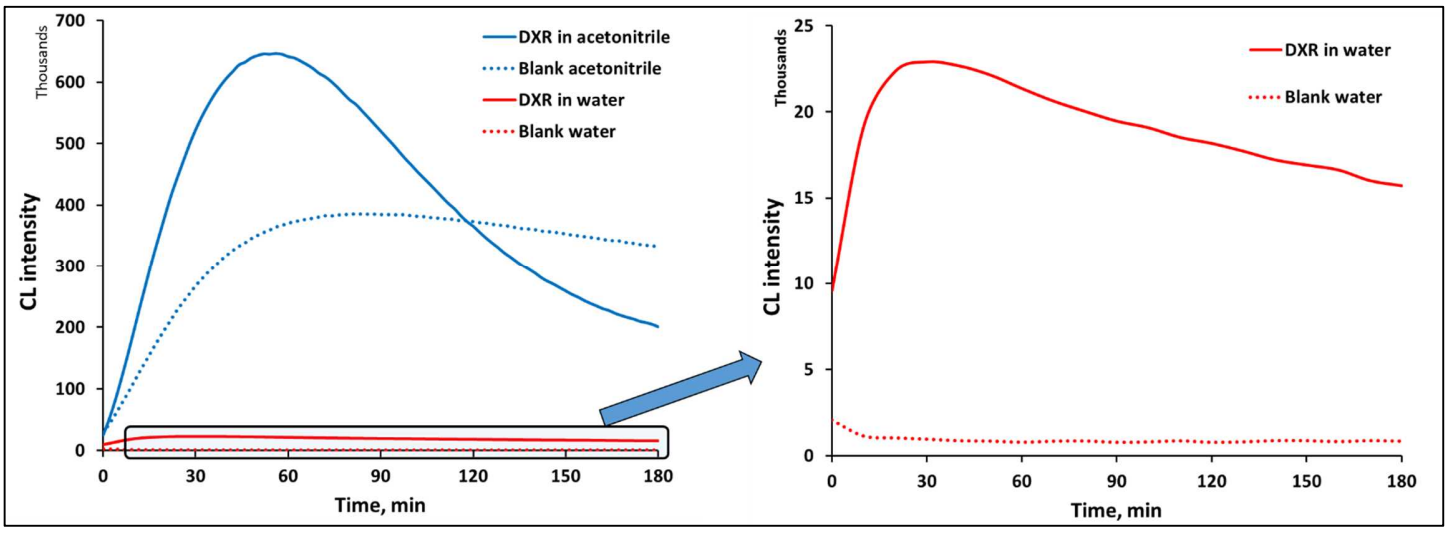


Fig. S4. The CL time profiles of blank and DXR standard (1 µm) dissolved water or acetonitrile upon its reaction with ABEI-LA

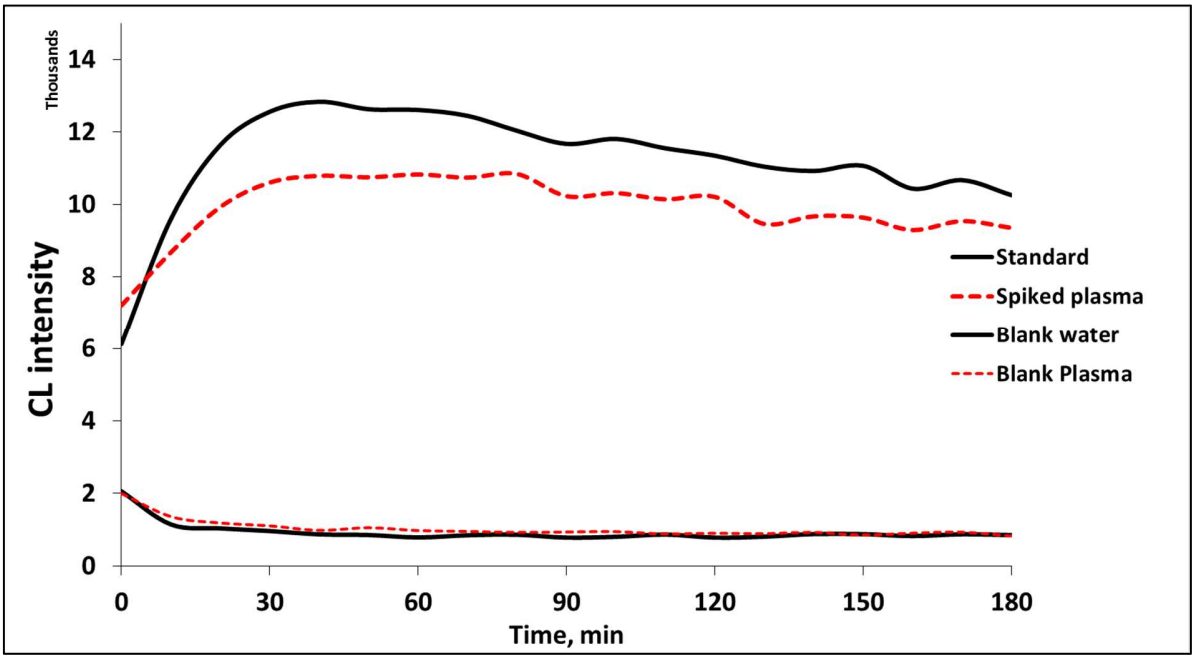


Fig. S5. The CL time profiles of blank water and blank extracted serum and DXR standard and extracted spiked serum, (200 nm) upon its reaction with ABEI-LA.