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Bioluminescence detection of superoxide anion using aequorin

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Abstract

Although the superoxide anion ($O_2^{-\bullet}$) is generated during normal cellular respiration and has fundamental roles in a wide range of cellular processes such as cell proliferation, migration, apoptosis and homeostasis, its dysregulation is associated with a variety of diseases. Regarding these prominent roles in biological systems, the development of accurate methods for quantification of superoxide anion has attracted tremendous research attentions. Here, we evaluated aequorin, a calcium-dependent photoprotein, as a potential bioluminescent reporter protein of superoxide anion. The mechanism is based on the measurement of aequorin bioluminescence, where the lower the concentration of coelenterazine under the oxidation of anion superoxide, the lower the aequorin regeneration, leading to a decrease in the bioluminescence. The bioluminescence intensity of aequorin was proportional to the concentration of superoxide anion in the range from 4 to 40000 pM with the detection limit (S/N = 3) of 1.2 pM which was 1000-fold lower than that of chemiluminescence methods. The proposed method exhibited high sensitivity and has been successfully applied to the determination of superoxide anion in the plant cell samples. The results could suggest photoprotein-based bioluminescence system as a highly sensitive, specific and simple bioluminescent probe for *in vitro* detection of superoxide anion.

Keywords**:** Aequorin; Photoprotein; Bioluminescent; Coelenterazine; Superoxide anion; Reactive oxygen species (ROS)

Introduction

In a biological context, reactive oxygen species (ROS), as natural by-products of the normal metabolism of oxygen, have significant roles in cell signaling and subsequent a wide range of cellular processes including cell proliferation, migration, apoptosis and homeostasis.[1–4] However, some environmental stresses (e.g., UV or heat exposure), lead to a significant increase in ROS level.[5] This may cause serious damage to cellular structures and predispose cells to malignant transformation.[6–10] Taken together, this is known as oxidative stress which is thought to play a significant role in the initiation and progression of neoplastic, cardiovascular, renal and neurodegenerative diseases.[11] Moreover, ROS production in plants is strongly influenced by some harsh environmental conditions, including salinity stress, drought, nutrient deficiency, metal toxicity, chilling, UV-B and ionizing radiation.[12,13]

Enzymatic electron transfer from a reductant to molecular oxygen leads to production of oxygen-derived free radicals in the body. As the products of a one-electron reduction of oxygen, superoxide anions ($O_2^{-\bullet}$), are the precursors of most other ROS. The reaction of $O_2^{-\bullet}$ with nitric oxide yields the highly aggressive oxidant peroxynitrite ($ONOO^-$) which largely limits nitric oxide bioavailability. Superoxide dismutases (SOD) gently catalyze the dismutation of $O_2^{-\bullet}$ to hydrogen peroxide (H_2O_2) in the body. In the presence of trace metals, specially copper and iron, H_2O_2 and $O_2^{-\bullet}$ can produce hydroxyl radicals ($OH^\bullet$) which excessively react with every biological molecule.[14] In various pathological conditions, several enzyme complexes, such as xanthine and NAD(P)H oxidases can be triggered in many cellular systems to generate large amounts of superoxide.[15,16] Due to the short half-life and high reactivity of ROS, direct *in vivo* detection of these radicals is really challenging. However, some molecules have been previously reported that react specifically with ROS and can be applied for tracing of them.[17] Enzyme activity assays,

fluorimetric, colorimetric, and luminescence-based assays are used to detect radicals.[18] Luminometric assays, in which light emission is enhanced in the presence of the studied radicals as inducer, are more sensitive methods and allow temporal resolution of the signal.[19,20] One of the main problems for detecting radicals is the lack of specific and sensitive methods to measure the oxidative stresses *in vivo* and *in vitro*. Different assays are developed to evaluate the generation of free radicals, However, each method shows significant limitations.[11]

As the prosthetic group of coelenterate and ctenophore photoproteins (e.g. aequorin and mnemiopsin), coelenterazine, could also act as a conventional luciferin in certain mysids, decapod shrimps, copepods, squid and fish.[21–23] Coelenterazine, an imidazopyrazinone molecule, is sequestered in the hydrophobic cavity of aequorin in a 2-hydroperoxycoelenterazine state.[24,25] Calcium binding to three Ca^{2+}-binding EF hand loops of aequorin causes a slight conformational change, leading to oxidation of the hydroperoxycoelenterazine to an excited coelenteramide. Relaxation of the excited coelenteramide proceeds radiatively, emitting the blue light at maximum wavelength of 470 nm.[26] The bioluminescence activity of these photoproteins depends on the presence of a tightly but non-covalently bound coelenterazine.[26] Since calcium ion directly contributes to the reaction at saturating concentration, the emission of light is proportional to the amount of the photoprotein. Photoproteins can be detected down to attomole levels with a range of linear response extending for several orders of magnitude.[27,28] Recombinant apophotoproteins are converted into photoproteins by incubation with coelenterazine without any folding problems. All these factors participated in the rapid development of photoproteins as bioluminescent reporters.[29–31] As a well-known bioluminescent photoprotein, aequorin is extensively used for a variety of analytical and biological applications, including as an intracellular calcium index,[32] gene expression studies,[33] drug discovery,[34] and as

a very sensitive reporter in immunological and binding assays.[35] Due to its negligible bioluminescence background, aequorin offers detection at very low concentration and no interference with biological fluids and matrices, resulting in very low detection limit. regarding this feature together with its non-toxicity, aequorin offer an advantage over the fluorescent or colorimetric alternatives in certain types of studies.[32]

The small molecule coelenterazine is known to react with the superoxide anion and peroxynitrite and generate a chemiluminescent signal.[23,36–39] Like bioluminescence, chemiluminescence generates its own emission and does not require excitation light. Accordingly, there is no background fluorescence except in the case of emission. Previous studies have indicated that chemiluminescence from coelenterazine can be used to measure physiological superoxide anion produced in living animal cells and in the cultured cells as well as the neutrophilic oxidative burst *in vitro*.[23,40,41] Coelenterazine is the substrate for the Gaussia and Renilla luciferases and often administered for bioluminescence imaging *in vivo.* Taken together, *in vivo* delivery of coelenterazine is considered safe.[42–44] However, due to the low chemiluminescence signal intensity, coelenterazine is not sensitive enough to detect low concentrations of superoxide anion. To overcome this limitation, a relatively high concentration of coelenterazine should be used. Here, we tried to improve the sensitivity of the method for measurement of superoxide anion using photoprotein-based bioluminescence system. As the most convenient method of generating $O_2^{-\bullet}$, xanthine/xanthine oxidase system was applied here to produce superoxide anions which convert coelenterazine to coelenteramide, followed by measuring the changes in effective concentration of coelenterazine by aequorin. More precisely speaking, coelenteramide, despite being capable of binding to apoaequorin, cannot produce bioluminescence. Consequently, the sensitivity of the assay can be increased by trapping the superoxide anion with coelenterazine

and then measuring this reduction by aequorin. The higher the superoxide anion produces in the reaction mixture, the lower the effective concentration of coelenterazine, leading to decrease in the bioluminescence of aequorin (scheme 1). This feature of the proposed method has also solved one of the most common problems in the measurement of superoxide anion which is very short half-life and conversion to other forms of ROS.

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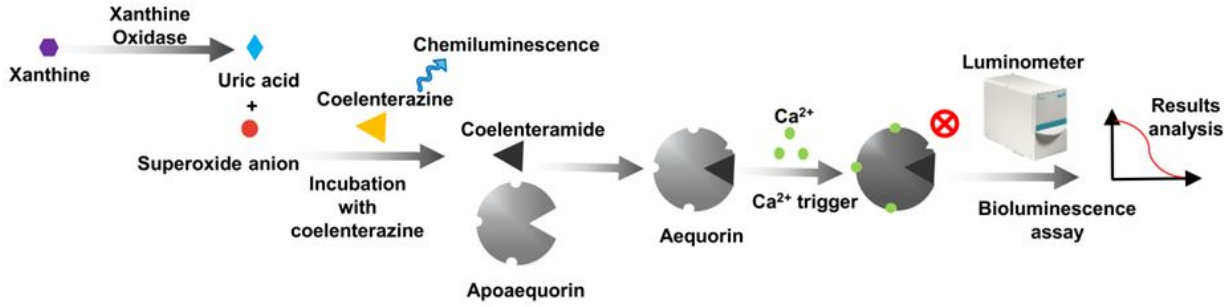

Scheme 1. Schematic illustration of superoxide anion assay using aequorin-based bioluminescence system. Considering that coelenterazine concentrations are important in the number of regenerated aequorin, any changes in the effective concentration of coelenterazine leads to change in the bioluminescence intensity.

Materials and Methods

Chemicals and reagents

Xanthine, xanthine oxidase and superoxide dismutase (SOD) were acquired from Sigma (St. Louis, MO, USA). The *cp*-coelenterazine was obtained from Resem BV (The Netherlands). Ampicillin and isopropyl-D-thiogalactopyranoside (IPTG) were purchased from Bio Basic Incorporation (Markham, Ontario, Canada). The Ni-NTA agarose was provided by Qiagen

(Qiagen, Hilden, Germany). All the other chemicals were obtained from Merck (Darmstadt, Germany).

Expression and purification of apoaequorin

The recombinant pET21a containing apoaequorin gene was transformed into *Escherichia coli* BL21 (DE3) expression host. Following induction of His-tagged protein expression by adding 1mM IPTG for 4 h at 20 ˚C, cells were harvested by centrifugation and lysed by sonication. Afterwards, the recombinant protein was purified by Ni-NTA agarose column described previously.[45]

Preparation of semi-synthetic aequorin

To generate semi-synthetic aequorin from recombinant apoaequorin, the purified apoaequorin was diluted with 50 mM Tris buffer (pH 7.6) containing 5 mM EDTA (buffer 1). This solution was mixed with a given volume of *cp*-coelenterazine at a final concentration of 0.1 μM. The regenerated mixture was briefly mixed and then incubated at 4 ˚C for 16 h.

Bioluminescence measurement

Bioluminescence emission intensities were recorded using Sirius L Tube Luminometer (Titertek-Berthold, Germany) and the peak emission intensity was plotted. 100 μL of semi-synthetic aequorin in 50 mM Tris buffer with pH 7.6 (buffer 2) at a concentration of 0.1 μM was pipetted into a 1.5 ml microtube. The luminescence intensity was measured after injecting 100 μL of buffer containing 50 mM Tris and 100 mM $CaCl_2$ (buffer 3) for 5 sec with 0.05 sec time interval.

Production and measurement of superoxide anion

Xanthine/xanthine oxidase system was used to produce superoxide anion. Reaction mixtures contained 0.1-0.5 U/ml of xanthine oxidase and 0.0006-600 μM of xanthine in 50 mM Tris buffer (pH 7.0). The production of uric acid was measured as a function of time by monitoring the increase in absorption at 290 nm using a UV S-2100 spectrophotometer. The concentration of superoxide anion was determined according to the stoichiometry. To prepare various concentrations of superoxide anion, different concentrations of xanthine (up to 150 μM and 0.6 mM for spectroscopic and chemiluminescence methods, respectively) were used. After the addition of 0.5 U/ml xanthine oxidase, the concentrations of enzyme and substrate were optimized to complete the reaction after 10 minutes.

Chemiluminescence detection of superoxide anion using coelenterazine

Chemiluminescence detection of superoxide anion was carried out according to the method of Saleh and Plieth.[37] 50 μM coelenterazine was added to the reaction mixture containing different concentrations of xanthine (0.6 nM to 0.6 mM) and 7 mU/ml xanthine oxidase, followed by measuring the bioluminescence intensity over 5 min.

Bioluminescence detection of superoxide anion using aequorin

In this method, the concentrations of xanthine and xanthine oxidase were the same as mentioned in the chemiluminescence method. Coelenterazine was added at a final concentration of 0.1 μM to the reaction mixture. After completing the enzyme reaction in 10 min, apoaequorin was added to the reaction mixture at a final concentration of 0.1 μM and incubated at 4 °C for 16 h. The bioluminescence intensity of aequorin was measured by injecting 100 μl of buffer 3.

Measurement of superoxide anion in real sample

The applicability of the method for evaluating the concentration of superoxide anions in real samples was assessed using *Nicotiana tabacum* cell. A magnetic field of 0.2 Tesla was used to induce the production of superoxide anion.[46] 200 mg of plant cell was ground into fine powder with liquid nitrogen. Further grinding was done in buffer 1. Following centrifugation of the homogenates (14000 g, 15 min at 4 °C), 100 µl of the supernatant was mixed with coelenterazine at final concentration of 0.1 µM. Apoaequorin was added to the reaction mixture after 10 min, and the concentration of superoxide anion was then evaluated according to the chemiluminescence and bioluminescence method described above. Also, as control, 25 unit/mL of SOD were added to the supernatant of treated sample and after 20 min, the coelenterazine was added to the mixture and the assay was performed as before.

Anion superoxide detection in spiked samples

To verify the accuracy and precision of the method, a certain concentration of anion superoxide (at a final concentration of 1 nM) produced by xanthine/xanthine oxidase system (3 nM xanthine and 0.5 unit/mL of xanthine oxidase) was spiked into the supernatant of untreated plant cell specimen in three replicates. The samples were analyzed following the assay procedure described above and the average recovery and variation coefficient value were calculated.

Interference studies

Different concentration of hydrogen peroxide (1 nM to 1 mM) was added to the reaction mixture. The concentration of superoxide anion was measured according to the method described above.

Effect of SOD on chemiluminescence and bioluminescence-based assay of superoxide anion

25 - 2.5 mU/ml of SOD was added to the reaction mixture, and all other assay conditions were the same as mentioned above.

Statistical analysis

The statistical analysis and curve fitting were performed using GraphPad Prism software (San Diego, CA). To analyze dose-response curves, a three parameters logistic equation (sigmoid) was used ($Y=Bottom + (Top-Bottom)/(1+10^{((X-LogIC_{50}))})$), where X and Y are the logarithm of concentration and response, respectively. Data are presented as the mean ± standard deviation (SD). Reproducibility of the data presented in this paper was confirmed by repeating the experiments at least three times.

Results and discussion

Optimization of superoxide anion production

In this study, the xanthine/xanthine oxidase system was used as the source of superoxide anion radicals (Figure 1A). The rate of the formation of uric acid from xanthine can be evaluated photometrically on the basis of UV absorbance at 290 nm.[47] Due to the mechanism of the reaction and the type of electron transfer, for every six uric acid molecules, two molecules of superoxide anion are produced, which is considered in the calculation of the superoxide anion concentration.[48] The production of superoxide anion was optimized considering to various parameters, including the reaction time, substrate and enzyme concentration to gain the maximum sensitivity of the bioluminescence system. Accordingly, the reaction was followed at 150 μM substrates and 0.1-0.5 U/ml enzymes in 30 min (Figure 1B). The results demonstrated that by using 150 μM xanthine and 0.5 U/ml xanthine oxidase, the reaction was completed in 10 min.

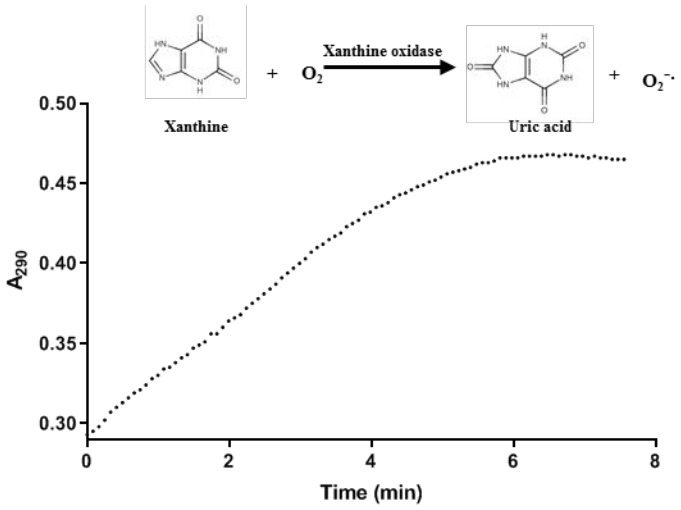

Figure 1. Progress curve of Xanthine oxidase and production of superoxide anion radicals by using xanthine as substrate. Oxidation of xanthine, to uric acid (UA) was catalyzed by xanthine oxidase, leading to formation of superoxide ($O_2^{-\bullet}$) radical. Xanthine oxidase utilizes xanthine and O_2 as substrate and produces uric acid and superoxide ($O_2^{-\bullet}$). The progress curve of the xanthine oxidase reaction in the presence of 150 μM xanthine and 0.1 U/ml xanthine oxidase by measuring the absorption of uric acid.

Superoxide anion assay by chemiluminescence method

As an alternative method for the above mentioned absorption assay and as a more sensitive method, chemiluminescence can be used for measuring superoxide anion.[37] Various parameters including reaction time, substrate and enzyme concentration were also investigated and optimized to perform the chemiluminescence method. As previously, the results show that when the superoxide anion reaches its highest level, the chemiluminescence of coelenterazine increases to maximum intensity (Figure 2A).

To determine the optimal concentration of coelenterazine and to investigate the linear relationship between the concentrations of superoxide anion and chemiluminescence intensity, different concentrations (25, 10, 5 and 1 μM) of coelenterazine were used. The results show that chemiluminescence intensity increases with increasing the concentration of coelenterazine, however, the concentration-dependent relationship does not differ significantly (Figure 2B). Finally, the lowest concentration of coelenterazine (1 μM), which showed the lowest EC_{50} (208 nM), was used as the optimal concentration for subsequent experiments. It should be pointed out that, the signal-to-noise ratio increases by reducing the concentration of coelenterazine, which decreases the quality of the measurement.

As depicted in Figure 2C, an obvious linear relationship has been observed between the concentration of superoxide anion (20-20000 nM) and the chemiluminescence intensity of coelenterazine (LOD 6 nM). As previously reported by Saleh et al, the activity of superoxidase was successfully measured by this method.[37] Since the signal intensity in this method depends on the concentration of coelenterazine, the relatively high concentrations of coelenterazine and high sensitive devices should be used for the assay, thereby limiting the use of this approach. In this study, to overcome the limitations of this method as well as to increase sensitivity, superoxide anion was measured using aequorin-based bioluminescence system.

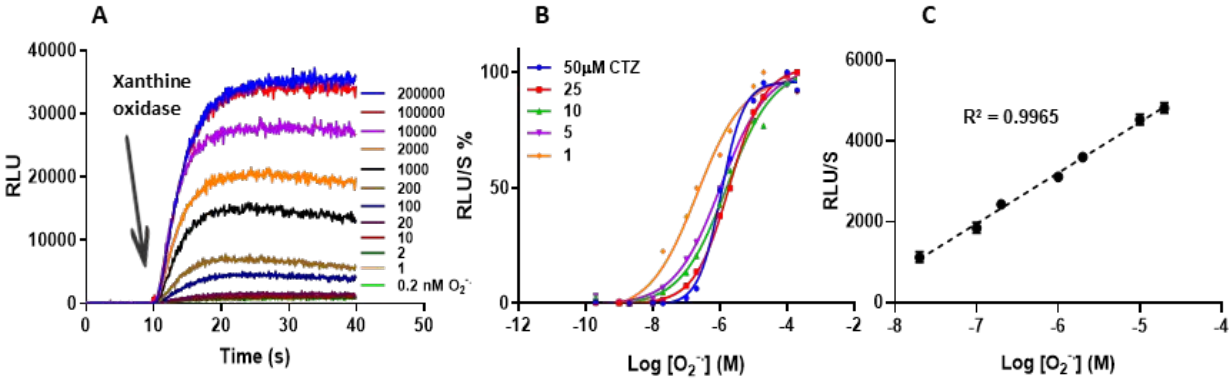

Figure 2. (A) Chemiluminescence kinetic curve of coelenterazine (25 μM) in the presence of different concentrations of superoxide anion. (B) A dose-response curve of coelenterazine chemiluminescence against different concentrations of superoxide anion produced by the xanthine/xanthine oxidase system. Chemiluminescence values of all samples were expressed as the percentage of the maximum chemiluminescence intensity (CTZ: coelenterazine). (C) The standard curve represents a linear relationship between RLU/S and different concentrations of superoxide anion for the optimal concentration of coelenterazine (1 μM).

Superoxide anion assay using photoproteins-based bioluminescence system

Given that the basis of the proposed method is the dependency of aequorin bioluminescence on coelenterazine concentration and by virtue of the applicability and suitability of coelenterazine for detection of superoxide anion, we next aimed to determine if aequorin-based bioluminescence system could detect changes in superoxide anion concentrations. Different amounts of superoxide anion were added to coelenterazine, followed by the addition of apoaequorin to form a holo-aequorine. The bioluminescence reaction could be triggered by the addition of calcium to the sample. Obviously, the more superoxide anion is produced in the reaction medium, the lower effective concentration of coelenterazine, leading to a decrease in the aequorin bioluminescence.

An inhibition curve for the superoxide anion was constructed by plotting bioluminescence inhibition of aequorin against the target concentration (Figure 3A). The IC_{50} of the bioluminescence was achieved at 430 pM; the limit of detection (LOD), which was defined as the inhibition concentration of 10% (IC_{10}), was 1.2 pM. The dynamic range was determined as

the analyte concentration generating between 20% and 80% of maximal inhibition. As shown in Figure 4B, the method exhibits excellent linearity (a correlation coefficient of 0.9834) within the range of 4–40000 pM with a calibration curve of Y = -61845X-453148. The sensitivity of the proposed bioluminescence method is approximately 1000-fold higher than those of the previously reported chemiluminescence-based method.

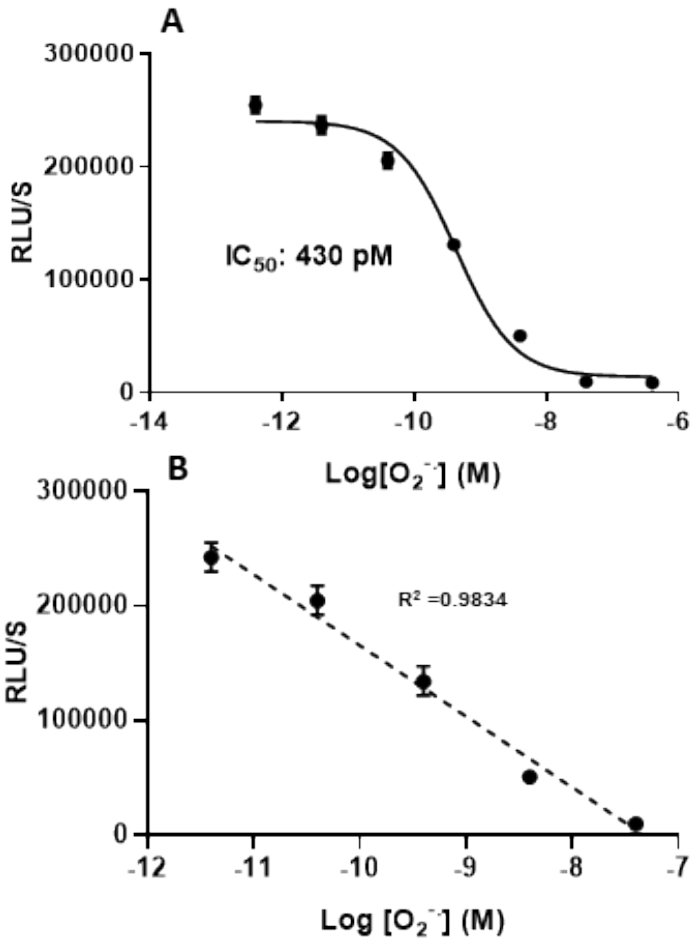

Figure 3. (A) Effect of different concentrations of superoxide anion on the bioluminescence intensity of aequorin. (B) The method is able to detect the superoxide anion in a linear range from 4 to 40000 pM.

The effect of SOD on chemiluminescence and bioluminescence-based assay of superoxide anion

Since the SOD specifically converts the superoxide anion to hydrogen peroxide, it can be used to demonstrate the specificity of the method after the production of superoxide anion by the xanthine/xanthine oxidase system in both chemiluminescence and bioluminescence methods. In the bioluminescence method, SOD suppresses its reaction with coelenterazine, converting it to coelenteramide and reducing the photoprotein emission by consuming anion superoxide. Thereby the effect of SOD on bioluminescence and chemiluminescence-based assay of superoxide anion was investigated to confirm that the changes of aequorin bioluminescence and coelenterazine chemiluminescence intensity is due to the increase in superoxide anion concentration not the other species. In chemiluminescence-based assay, after initiating the enzymatic reaction of superoxide anion production and reaching the maximum of the coelenterazine chemiluminescence intensity, different concentration of SOD was injected into the reaction mixture. As shown in Figure 4, immediately after the injection of SOD, a significant drop in chemiluminescence of coelenterazine occurs, which is proportional to the amount of enzyme concentration. In bioluminescence-based assay when coelenterazine was mixed with a reaction mixture containing xanthine and xanthine oxidase, the addition of aequorin led to a bioluminescent reaction. Since the consumption of superoxide anion by SOD prevents its effect on coelenterazine, the bioluminescent intensity of aequorin increases. Figure 5B shows the effect of different concentrations of SOD (25-0.0025 U/mL) on the reaction mixture of xanthine and xanthine oxidase. So the sensitivity of methods to SOD suggests that the changes in luminescent intensity are due to the increase in the superoxide anion concentration.

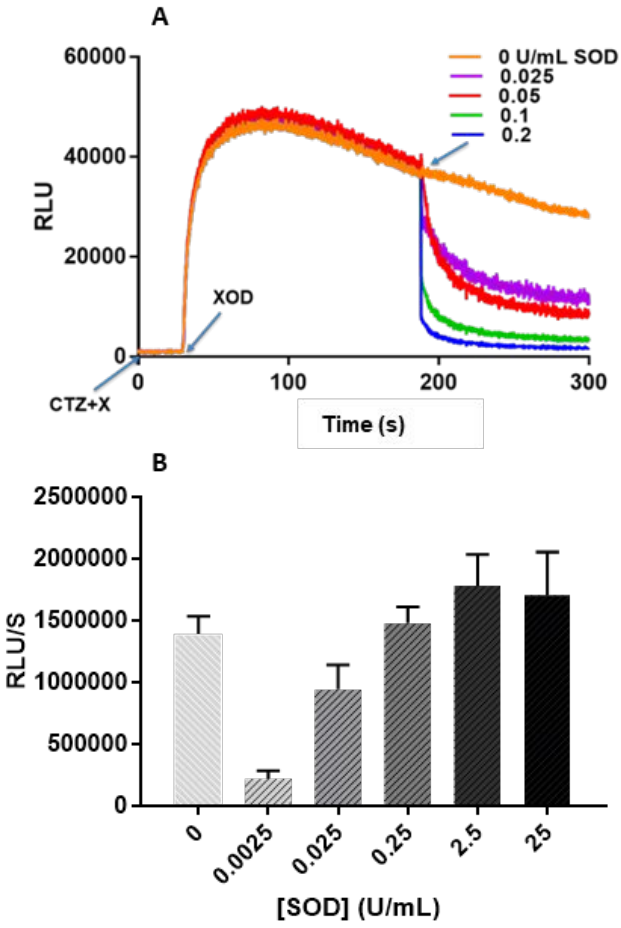

Figure 4. The effect of different amounts of SOD on (A) chemiluminescence and (B) bioluminescence-based assay of superoxide anion (CTZ: coelenterazine).

Interference study

Since the biological environments contain many other types of ROS in addition to superoxide anion, the specificity of the detection methods to measure each of these radical species has a great deal of importance. Here, the interference of H_2O_2, as the most common type of ROS, was investigated in the bioluminescence detection of superoxide anion. The results showed that H_2O_2 had no effect on aequorin bioluminescence intensity up to the concentration of 100 μM. Taking

into account its usual range in biological samples, H_2O_2 could not interfere with the detection method (Figure 5). In summary, the method exhibited favorable selectivity for the determination of superoxide anion.

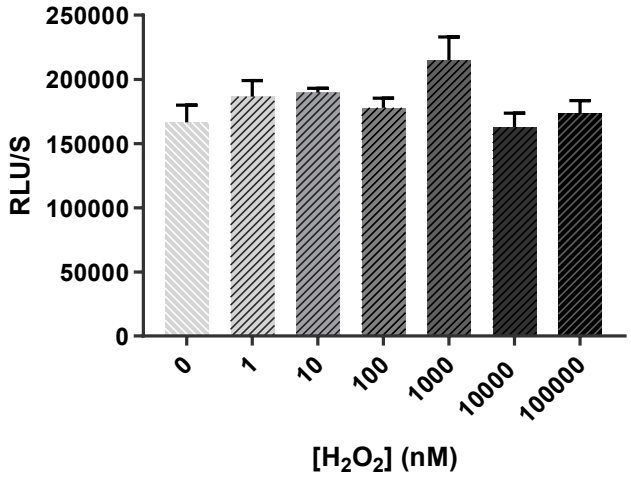

Figure 5. Effect of different concentrations of H_2O_2 on bioluminescence intensity of aequorin.

Determination of superoxide anion in real sample

To better understand the efficiency and feasibility of our approach versus chemiluminescence methods, incurred and negative plant cells of *Nicotiana tabacum* were analyzed using both the monitoring platforms. The magnetic field was applied to induce superoxide anion production in the cells as previous report [46]. Each sample was analyzed in triplicate and levels were determined using the calibration curve (Figure 3B). Initially, superoxide anion assay was performed using different concentrations of coelenterazine by the chemiluminescence method, which showed that the treated samples had higher chemiluminescence intensity than control due to the higher level of superoxide anion in these samples. As shown in Figure 6A, the higher the concentration of

coelenterazine, the greater the chemiluminescence intensity. However due to the lower sensitivity (LOD = 6 nM) of the chemiluminescence method, it is not possible to quantify this level of anion superoxide produced in the treated sample by this method because this value is not in the linear range of the chemiluminescence method and the level of superoxide anion in the treated and untreated groups can only be evaluated for qualitative comparison.

Subsequently, for comparative purpose, the amount of superoxide anion in samples was evaluated using aequorin-based bioluminescence method (Figure 6B). The concentration of superoxide anion in untreated and treated samples was determined 0.1 and 1.17 nM respectively by bioluminescence method. As shown in Figure 6B, the difference between treated and controlled samples can only be detected at high concentrations of coelenterazine in chemiluminescence method and the signal intensity is nearly 10 times lower than that of the proposed method. These results confirm the usefulness of aequorin as a very specific and sensitive bioluminescence probe for superoxide anion and this is one of the advantages of our method over the chemiluminescence methods that can detect this low level of anion superoxide.

Meanwhile, in both luminescence and bioluminescence methods the SOD-treated group was investigated to determine the contribution of superoxide anion to the produced signal. Interestingly, it has been shown that the major contribution of ROS produced in cells was due to superoxide anion.

Accuracy was next examined using the recovery of spiked sample. Parallel samples were prepared and determined by the method. Resulting levels in the spiked samples were deducted from the control sample and then quantified using the calibration curve. The calculated recovery value and CV are 90.5 ± 3.9% and 7.1%, respectively. These results revealed that the precision

of the aequorin-based bioluminescence method for superoxide anion quantification was acceptable.

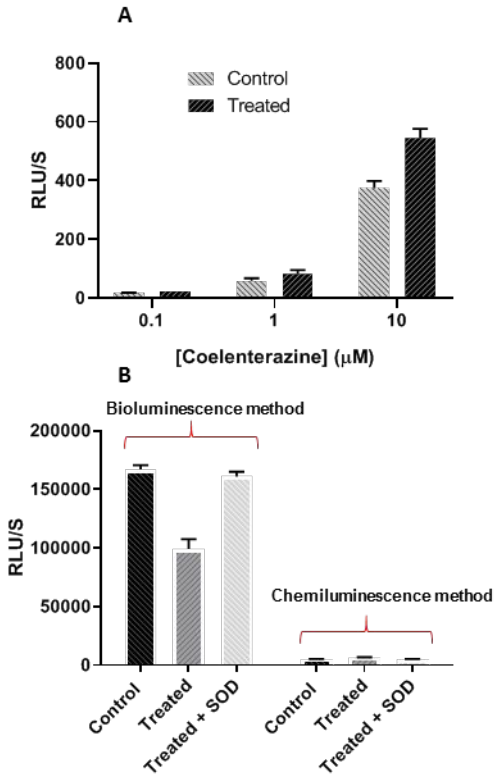

Figure 6. Superoxide anion determination by coelenterazine-based chemiluminescence assay using 10, 1 and 0.1 μM of coelenterazine (A) and aequorin-based bioluminescence method (B) in plant cells of *Nicotiana tabacum* under magnetic field stress versus untreated and SOD treated samples. The concentration of 0.1 μM coelenterazine was used for both methods.

Conclusion

Here, the dependency of aequorin bioluminescence to coelenterazine, was used to measure superoxide anion, as one of the common species of ROS. These results indicate the usefulness of aequorin as a bioluminescent indicator for assaying superoxide anion. The proposed method has

advantages like requiring neither high concentrations of coelenterazine nor a large sample volume. Furthermore, it seems to be specific to superoxide anion, as may be concluded from the influence by SOD. The plotted parameters are linearly correlated when the concentration of superoxide anion is between 4-40000 pM with LOD of 1.2 pM. Sensors for biological molecules are most useful when they can be applied in biological environments. The photoproteins-based bioluminescence system provides that opportunity by measuring the superoxide anion in plant cells in which superoxide anion was produced through a magnetic field. Comparison between the linear range of bioluminescence and chemiluminescence methods shows that the proposed method is more sensitive for determining the concentration of superoxide anion. Also, due to the use of lower concentrations of coelenterazine, our method seems to be more cost-effective than the chemiluminescence method so that the production signals are increased by aequorin. Collectively, these results establish a new method allowing simple, selective and quantitative detection of superoxide anion which can easily be applicable for real samples.

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References

(1) Arnold, R. S.; Shi, J.; Murad, E.; Whalen, A. M.; Sun, C. Q.; Polavarapu, R.; Parthasarathy, S.; Petros, J. A.; Lambeth, J. D. Hydrogen Peroxide Mediates the Cell Growth and Transformation Caused by the Mitogenic Oxidase Nox1. *Proc. Natl. Acad. Sci.* **2001**, *98*, 5550–5555.

(2) Ray, P. D.; Huang, B.-W.; Tsuji, Y. Reactive Oxygen Species (ROS) Homeostasis and Redox Regulation in Cellular Signaling. *Cell. Signal.* **2012**, *24*, 981–990.

(3) Dickinson, B. C.; Chang, C. J. Chemistry and Biology of Reactive Oxygen Species in Signaling or Stress Responses. *Nat. Chem. Biol.* **2011**, *7*, 504.

(4) Burhans, W. C.; Heintz, N. H. The Cell Cycle Is a Redox Cycle: Linking Phase-Specific Targets to Cell Fate. *Free Radic. Biol. Med.* **2009**, *47*, 1282–1293.

(5) Devasagayam, T. P. A.; Tilak, J. C.; Boloor, K. K.; Sane, K. S.; Ghaskadbi, S. S.; Lele, R. D. Free Radicals and Antioxidants in Human Health: Current Status and Future Prospects. *Japi* **2004**, *52*, 4.

(6) Maynard, S.; Schurman, S. H.; Harboe, C.; de Souza-Pinto, N. C.; Bohr, V. A. Base Excision Repair of Oxidative DNA Damage and Association with Cancer and Aging. *Carcinogenesis* **2008**, *30*, 2–10.

(7) Bartsch, H.; Nair, J. Potential Role of Lipid Peroxidation Derived DNA Damage in Human Colon Carcinogenesis: Studies on Exocyclic Base Adducts as Stable Oxidative Stress Markers. *Cancer Detect. Prev.* **2002**, *26*, 308–312.

(8) Bartsch, H.; Nair, J. Oxidative Stress and Lipid Peroxidation-Derived DNA-Lesions in Inflammation Driven Carcinogenesis. *Cancer Detect. Prev.* **2004**, *28*, 385–391.

(9) Liou, G.-Y.; Storz, P. Reactive Oxygen Species in Cancer. *Free Radic. Res.* **2010**, *44*, 479–496.

(10) Waris, G.; Ahsan, H. Reactive Oxygen Species: Role in the Development of Cancer and Various Chronic Conditions. *J. Carcinog.* **2006**, *5*, 14.

(11) Brandes, R. P.; Janiszewski, M. Direct Detection of Reactive Oxygen Species Ex Vivo. *Kidney Int.* **2005**, *67*, 1662–1664.

(12) Kroneck, P. M. H.; Torres, M. E. S. *Sustaining Life on Planet Earth: Metalloenzymes Mastering Dioxygen and Other Chewy Gases*; Springer, 2015.

(13) Sosa, M. E. T.; Saucedo-Vázquez, J. P.; Kroneck, P. M. The Magic of Dioxygen. *Met. Ions Life Sci.* **2015**, *15*, 1–12.

(14) Fridovich, I. Fundamental Aspects of Reactive Oxygen Species, or What's the Matter with Oxygen? *Ann. N. Y. Acad. Sci.* **1999**, *893*, 13–18.

(15) Pacher, P.; Nivorozhkin, A.; Szabó, C. Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol. *Pharmacol. Rev.* **2006**, *58*, 87–114.

(16) Griendling, K. K.; Sorescu, D.; Ushio-Fukai, M. NAD (P) H Oxidase: Role in Cardiovascular Biology and Disease. *Circ. Res.* **2000**, *86*, 494–501.

(17) Mayne, S. T. Antioxidant Nutrients and Chronic Disease: Use of Biomarkers of Exposure and Oxidative Stress Status in Epidemiologic Research. *J. Nutr.* **2003**, *133*, 933S–940S.

(18) Tarpey, M. M.; Fridovich, I. Methods of Detection of Vascular Reactive Species: Nitric Oxide, Superoxide, Hydrogen Peroxide, and Peroxynitrite. *Circ. Res.* **2001**, *89*, 224–236.

(19) Baker, M. A.; Krutskikh, A.; Curry, B. J.; McLaughlin, E. A.; Aitken, R. J. Identification of Cytochrome P450-Reductase as the Enzyme Responsible for NADPH-Dependent Lucigenin and Tetrazolium Salt Reduction in Rat Epididymal Sperm Preparations. *Biol. Reprod.* **2004**, *71*, 307–318.

(20) Janiszewski, M.; Souza, H. P.; Liu, X.; Pedro, M. A.; Zweier, J. L.; Laurindo, F. R. M. Overstimation of Verestimation of NADH-Driven Vascular Oxidase Activity Due to Lucigenin Artifacts. *Free Radic. Biol. Med.* **2002**, *32*, 446–453.

(21) Campbell, A. K.; Patel, A. K.; Razavi, Z. S.; McCAPRA, F. Formation of the Ca2+-Activated Photoprotein Obelin from Apo-Obelin and MRNA inside Human Neutrophils. *Biochem. J.* **1988**, *252*, 143–149.

(22) Shimomura, O. Preparation and Handling of Aequorin Solutions for the Measurement of Cellular Ca2+. *Cell Calcium* **1991**, *12*, 635–643.

(23) Lucas, M.; Solano, F. Coelenterazine Is a Superoxide Anion-Sensitive Chemiluminescent Probe: Its Usefulness in the Assay of Respiratory Burst in Neutrophils. *Anal. Biochem.* **1992**, *206*, 273–277.

(24) Stepanyuk, G. a.; Golz, S.; Markova, S. V.; Frank, L. a.; Lee, J.; Vysotski, E. S. Interchange of Aequorin and Obelin Bioluminescence Color Is Determined by Substitution of One Active Site Residue of Each Photoprotein. *FEBS Lett.* **2005**, *579*, 1008–1014.

(25) Deng, L.; Vysotski, E. S.; Markova, S. V; Liu, Z.; Lee, J.; Rose, J.; Wang, B. All Three Ca2+-binding Loops of Photoproteins Bind Calcium Ions: The Crystal Structures of Calcium-loaded Apo-aequorin and Apo-obelin. *Protein Sci.* **2005**, *14*, 663–675.

(26) Rowe, L.; Rothert, A.; Logue, C.; Ensor, C. M.; Deo, S. K.; Daunert, S. Spectral Tuning of Photoproteins by Partnering Site-Directed Mutagenesis Strategies with the Incorporation of Chromophore Analogs. *Protein Eng. Des. Sel.* **2008**, *21*, 73–81.

(27) Illarionov, B. A.; Frank, L. A.; Illarionova, V. A.; Bondar, V. S.; Vysotski, E. S.; Blinks, J. R. Recombinant Obelin: Cloning and Expression of cDNA, Purification, and Characterization as a Calcium Indicator. *Methods Enzymol.* **2000**, 305, 223-249.

(28) Illarionov, B. A.; Bondar, V. S.; Illarionova, V. A.; Vysotski, E. S. Sequence of the cDNA Encoding the Ca 2+-Activated Photoprotein Obelin from the Hydroid Polyp Obelia Longissima. *Gene.* **1995**, *153*, 273–274.

(29) Deo, S. K.; Daunert, S. Luminescent Proteins from Aequorea Victoria: Applications in Drug Discovery and in High Throughput Analysis. *Fresenius. J. Anal. Chem.* **2001**, *369*, 258–266.

(30) Frank, L. a.; Petunin, A. I.; Vysotski, E. S. Bioluminescent Immunoassay of Thyrotropin and Thyroxine Using Obelin as a Label. *Anal. Biochem.* **2004**, *325*, 240–246.

(31) Frank, L.; Markova, S.; Remmel, N.; Vysotski, E.; Gitelson, I. Bioluminescent Signal System: Bioluminescence Immunoassay of Pathogenic Organisms. *Lumin. J. Biol. Chem. Lumin.* **2007**, *22*, 215–220.

(32) McConkey, D. J.; Nutt, L. Measurement of Changes in Intracellular Calcium during Apoptosis. In *Apoptosis Methods and Protocols*. **2004**; pp 117–130.

(33) Tanahashi, H.; Ito, T.; Inouye, S.; Tsuji, F. I.; Sakaki, Y. Photoprotein Aequorin: Use as a Reporter Enzyme in Studying Gene Expression in Mammalian Cells. *Gene.* **1990**, *96*, 249–255.

(34) Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence-and Luminescence-Based Methods for the Determination

of Affinity and Activity of Neuropeptide Y2 Receptor Ligands. *Eur. J. Pharmacol.* **2006**, *551*, 10–18.

(35) Rowe, L.; Dikici, E.; Daunert, S. Engineering Bioluminescent Proteins: Expanding Their Analytical Potential. ACS Publications 2009.

(36) Tarpey, M. M.; White, C. R.; Suarez, E.; Richardson, G.; Radi, R.; Freeman, B. A. Chemiluminescent Detection of Oxidants in Vascular Tissue: Lucigenin but Not Coelenterazine Enhances Superoxide Formation. *Circ. Res.* **1999**, *84*, 1203–1211.

(37) Saleh, L.; Plieth, C. A Coelenterazine-Based Luminescence Assay to Quantify High-Molecular-Weight Superoxide Anion Scavenger Activities. *Nat. Protoc.* **2010**, *5*, 1635–1641.

(38) Teranishi, K.; Shimomura, O. Coelenterazine Analogs as Chemiluminescent Probe for Superoxide Anion. *Anal. Biochem.* **1997**, *249*, 37–43.

(39) Information, P. Coelenterazine and Coelenterazine Derivatives. **2001**, *373*, 1–3.

(40) Bronsart, L.; Nguyen, L.; Habtezion, A.; Contag, C. Reactive Oxygen Species Imaging in a Mouse Model of Inflammatory Bowel Disease. *Mol. Imaging Biol.* **2016**, *18*, 473–478.

(41) Bronsart, L. L.; Stokes, C.; Contag, C. H. Multimodality Imaging of Cancer Superoxide Anion Using the Small Molecule Coelenterazine. *Mol. Imaging Biol.* **2016**, *18*, 166–171.

(42) Negrin, R. S.; Contag, C. H. In Vivo Imaging Using Bioluminescence: A Tool for Probing Graft-versus-Host Disease. *Nat. Rev. Immunol.* **2006**, *6*, 484.

(43) Zhao, H.; Doyle, T. C.; Wong, R. J.; Cao, Y.; Stevenson, D. K.; Piwnica-Worms, D.; Contag, C. H. Characterization of Coelenterazine Analogs for Measurements of Renilla

Luciferase Activity in Live Cells and Living Animals. *Mol. Imaging.* **2004**, 43-54.

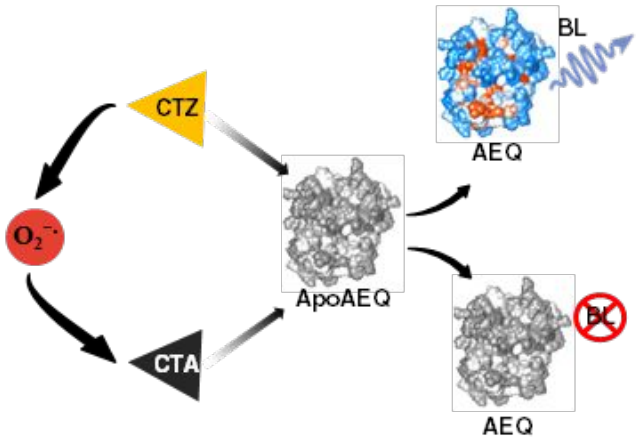
(44) Gross, S.; Piwnica-Worms, D. Spying on Cancer: Molecular Imaging in Vivo with Genetically Encoded Reporters. *Cancer Cell.* **2005**, *7*, 5–15.

(45) Jalilian, N.; Sajedi, R. H.; Shanehsaz, M.; Gharaat, M. CdTe Quantum Dots with Green Fluorescence Generated by Bioluminescence Resonance Energy Transfer from Aequorin. *Microchim. Acta.* **2016**, *184*, 753–762.

(46) Jouni, F. J.; Abdolmaleki, P.; Ghanati, F. Oxidative Stress in Broad Bean (Vicia Faba L.) Induced by Static Magnetic Field under Natural Radioactivity. *Mutat. Res. Toxicol. Environ. Mutagen.* **2012**, *741*, 116–121.

(47) Worthington, K., and Worthington, V. Worthington Enzyme Manual http://www.worthington-biochem.com/XO/assay.html.

(48) Gao, J. J.; Xu, K. H.; Tang, B.; Yin, L. L.; Yang, G. W.; An, L. G. Selective Detection of Superoxide Anion Radicals Generated from Macrophages by Using a Novel Fluorescent Probe. *FEBS J.* **2007**, *274*, 1725–1733.



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