

Direct Colorimetric Detection of Hydrogen Peroxide Using 4-Nitrophenyl Boronic Acid or Its Pinacol Ester

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Abstract

A colorimetric method for the direct determination of hydrogen peroxide in aqueous solution is described. H_2O_2 stoichiometrically converts 4-nitrophenyl boronic acid or 4-nitrophenyl boronic acid pinacol ester into 4-nitrophenol, which can be quantified by measuring the absorption at 400 nm in neutral or basic media. The reactions proceed fast under basic conditions and complete in 2 minutes to at pH 11 and 80°C. The linear range for the colorimetric method extends beyond 1.0 to 40 μM H_2O_2 , and the limit of detection is $\sim 1.0 \mu M$ H_2O_2 . This method offers a convenient and practical process for rapid determination of hydrogen peroxide in aqueous media. Compared to many other techniques in H_2O_2 detection, this process is a direct measurement of H_2O_2 , and is relatively unaffected by the presence of various salts, metal ions and the chelator EDTA.

Keywords: Hydrogen Peroxide Detection, 4-Nitrophenyl Boronic Acid, 4-Nitrophenyl Boronic Acid Pinacol Ester, 4-Nitrophenol, Colorimetric Method

1. Introduction

Hydrogen peroxide is an integral part of atmospheric chemistry and biological systems. In the atmosphere, it is an oxidant that is produced from the combination of hydroperoxyl radicals ($HO_2\cdot$) and their hydrated form [1]. Hydrogen peroxide is exceptionally soluble in water and it is thought to be the most efficient oxidant in the formation of H_2SO_4 from dissolved SO_2 [1]. This implies that hydrogen peroxide could have some role in the acidity of rainwater. Hydroperoxides are significant atmospheric sinks and temporary reservoirs for $HO_x\cdot$ and $RO_x\cdot$ radicals [1,2]. In biological systems, H_2O_2 is produced in reactions catalyzed by numerous bio-enzymes. It has also come forth as a recently recognized messenger in cellular signal transduction [3]. At high concentrations, aqueous solutions of hydrogen peroxide can irritate the eye and other organs, in addition to being a mutagen [4]. In the presence of redox active metal ions, H_2O_2 can be converted to $OH\cdot$ radicals via Fenton reactions [5]:



The extremely reactive hydroxyl radical ($OH\cdot$, half-life ≈ 1 ns) is highly toxic to cells and contributes to neurodegenerative diseases and the aging process. Fe^{III} can

be reduced to Fe^{II} by cellular reductants such as hydroascorbate ($AscH^-$) and nicotinicamide adenine dinucleotide (NADH), making the Fenton reactions catalytic.

More recently, peroxide based explosives have been involved in some recent terrorism incidents [6]. Thus simple and sensitive peroxide detection is also important in counterterrorism efforts. Various methods have been developed for H_2O_2 detection [1,7-14]. The horseradish peroxidase (HRP)-catalyzed reaction is one of the most popular enzymatic assays used for determination of H_2O_2 [8], however, this reaction is quenched or restrained from cations, surfactants, and organic solvents, and the reagents are expensive. Other methods for H_2O_2 determination include HPLC, colorimetric methods, amperometry and chemiluminescence. Titanium-based assays (Ti-PAPS reagents) were developed in the 1980's for spectrophotometric detection of H_2O_2 [9]. The Fox assay was developed in 1990's based on ferrous ion oxidation in the presence of the ferric ion indicator xylenol orange under acidic conditions [10]. Tanner and co-workers investigated the detection of H_2O_2 through a reaction with pyridine-2,6-dicarboxylic acid and vanadate(V) in acidic solution to form an orange-red complex chelate complex, oxo-peroxo-pyridine-2,6-dicarboxylato vanadate (V) detectable at 432 nm [11]. Recently, Luo and

co-workers developed a detection method based on oxidation of methyl orange using an iron-catalyzed Fenton reaction system under acidic conditions [12]. Other methods, such as fluorescent probes (e.g. Peroxy Green 1 and Peroxy Crimson 1) have recently been developed to monitor hydrogen peroxide production in living cells [13]. Chemiluminescence methods have also been developed recently [4,14]. These methods are highly sensitive, but they are limited by complicated apparatus setup and sensor preparation, interferences from metal ions, and inhibition from chelators, e.g. EDTA [2,4,14].

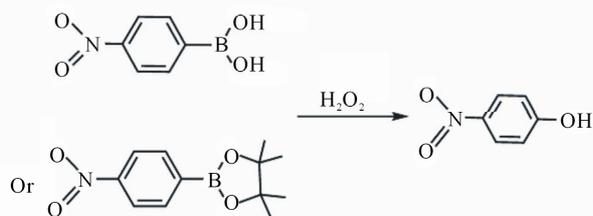
Despite the numerous methods for hydrogen peroxide detection available, it is still of interest to find a simple, direct, inexpensive technique that requires simple instrumentation and is free of numerous interferences. It has been shown that H_2O_2 may convert aromatic boronic acid or their pinacol esters to a hydroxyl group [13,15,16,17]. Based on the same principle, 4-nitrophenylboronic acid or 4-nitrophenylboronic acid pinacol ester may react with H_2O_2 to produce 4-nitrophenol (Scheme 1), a yellow colored compound detectable at 400 nm. Here we have investigated the possibility of utilizing 4-nitrophenylboronic acid or its pinacol ester to determine H_2O_2 directly in aqueous media.

2. Experimental

2.1. Reagents and Apparatus

4-nitrophenylboronic acid and 4-nitrophenylboronic acid pinacol ester were purchased from Boron Molecular Company (Raleigh, NC). Hydrogen peroxide was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other chemicals are commercially available and used without further purification.

UV-Vis spectroscopic studies were performed on a Perkin Elmer Lambda 25 Spectrophotometer. NMR spectra were recorded on a Bruker AC-300 NMR spectrometer. The pH value of all buffer solutions was determined with a Corning pH meter equipped with a Sigma-Aldrich micro combination electrode calibrated with Aldrich buffer solutions.



Scheme 1. 4-Nitrophenylboronic acid or its pinacol ester reacts with H_2O_2 to produce 4-nitrophenol.

2.2. Specifications of Reactions between H_2O_2 and 4-Nitrophenyl Boronic Acid or 4-Nitrophenyl Boronic Acid Pinacol Ester

Stock solutions of 4-Nitrophenyl boronic acid (1.0 mM) and 4-Nitrophenyl boronic acid pinacol ester (1.0 mM) were prepared in methanol. These were then diluted in 20 mM Tris-HCl buffer (pH 7.27) to make several 100 μ M samples. H_2O_2 was then added to the samples to create a final H_2O_2 concentration of 1.0 mM. The reactions were then monitored by UV-vis spectrometer at 10, 20, 30, 60, 90, 120, 150 and 180 minutes, respectively. Similar reactions were also investigated at pH values of 6.91, 8.06, 9.04, 9.96 and 10.95 in 20 mM Tris-HCl buffers, after incubation for 60 minutes at room temperature (25°C).

More detailed studies were carried out at pH 8 and pH 11 and at various temperatures. 20 mM Tris-HCl buffer, pH 8.06 was used for the pH 8 studies. 4-Nitrophenyl boronic acid (100 μ M) or 4-Nitrophenyl boronic acid pinacol ester was mixed with 1.0 mM H_2O_2 in the buffer. Samples were incubated in sealed tubes for varying amounts of time at 37°C, to simulate physiological relevant conditions, and the kinetics were monitored by a UV-vis spectrometer. The same was done for pH 11, but instead 1.0 mM NaOH (pH 11) was used. Analogous procedures were also carried out at 23°C, 55°C and 80°C.

2.3. Determining the Rate Constants and Activation Energy

The kinetic data at different temperatures was used to estimate the rate constants and activation energy of the reactions of 4-nitrophenylboronic acid and 4-nitrophenylboronic acid pinacol ester with H_2O_2 . For a first order reaction, a plot of the natural log of the reactant concentration vs. time will yield a linear relationship. At 10-fold excess of H_2O_2 , the reaction is pseudo 1st order. The initial concentration of reactant, $[A_0]$, is proportional to the concentration of product, $[P_f]$, after the reaction has completed since nearly all of the reactant has been converted to product,

$$[A_0] \propto [P_f] \quad (1)$$

In this way, the concentration of reactant at time t is proportional to $[P_f]$ minus the concentration of product at time t, $[P]$,

$$[A] \propto [P_f] - [P] \quad (2)$$

Using these substitutions, the first order rate law $\ln([A]/[A_0]) = -kt$ can be written as,

$$\ln\left(\frac{[P_f] - [P]}{[P_f]}\right) = -kt \quad (3)$$

The concentration of product is comparative to the absorbance, so $[P_f]$ can be related to the absorbance of the highest peak of the product at 400 nm, when the reaction has reached completion. The $[P]$ values can be interpreted as the absorbance at 400 nm for the intermediate times that is all the peaks below the highest. In this way, the rate constants for the reactions at different temperatures can be determined by plotting

$\ln\left(\frac{[P_f] - [P]}{[P_f]}\right)$ vs time, where the rate constant k is the negative of the resulting slope.

2.4. Standard Curves

Standard curves for H_2O_2 were determined for both 4-Nitrophenyl boronic acid and 4-Nitrophenyl boronic acid pinacol ester at pH 8 and pH 11. In each case, the concentration of boronic compounds was $50 \mu M$, and the H_2O_2 concentrations were 0.5, 1, 2, 5, 10, 20 and $40 \mu M$. For the trials at pH 8 (20 mM Tris-HCl buffer, pH 8.06), the samples were incubated at $37^\circ C$ for 2 hours and absorbances at 400 nm were recorded. For the pH 11 tests, the samples were incubated in 1 mM NaOH at $80^\circ C$ for 10 minutes and the absorptions at 400 nm were recorded.

2.5. Interferences from Salts, Reducing Agents, Metal Ions, and Chelators

The effect of varying concentrations of several salts, NaCl, K_2HPO_4 , K_2SO_4 and KNO_3 , on the H_2O_2 detection was tested. The samples, each containing $50 \mu M$ of 4-Nitrophenyl boronic acid (or 4-Nitrophenyl boronic acid pinacol ester), $40 \mu M$ of H_2O_2 and a salt concentration (0 μM , 10 μM , 100 μM , 500 μM , 1 mM or 10 mM) were incubated at pH 11 (1 mM NaOH) and $80^\circ C$ for 10 minutes, after which, the absorbance at 400 nm was recorded.

A similar procedure was used to test the interference from several metal ions, ascorbic acid, glutathione (GSH) and the chelator EDTA.

3. Results and Discussion

3.1. Kinetics of the Reactions of 4-Nitrophenyl Boronic Acid or Its Pinacol Ester with H_2O_2

The reaction of 4-nitrophenylboronic acid or 4-nitrophenylboronic acid pinacol ester with H_2O_2 were monitored by UV-vis spectroscopy in $100 \mu M$ solution, pH 7.27. The 4-nitrophenylboronic acid and 4-nitrophenylboronic acid pinacol ester are colorless and displayed absorption (λ_{max} at 290 nm) in UV region only. After the addition of H_2O_2 , the original absorption peak for 4-nitrophenylboronic acid pinacol ester (**Figure 1**) decreased intensity while a new peak centered at 405 nm appeared

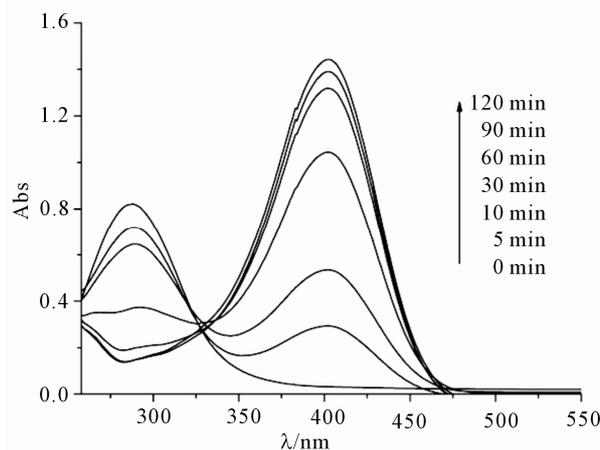


Figure 1. Kinetics of UV-vis spectra of $100 \mu M$ 4-Nitrophenyl boronic acid pinacol ester reaction with H_2O_2 (20 mM Tris-HCl buffer, pH 8.06, $37^\circ C$).

and increased in intensity with an isobestic point at 330 nm, and the color of the solution changed from colorless to yellow, implying the formation of 4-nitrophenol. The rate constant was determined to be $0.0586 s^{-1}$. Similar changes were observed for 4-nitro-phenylboronic acid.

To investigate whether the conversion is a clean chemistry and to confirm that 4-nitrophenol is the product, the reaction processes were monitored by 1H NMR spectroscopy. As shown in **Figure 2**, when H_2O_2 was added to 4-nitrophenyl boronic acid, the original proton resonances at 8.19 and 8.21 ppm (doublet, meta) and 7.90 ppm (broad, ortho) decreased in intensity with the later split into two peaks at 7.92 and 7.95 ppm. The splitting is probably due to the breaking of the hydrogen bonds in the 4-nitrophenyl boronic acid dimer [18]. Meanwhile, new peaks characteristic for 4-nitrophenol emerged and grew in intensity, at the expense of the resonances of the 4-nitrophenyl boronic acid. After 240 min, the 1H NMR spectrum (**Figure 2(c)**) is identical to that of the 4-nitrophenol standard (**Figure 2(d)**). This clearly confirmed that 4-nitrophenyl boronic acid had been cleanly converted to 4-nitrophenol. Similar experiments performed with 4-nitrophenyl boronic acid pinacol ester demonstrated a clean conversion to 4-nitrophenol by H_2O_2 but at a slower rate.

3.2. pH/Temperature Dependence

The pH dependence of the conversion of the boronic acid compounds by H_2O_2 was investigated over a pH range of 6.9 to 11.8 after incubation at room temperature ($25^\circ C$) for 60 min. As shown in **Figure 3**, improved conversion was achieved at more basic pH values with the best pH at ~ 11 for both the 4-nitrophenyl boronic acid and its pinacol ester.

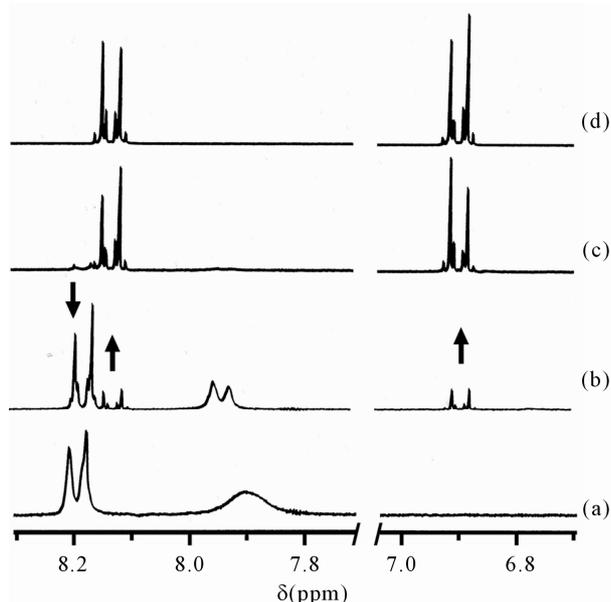


Figure 2. $^1\text{H-NMR}$ spectra of (a) 10 mM 4-Nitrophenyl boronic acid in $d^4\text{-MeOH}$, the mixture of 4-Nitrophenyl boronic acid (10 mM) and H_2O_2 (100 mM) incubated for 60 min (b) and 240 min (c), and (d) 10 mM 4-nitrophenol in $d^4\text{-MeOH}$.

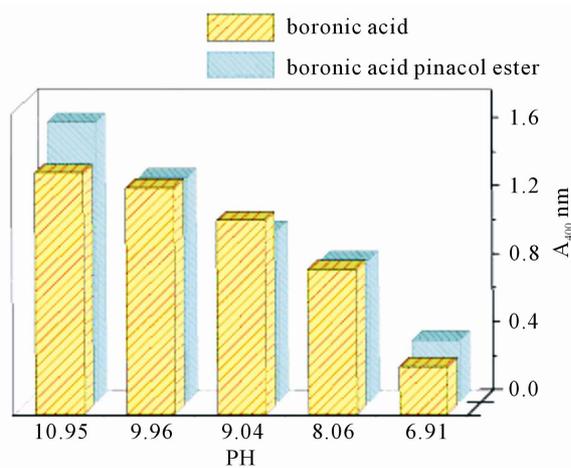


Figure 3. pH profile of absorbance (400 nm) of the reaction of H_2O_2 incubated with 4-Nitrophenyl boronic acid or its pinacol ester (100 μM) for 60 min.

The effect of temperature was also investigated. As expected, the reaction rate increased with increasing temperature. At pH 11 and 37°C , the reaction reached completion in 60 minutes, about half the time as the room temperature and pH 8 reaction. Increasing the temperature further to 55°C decreased reaction time to about 15 minutes. And at 80°C , the reaction finished within 2 minutes. The rate constants for 4-nitrophenylboronic acid and its pinacol ester at pH 11 and varying temperatures were determined and shown in Table 1.

Table 1. Rate constants for 4-nitrophenyl boronic acid and its pinacol ester at pH 11 (s^{-1}).

	25°C	37°C	55°C	80°C
4-Nitrophenylboronic acid	0.0315	0.1318	0.2977	ND
4-Nitrophenylboronic acid pinacol ester	0.0586	0.144	0.3292	0.7155

The rate constants at different temperatures were used to estimate the activation energy. According to the Arrhenius equation, $k = Ae^{E_a/RT}$ [19], a plot of $\ln(k)$ vs. $1/T$ will give a linear plot with slope of $-E_a/R$. The activation energies at pH 11 were calculated to be 82.4 kJ/mol and 59.5 kJ/mol for 4-nitrophenylboronic acid pinacol ester and 4-nitrophenylboronic acid, respectively.

3.3. Standard Curves and Detection Limit

Experiments were performed with 50 μM 4-Nitrophenyl boronic acid or the boronic acid ester and a range of H_2O_2 concentrations from 0.5 μM to 40 μM . A linear trend was observed for H_2O_2 concentration in the range of 1 μM to 40 μM (Figure 4), indicating reliable detection. Similar tests were carried out for physiological conditions, pH 8 and 37°C , which exhibited comparable results. The 4-Nitrophenyl boronic acid reaction with H_2O_2 displayed the potential to be accurate to a $[\text{H}_2\text{O}_2]$ of about 1 μM .

3.4. Interference

It is of interest to study the ability of 4-Nitrophenyl boronic acid or its pinacol ester to maintain accurate detection of H_2O_2 in the presence of other compounds. Several

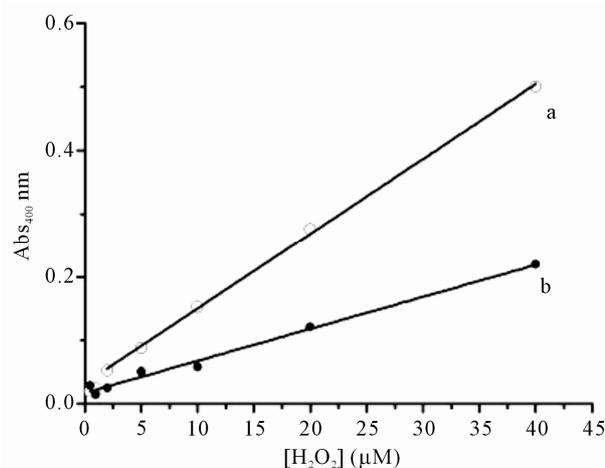


Figure 4. Standard curves of detection of H_2O_2 with (a) 50 μM 4-Nitrophenyl boronic acid pinacol ester at pH 11, 37°C or with (b) 50 μM 4-Nitrophenyl boronic acid at pH 8.0, 37°C .

potential interfering compounds were investigated, including various salts, reductants, metal ions, and chelators. The salts (0 to 10 mM) NaCl, K₂HPO₄, K₂SO₄, and KNO₃ were all tested with the reaction and, as expected, showed no significant affect on the ability to detect H₂O₂.

The effect of the chelator EDTA was investigated. It appears that at low concentrations EDTA does not have a significant effect on H₂O₂ detection (**Figure 5**). The absorbance remained relatively steady, and began to decrease slightly only at the higher concentrations of about 1 mM and above. At 10 mM EDTA, the absorbance decreased by ca. 34%. This is a significant improvement over the chemiluminescence method where EDTA significantly inhibits detection at the 20 μM level and above, reducing the signal to near zero by 100 μM [2].

The influence of transition metal ions was examined by analyzing the absorbance deviation at 400 nm of a solution of 50 μM boronic acid or its picanol ester incubated with 50 μM H₂O₂ for 45 min at pH 11.0, to which varying concentrations of Pb²⁺, Mn²⁺, Ni²⁺, Fe³⁺, Cu²⁺ and Zn²⁺ was added. As shown in **Figure 6**, the interference caused by Pb²⁺ is almost negligible under all tested concentrations from 5 μM to 50 μM. Ions of Mn²⁺, Ni²⁺ and Cu²⁺ show little interference up to concentration of 20 μM; however, a decrease in absorbance by ca. 12% was observed at 50 μM. Zn²⁺ demonstrated mild interference on 4-Nitrophenyl boronic acid or its pinacol ester detection (increasing absorbance by ca. 2% - 5%) in the tested concentration range. Fe³⁺ displayed more serious interference in both the systems with the absorbance increased by ca. 11% at an Fe³⁺ concentration of 20 μM, probably due to the absorbance from iron-hydroxide complexes formed in the solution.

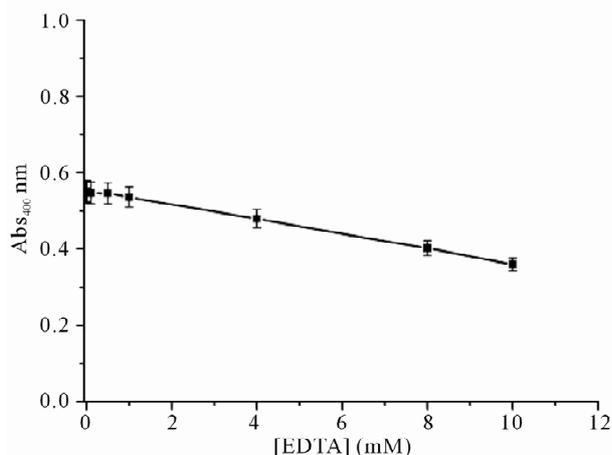


Figure 5. Effect of EDTA at varying concentrations on the absorbance (400 nm) of solution of 50 μM boronic acid and 40 μM H₂O₂.

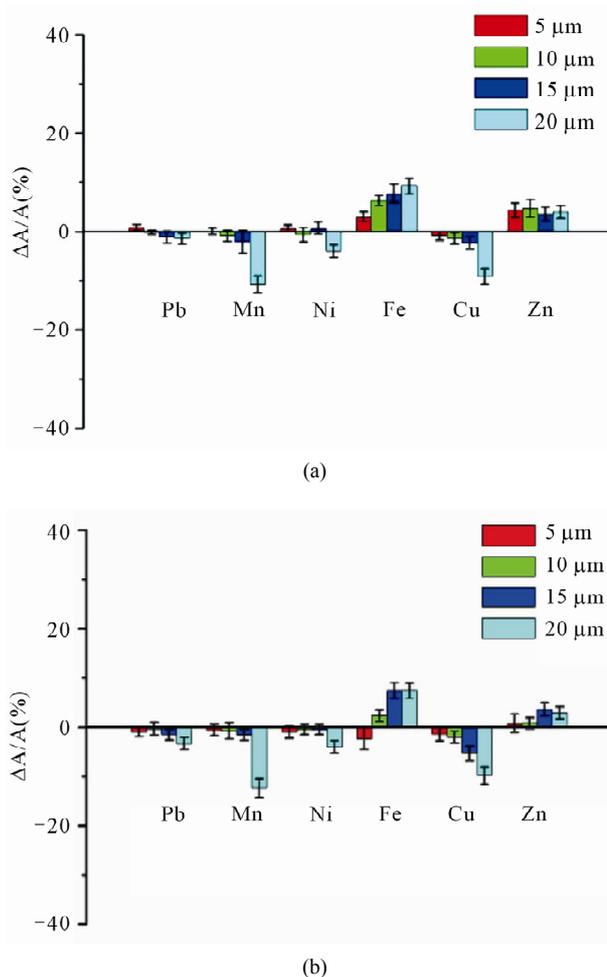


Figure 6. Interference of Pb²⁺, Mn²⁺, Ni²⁺, Fe³⁺, Cu²⁺ and Zn²⁺ on H₂O₂ detection achieved with 4-Nitrophenyl boronic acid (a) and its pinacol ester (b) at pH 11.

4. Conclusions

The reaction of 4-nitrophenyl boronic acid or 4-nitrophenyl boronic acid pinacol ester with hydrogen peroxide is a useful method for hydrogen peroxide detection. The reaction runs under neutral to basic conditions, with maximum kinetics at pH 11 and high temperatures. This method is able to detect H₂O₂ to a concentration of about 1 μM. It is unaffected by the presence of various salts, or low levels of the metal ions of Pb²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, Mn²⁺ and Fe³⁺, and the chelator EDTA. The reaction of boronic acid with H₂O₂ is a direct measurement of H₂O₂ as compared to many other methods; it requires only simple instrumentation and preparation, and is very inexpensive. It could prove useful in detecting H₂O₂ in the environment and in biological systems and this chemistry may be harnessed to develop novel devices for H₂O₂ detection.

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