JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Nitrophenylboronic Acids as Highly Chemoselective Probes To Detect Hydrogen Peroxide in Foods and Agricultural Products

Chun-Ping Lu,[†] Chieh-Ti Lin,[†] Ching-Ming Chang,[‡] Shih-Hsiung Wu,[*,†] and Lee-Chiang Lo[*,§]

[†]Institute of Biological Chemistry and [‡]Taiwan International Graduate Program, Academia Sinica, Taipei 115, Taiwan
[§]Department of Chemistry, National Taiwan University, Taipei 106, Taiwan

ABSTRACT: Hydrogen peroxide is commonly used in the food processing industry as a chlorine-free bleaching and sterilizing agent, but excessive amounts of residual hydrogen peroxide have led to cases of food poisoning. Here we describe the development of a novel nonenzymatic colorimetric method for the determination of residual hydrogen peroxide in foods and agricultural products. Nitrophenylboronic acids chemoselectively react with hydrogen peroxide under alkaline conditions to produce yellow nitrophenolates. Of the three nitrophenylboronic acid isomers tested, the *p*-isomer displayed the highest sensitivity for hydrogen peroxide and the fastest reaction kinetics. The reaction product, *p*-nitrophenolate, has an absorption maximum at 405 nm and a good linear correlation between the hydrogen peroxide concentration and the A_{405} values was obtained. We successfully applied this convenient and rapid method for hydrogen peroxide determination to samples of dried bean curds and disposable chopsticks, thereby demonstrating its potential in foods and agricultural industries.

KEYWORDS: hydrogen peroxide, nitrophenylboronic acid, nitrophenol, dried bean curd, disposable chopsticks

INTRODUCTION

The oxidizing agent hydrogen peroxide is commonly used in the food processing industry as a chlorine-free bleaching and sterilizing agent. Hydrogen peroxide has been used to prevent microbial proliferation in foods such as soybean products, noodles, fish products, fresh-cut fruits, and dairy products.[1−6] Also dining utensils, especially chopsticks made of bamboo or wood, are often treated with H_2O_2 for bleaching and sterilization.[7] A negative side effect of hydrogen peroxide treatment is the occasional food poisoning caused by excessive amounts of residual H_2O_2 in processed foods such as udon and noodles.[8] Food regulations in Taiwan do not allow any residual H_2O_2 in the food.[9] In the USA, residual H_2O_2 must be removed from dairy foods after bleaching, and the allowance level is 0.05% (w/w) according to US FDA regulations.[10] The increasing concern about residual H_2O_2 in foods and other items used in daily life has therefore made the development of convenient and rapid methods for H_2O_2 detection a high priority task.

To date, numerous methods have been developed for H_2O_2 determination in food science and industry.[11−16] Despite their success in various applications, limiting factors associated with the individual method, e.g., availability and cost of the instrument, ease of derivatization steps, stability of the enzymes and related reagents, and possible interference of the reagents or the analytical samples, have to be evaluated and investigated when exploring new applications. Therefore, it would be of great advantage to expand the repertoire of the methods by exploiting a novel sensing mechanism.

Arylboronic acids react with H_2O_2 under mild alkaline conditions to generate the corresponding phenolates.[17,18] We previously demonstrated that this unique chemistry could be utilized to design highly chemoselective probes for H_2O_2.[19] The working principle was later extensively adopted by Chang's group to develop a series of highly fluorescent imaging probes for cellular H_2O_2.[20,21] However, most probes in this category had to be acquired by a multistep synthesis. To explore new applications of this class of probes in food sciences, we envisioned that nitrophenylboronic acids (NPBAs) would be excellent candidates worth exploring because they are commercially available and inexpensive, and more importantly because the transformed products are the corresponding nitrophenolates (NPs), which have been widely used as chromophoric reporter groups in biochemical assays. Here we studied the reactions of NPBAs with H_2O_2, evaluated their performance, and utilized *p*-NPBA to establish a convenient assay that we successfully applied to determine residual H_2O_2 in dried bean curds and disposable chopsticks.

MATERIALS AND METHODS

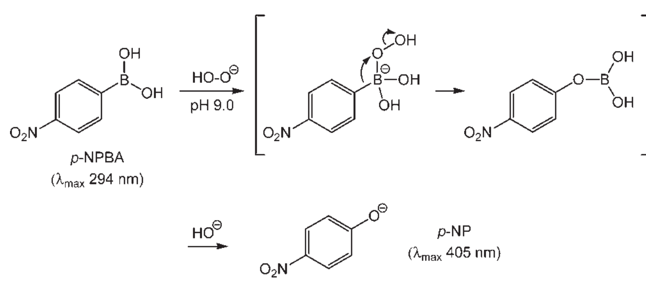
Reagents and Chemicals. All reagents were analytical grade and used without further purification. Dimethyl sulfoxide (DMSO) was from J. T. Baker (Phillipsburg, NJ, USA); 30% (w/w) H_2O_2 solution was from Sigma-Aldrich (St. Louis, MO, USA) and was standardized by the potassium permanganate titration method.[22] Working solutions of H_2O_2 were prepared by diluting the standardized solution with distilled water immediately before experiments. *o*-Nitrophenylboronic acid (*o*-NPBA), *m*-NPBA, *p*-NPBA, and *m*-nitrophenol (*m*-NP) were from Aldrich (St. Louis, MO, USA). *o*-NP and *p*-NP were from Fluka (St. Louis, MO, USA). Stock solutions (100 mM) of all NPBAs and NPs were prepared in DMSO, and working solutions were prepared by appropriate dilution with 150 mM sodium carbonate/bicarbonate buffer, pH 9.0.

Spectroscopic Monitoring of the Model Reaction of *p*-NPBA with H_2O_2. The reaction solution was prepared by mixing

Received: July 18, 2011
Accepted: October 3, 2011
Revised: September 23, 2011
Published: October 03, 2011

ACS Publications © 2011 American Chemical Society

Scheme 1. Mechanism of the Reaction of *p*-NPBA with H_2O_2 under Alkaline Conditions To Generate *p*-NP



200 μM *p*-NPBA with an equal volume of 1 mM H_2O_2 at room temperature. Absorption spectra in the wavelength range of 230−550 nm were recorded at 5, 15, 25, and 35 min with a UV/visible spectrophotometer (Ultrospec 4000, Pharmacia Biotech, USA).

Determination of the Molar Extinction Coefficient of *o*-NP, *m*-NP, and *p*-NP. Stock solutions of *o*-NP, *m*-NP, and *p*-NP were diluted with 150 mM carbonate/bicarbonate buffer, pH 9.0, to give NP concentrations of 100, 500, and 50 μM. The absorption at 405 nm was measured using an Ultrospec 4000 UV/visible spectrophotometer. The molar extinction coefficient (ε) of each compound was calculated using the Lambert−Beer law: $A_\lambda = \varepsilon$ (cm^{-1} M^{-1}) × concentration (M) × length of light path (cm).

General Procedure for the Reactions of NPBAs with H_2O_2. Reaction solutions containing 100 μL of the corresponding NPBA working solution and equal volume of H_2O_2-containing solution were placed in a 96-well microplate. The absorbance in each well was measured at 405 nm over a designated period of time with a microplate reader (PARADIGM Detection Platform, Beckman). For the comparative reactions of H_2O_2 with the three NPBA isomers, the reaction solutions contained 50 μM H_2O_2 and 5 mM NPBA. The absorbance was recorded over a period of 60 min. The effect of the *p*-NPBA concentration on the reaction with H_2O_2 was evaluated using 1, 2, or 5 mM *p*-NPBA in a reaction with 50 μM H_2O_2. The absorbance was monitored over 30 min.

Standard Calibration Curve of H_2O_2. Reaction solutions contained 2 mM *p*-NPBA and serially diluted H_2O_2 solutions (3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 μM), and the absorbance of the reaction mixtures was then recorded as described above. The averaged absorbance values recorded between 15 and 20 min were plotted against the hydrogen peroxide concentrations.

Measurement of Residual H_2O_2 in Chopsticks and Dried Bean Curds. Three samples of disposable chopsticks and three samples of dried bean curds were obtained from restaurants and traditional markets, respectively, in Taipei, Taiwan. These samples were extracted with deionized water according to modified literature procedures.[2,23] The disposable chopsticks were sliced, and the dried bean curds were diced; the samples were then extracted with deionized water [chopsticks, 70 mg per mL of water (w/v); dried bean curds, 1 g of curd per mL of water (w/v)] at 4 °C for 24 h. The solutions were then transferred to 30 μm centrifuge columns (Pierce, USA) and centrifuged at 15000*g* for 1.5 min at 4 °C. The filtrates from the dried bean curd samples were filtered through 0.22 μm disk filters. Hydrogen peroxide in each sample was assayed in duplicate in a 96-well microplate using the assay protocol described above; the values reported are the averages of the two measurements. Briefly, each well contained 100 μL of 4 mM *p*-NPBA and 100 μL of each sample. Blank solutions were prepared by mixing 100 μL of the sample solutions with an equal volume of 150 mM carbonate/bicarbonate buffer, pH 9.0. The plates were vortexed, and the reactions were then allowed to react for 20 min at room temperature. The absorbance at 405 nm was recorded. The absorbance of the blanks was subtracted from absorbance of the respective samples.

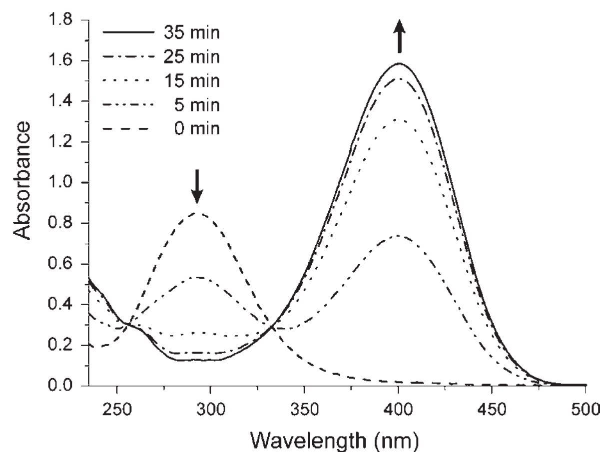


Figure 1. Time-course UV−vis spectra of the reaction of *p*-NPBA (100 μM) with H_2O_2 (500 μM).

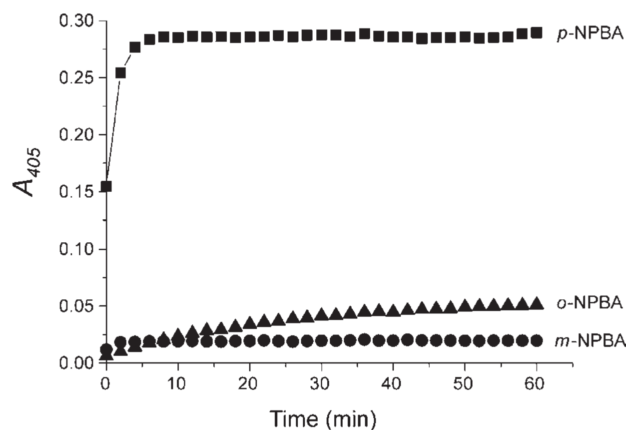


Figure 2. Relative reactivity and sensitivity of NPBA isomers toward H_2O_2. *o*-NPBA, *m*-NPBA, and *p*-NPBA (5 mM) were each incubated with H_2O_2 (50 μM) at 25 °C in 75 mM carbonate/bicarbonate buffer, pH 9.0. The progress of the reactions (increase in the respective NP product) was monitored at 405 nm.

RESULTS AND DISCUSSION

Reaction of *p*-NPBA with H_2O_2. The hydrogen-peroxide-induced conversion of arylboronic acids to phenols is rapid under alkaline condition.[18] To verify the reactions between NPBAs and H_2O_2 as well as to observe the formation of NPs, we carried out a time-course spectroscopic study using *p*-NPBA as a model (Scheme 1). *p*-NPBA displayed a λ_{max} at 294 nm in 75 mM carbonate/bicarbonate buffer, pH 9.0 (Figure 1). When it reacted with excess H_2O_2, the absorption peak at 294 nm decreased and a new peak with λ_{max} at 405 nm concomitantly increased, which supported the formation of *p*-NP. The isosbestic point at 333 nm confirmed the presence of two major interconverting species in the mixture. The absorption maximum of *p*-NP (405 nm) was well separated from that of *p*-NPBA (294 nm), which makes *p*-NP an excellent reporter for the H_2O_2 assays.

Relative Reactivity and Sensitivity of *o*-NPBA, *m*-NPBA and *p*-NPBA toward H_2O_2. We studied the reactivity and sensitivity of the three different NPBA isomers toward hydrogen peroxide. We spectrophotometrically monitored (A_{405}) the

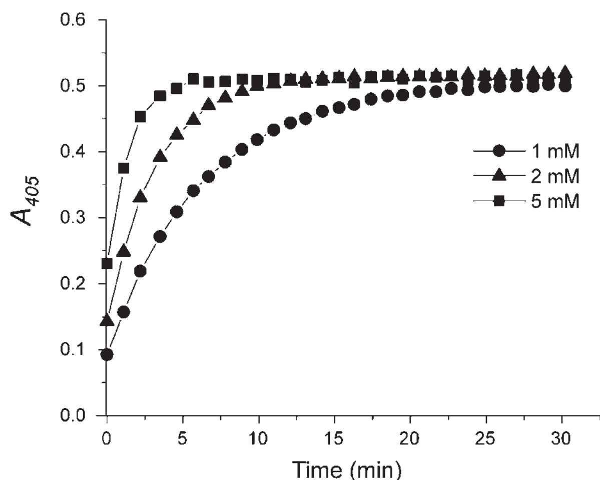


Figure 3. Effect of the *p*-NPBA concentration on the reaction with H_2O_2. The progress of the reactions of *p*-NPBA (1, 2, and 5 mM) with 50 μM H_2O_2 in 75 mM carbonate/bicarbonate buffer, pH 9.0, was monitored at 405 nm.

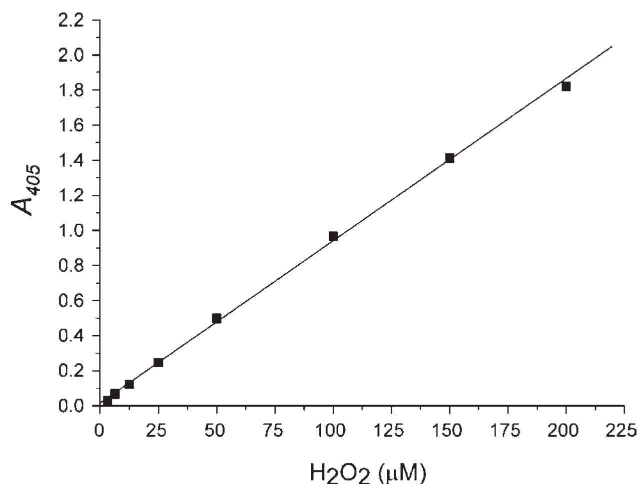


Figure 4. Linear correlation of released *p*-NP (A_{405}) in reactions of *p*-NPBA (2 mM) with 3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 μM H_2O_2 in 75 mM carbonate/bicarbonate buffer, pH 9.0, for 20 min.

production of the NP isomers from the reactions of H_2O_2 (50 μM) with the respective isomers of NPBA (5 mM) in carbonate/bicarbonate buffer (75 mM, pH 9.0) over time. The absorbance of the reaction mixture with *p*-NPBA reached a plateau in 5 min. In contrast, the absorbance of the reactions with *o*- and *m*-NPBA reached a plateau not only much more slowly but also at a much lower intensity (Figure 2).

Since the dramatic difference in the absorbance intensities might be attributed to the extinction coefficients (ε value) of the NPs, we measured the ε values of the three NP isomers at 405 nm. *p*-NP had the highest ε value, about 4- and 11-fold higher than that of *o*- and *m*-NP, respectively (*o*-NP, 4,720 cm^{-1} M^{-1}; *m*-NP, 1,740 cm^{-1} M^{-1}; and *p*-NP, 19,400 cm^{-1} M^{-1}). We therefore chose *p*-NPBA for further studies.

We subsequently studied the effect of the *p*-NPBA concentration on the reaction with H_2O_2. A fixed, limiting amount of H_2O_2 (50 μM) was allowed to react with three different concentrations of *p*-NPBA (1, 2, and 5 mM) in 75 mM carbonate/bicarbonate buffer, pH 9.0. The reaction with 1 mM *p*-NPBA required more than 30 min to go to completion (Figure 3). With higher concentrations of *p*-NPBA, the reactions proceeded faster. For example, the assays could be completed within 12−15 min with 2 mM *p*-NPBA and could be further shortened to 5 min with 5 mM *p*-NPBA. Although we chose 2 mM *p*-NPBA for subsequent experiments, these results suggested that higher concentrations of *p*-NPBA (>2 mM) would also be appropriate for the assays.

Calibration Curve. We then determined a calibration curve for the H_2O_2 assay using 2 mM *p*-NPBA and various concentrations of H_2O_2 (3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 μM). The released *p*-NP (A_{405}) was plotted against the H_2O_2 concentrations, and the resulting curve displayed a good linear correlation with a correlation coefficient (R^2) of 0.9998 (Figure 4). The results also indicated that this method is applicable to detect micromolar levels of H_2O_2.

Determination of H_2O_2 in Samples of Dried Bean Curds and Disposable Chopsticks. We tested our method in the determination of residual H_2O_2 in two commonly encountered food and agricultural products: dried bean curds (A) and disposable chopsticks (B). Three samples of each were examined. After aqueous extraction of the chopped samples and appropriate dilution with distilled water, the H_2O_2 content of each sample was determined as described above using *p*-NPBA. The calculated H_2O_2 contents in each sample are listed in Table 1. One of three dried bean curds (A3) contained a surprisingly high level of residual H_2O_2 (437 ppm). The residual H_2O_2 in the other two samples was either a trace amount (<2 ppm for A1) or nondetectable (A2). We also observed wide differences in the results with the disposable chopsticks samples: sample B3 yielded 120 ppm of H_2O_2, whereas the other two samples (B1 and B2) contained relatively low levels of H_2O_2 (16−20 ppm). As a comparison, we examined samples of unprocessed bamboo, either withered (brown in color) or fresh (green in color). As expected, neither bamboo sample contained detectable amounts of H_2O_2.

Table 1. Results of the H_2O_2 Content in Dried Bean Curds and Disposable Chopsticks

item	sample	H_2O_2 content[a] (ppm)
dried bean curds (A)	A1	<2
	A2	ND[b]
	A3	437
disposable chopsticks (B)	B1	20
	B2	16
	B3	120

[a] Each value represents an average of duplicate analyses. [b] ND, nondetectable.

In order to further verify the method presented in this paper, we performed a comparative analysis using a commercially available hydrogen peroxide assay kit (HRP-OxiRed) [24] on a dried bean curd sample solution spiked with 200 ppm H_2O_2. As a result, we obtained consistent data from both methods and the difference was less than 2%. We have also investigated the potential interfering effect of some food ingredients and additives. EDTA, potassium bromate, ascorbic acid, BHT, sodium dithionite, cysteine, and *tert*-butyl hydroperoxide were tested at both 10 and 100 μM. They were allowed to react with 2 mM *p*-NPBA, and the absorption at 405 nm was monitored. The results

showed that all these materials produced no significant changes at low concentrations (10 μM). Even at higher concentrations (100 μM), all of the above materials, except ascorbic acid, still gave negative responses. In the case of 100 μM ascorbic acid, we observed a slow yet steady increase in the absorbance (<0.0025 OD_{405}/min). Although the nature of this increase in A_{405} was not characterized, it does not constitute a serious interference in the assays as we could suppress its effect by starting with higher concentrations of *p*-NPBA. It is interesting to note that EDTA, potassium bromate, and hydroperoxides, which are known interfering materials to the existed methods, all gave negligible effects in this study, confirming again that *p*-NPBA has excellent chemoselectivity toward H_2O_2. It also demonstrated that the feature of adopting a novel sensing mechanism for H_2O_2 detection would provide a great opportunity for future applications.

In summary, we studied the reaction of NPBA isomers with H_2O_2 and demonstrated that *p*-NPBA is well suited as a highly specific reagent for H_2O_2 determination. Our method using *p*-NPBA does not require any reagents to be synthesized, and is convenient, rapid, and inexpensive. More importantly, *p*-NP, which serves as an ideal chromophoric reporter with a λ_{max} at 405 nm, can be conveniently monitored with a standard spectrophotometer, available in most laboratories. We believe that the method for H_2O_2 determination described here would be of great value in food and agricultural industry.

AUTHOR INFORMATION

Corresponding Author

*S.-H.W.: e-mail, shwu@gate.sinica.edu.tw; tel and fax, 886-2-26539142. L.-C.L.: e-mail, lclo@ntu.edu.tw; tel and fax, 886-2-33661669.

Funding Sources

We thank Academia Sinica and National Science Council (NSC 98-2113-M-002-009-MY3 to L.-C.L.) for financial support.

REFERENCES

(1) Tanaka, A.; Iijima, M.; Kikuchi, Y. Determination of Hydrogen-peroxide in fish products and noodles (Japanese) by gas-liquid-chromatography with electron-capture detection. *J. Agric. Food Chem.* **1990**, *38*, 2154–2159.

(2) Chang, H.-W.; Chen, W.-L. Effects of the application of hydrogen peroxide on the preservation of firm tofu. *J. Food Drug Anal.* **1999**, *7*, 215–223.

(3) Ansari, M. I. A.; Datta, A. K. An overview of sterilization methods for packaging materials used in aseptic packaging systems. *Food Bioprod. Process.* **2003**, *81*, 57–65.

(4) Ukuku, D. O. Effect of hydrogen peroxide treatment on microbial quality and appearance of whole and fresh-cut melons contaminated with Salmonella spp. *Int. J. Food Microbiol.* **2004**, *95*, 137–146.

(5) Kim, H. J.; Fonseca, J. M.; Kubota, C.; Choi, J. H. Effect of hydrogen peroxide on quality of fresh-cut tomato. *J. Food Sci.* **2007**, *72*, S463–S467.

(6) Kang, E. J.; Campbell, R. E.; Bastian, E.; Drake, M. A. Invited review: Annatto usage and bleaching in dairy foods. *J. Dairy Sci.* **2010**, *93*, 3891–3901.

(7) Chu, C.-M.; Chang, M.-H.; Kao, Y.-M.; Shin, Y.-C. Survey on residues of sulfur dioxide, hydrogen peroxide and biphenyl in disposable chopsticks. *Ann. Rep. BFDA (in Chinese)* **2009**, *27*, 230–236.

(8) Sui, S.-C.; Chou, S.-S.; Chang, P.-C.; Hwang, D.-F. Identification of hydrogen peroxide as a causative agent in noodles implicated in food poisoning. *J. Food Drug Anal.* **2001**, *9*, 220–223.

(9) TFDA. Laws & Regulations of Food No.2 Code 02003: Hydrogen peroxide. Accessed July 5, 2011.

(10) US FDA. 21 CFR 184.1366: Hydrogen peroxide. http://www.accessdata.fda.gov/scripts/ cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1366. Accessed Feb. 3, 2010.

(11) Amin, V. M.; Olson, N. F. Spectrophotometric determination of hydrogen peroxide in milk. *J. Dairy Sci.* **1967**, *50*, 461–464.

(12) Şansal, Ü.; Somer, G. Detection of H_2O_2 in food samples by FTIR. *Food Chem.* **1999**, *65*, 259–261.

(13) Nogueira, R. F. P.; Oliveira, M. C.; Paterlini, W. C. Simple and fast spectrophotometric determination of H_2O_2 in photo-Fenton reactions using metavanadate. *Talanta* **2005**, *66*, 86–91.

(14) Liang, K. Z.; Mu, W. J. ZrO_2/DNA-derivated polyion hybrid complex membrane for the determination of hydrogen peroxide in milk. *Ionics* **2008**, *14*, 533–539.

(15) Wei, H.; Wang, E. Fe_3O_4 Magnetic nanoparticles as peroxidase mimetics and their applications in H_2O_2 and glucose detection. *Anal. Chem.* **2008**, *80*, 2250–2254.

(16) Abbas, M. E.; Luo, W.; Zhu, L.; Zou, J.; Tang, H. Fluorometric determination of hydrogen peroxide in milk by using a Fenton reaction system. *Food Chem.* **2010**, *120*, 327–331.

(17) Kuivila, H. G. Electrophilic displacement reactions. 3. Kinetics of the reaction between hydrogen peroxide and benzeneboronic acid. *J. Am. Chem. Soc.* **1954**, *76*, 870–874.

(18) Kuivila, H. G.; Armour, A. G. Electrophilic displacement reactions. 9. Effects of substituents on rates of reactions between hydrogen peroxide and benzeneboronic acid. *J. Am. Chem. Soc.* **1957**, *79*, 5659–5662.

(19) Lo, L.-C.; Chu, C.-Y. Development of highly selective and sensitive probes for hydrogen peroxide. *Chem. Commun.* **2003**, 2728–2729.

(20) Dickinson, B. C.; Huynh, C.; Chang, C. J. A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *J. Am. Chem. Soc.* **2010**, *132*, 5906–5915.

(21) Van de Bittner, G. C.; Dubikovskaya, E. A.; Bertozzi, C. R.; Chang, C. J. *In vivo* imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 21316–21321.

(22) Klassen, N. V.; Marchington, D.; McGowan, H. C. E. H_2O_2 determination by the I_3^- method and by $KMnO_4$ titration. *Anal. Chem.* **1994**, *66*, 2921–2925.

(23) Su, S.-C.; Liu, C.-H.; Chen, H.-C.; Chang, P.-C.; Chou, S.-S. Studies on the determination of hydrogen peroxide and its dissipition in foods. *J. Food Drug Anal.* **1999**, *7*, 131–142.

(24) Towne, V.; Will, M.; Oswald, B.; Zhao, Q. Complexities in horseradish peroxidase-catalyzed oxidation of dihydroxyphenoxazine derivatives: appropriate ranges for pH values and hydrogen peroxide concentrations in quantitative analysis. *Anal. Biochem.* **2004**, *334*, 290–296.