

Determination of glutamate using paper-based microfluidic devices with colorimetric detection for food samples

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Abstract

A paper-based device (PAD) capable of colorimetric detection was developed to determine the presence of glutamate in various food samples. The PAD employs an enzymatic reaction with glutamate followed by an oxidation reaction with N-benzoyl leucomethylene blue (BLMB) in the presence of horseradish peroxidase. The designed PAD consists of a sample introduction zone connected to a channel that transports a sample solution to three detection zones. The detection zones contain pre-deposited reagents: glutamate oxidase, horseradish peroxidase, BLMB, a phosphate buffer, and poly(acrylic acid). The PAD is perpendicularly immersed into a sample solution and bent at a right angle using a 3D-printed holder to allow the sample to simultaneously flow into three different detection zones. When the PAD is immersed into a sample containing glutamate, glutamate oxidase produces hydrogen peroxide, which changes the pale blue color of BLMB to a deep blue color in the presence of horseradish peroxidase. Under the optimum conditions, the calibration curve between the logarithm of the glutamate concentrations and the color intensity was linear within a range of from 5×10^{-6} mol L⁻¹ to 10^{-2} and with a correlation coefficient of 0.994. Using this system, the PAD successfully determined glutamate in soup stocks, sauces, snacks, and tomato juice without the need of complicated sample pretreatment. These results agreed with those of a commercially available glutamate assay kit, which was employed as a certification method ($t_{\text{stat}} = 1.95$, $t_{\text{crit}} = 2.57$). The developed PAD is simple, easy to fabricate, portable, and could be used outside of equipped laboratories to determine the presence of glutamate in food samples.

Keywords Glutamate, Paper-based analytical device, Enzymatic reaction

1. Introduction

Food flavor comes when specific molecules stimulate the chemical senses of taste and smell in humans. Humans usually use all three of their natural food receptacles — the tongue, mouth, and throat — to sense differences in taste. Moreover, the flavoring molecules in food stimulate the olfactory cells in the nasal cavity, which enhances the flavor of food [1].

One of the current culinary challenges is the need to satisfy individuals who desire different tastes. Therefore, the obsession with taste discrimination seems to be increasing, and the food industry that caters to it has grown dramatically to meet the demand for delicious and/or healthy foods to satisfy the varied world of human taste. In order to satisfy the appetites of humans, flavor enhancers are being developed by the food industry. Flavor enhancers are chemicals added to foods to supplement, enhance, and magnify flavor [2].

Monosodium L-glutamate (MSG) is the salt of an amino acid, glutamate, which is widely used as a flavor enhancer similar to naturally occurring free glutamate in food [3]. Even though glutamic acid occurs naturally, commercial MSG is also produced using molasses, starch, corn sugar, sugar cane, and fermented sugar beets [4]. The unique flavor of MSG is referred to as “umami” taste by the Japanese, and is a fifth taste sensation in addition to sweet, salty, bitter, and sour. Umami taste is popular in Japanese, Korean, and Chinese food [5-6]. In 1991, the average intake of MSG in the United Kingdom was 580 mg/day for the general population and 4.68 g/day for extreme users [7]. The estimated average daily MSG intake per person in industrialized countries is 0.3–1.0 g, depending on the MSG content in prepared foods and on individual taste preferences [8]. A typical Chinese restaurant meal contains MSG in a range of from 10 to 1,500 mg per 100 g, according to information from a 2003 joint inquiry by the governments of Australia and New Zealand [9].

While glutamate is a non-essential amino acid in our body, it can be an energy source for specific tissues as well as a substrate for glutathione synthesis [10]. In 1959, the Food and Drug Administration (FDA) classified MSG as a "generally recognized as safe" (GRAS) substance. Nevertheless, in 1995, the FDA reported that an unknown population could develop a complex of MSG symptoms resulting in headaches, nausea, quick heartbeat, bronchospasm, chest pain, drowsiness, weakness, and sweating [11]. Moreover, a high concentration of MSG in food could trigger allergies or, and much worse, Parkinson's and Alzheimer's diseases in serious cases [12]. Therefore, a simple method to assess the amount of MSG is crucial for the food industry, which includes both suppliers and consumers.

Several methods for determining MSG have been reported by different research groups. Fluorescence spectrophotometry utilizes the reaction of fluorescamine with glutamic acid under alkaline conditions to provide a stable fluorophore and nonfluorescent hydrolysis product [13]. Spectrophotometry employs a substrate-cycling reaction between L-glutamate dehydrogenase (GDH) and the novel enzyme d-phenylglycine aminotransferase (d-PhgAT) to generate two accumulating products that strongly absorb UV light at the same wavelength [14]. Moreover, there are reports of the chemiluminescence detection of L-glutamate via a reaction of luminol and the H_2O_2 that is produced by enzymatic reactions of glutamate [15]. Electrochemistry has also been applied to glutamate determination as seen in amperometric [16-19], potentiometric [20-22], and conductometric [23] forms of detection. Capillary electrophoresis permits the determination of glutamate with derivatization using o-phthalaldehyde in the presence of 3-mercaptopropionic acid [24]. Another popular technique for glutamate determination is chromatographic separation such as in gas chromatography [25-26], high-performance liquid chromatography [27-31], and high-performance thin-layer chromatography [32].

Although many analytical instruments permit the determination of glutamate, paper-based analytical devices (PADs) are attractive for routine chemical analysis outside of laboratory settings because the process is inexpensive, transportable, easy to operate, and the gathered data are simple to process. The PADs are fabricated by patterning hydrophobic barriers to define and control the hydrophilic areas on paper. A paper substrate is useful to manipulate small volumes of 10^{-6} - 10^{-9} L by capillary action that achieves spontaneous liquid flow without the need of a pumping system. There are several ways to fabricate the PADs: wax printing, ink-jet printing, screen printing, movable-type printing, and cutting. The most prevalent techniques are wax printing and ink-jet printing because hydrophilic channels can be fabricated simply and precisely on paper [33-35].

In this research, we developed a PAD to determine glutamate in food samples. A dip-and-read type PAD was designed to achieve a simple operation that required no use of micropipettes for sample introduction. The PADs are equipped with a sample introduction zone and three detection zones connected by flow channels. The detection zones consist of L-glutamate oxidase, horseradish peroxidase, N-benzoyl leucomethylene blue (BLMB), poly(acrylic acid), and a phosphate buffer. L-glutamate oxidase generates hydrogen peroxide in the reaction with glutamate, and then hydrogen peroxidase oxidizes BLMB in the presence of horseradish peroxidase [36]. The reactions convert BLMB to methylene blue in the detection zones. After drying the PAD, the images of the PADs are

captured by a scanner to obtain the color intensity for quantitative analysis. The PADs were applied to the measurement of glutamate in several food samples.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade. Deionized water (DI water, resistivity >18 MΩ cm) from the Elix water purification system (Direct-Q UV3, Merck, Darmstadt, Germany) was employed to prepare all solutions. Sodium dihydrogen phosphate, disodium hydrogen phosphate, dimethyl sulfoxide, L-glutamic acid, and a 25% poly(acrylic acid) solution (8,000-12,000 cP at 30 °C) were obtained from Wako Pure Chemical Corporation (Osaka, Japan). N-Benzoyl leucomethylene blue was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and was dissolved in dimethyl sulfoxide. Horseradish peroxidase and L-glutamate oxidase were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). A Glutamate Assay Kit-WST was obtained from Dojindo Laboratories (Kumamoto, Japan). Phosphate buffer solutions with different pHs were prepared by mixing 0.05 mol L⁻¹ sodium dihydrogen phosphate and 0.05 mol L⁻¹ disodium hydrogen phosphate at different ratios. A poly(acrylic acid) solution with a concentration of 2.5% v/v was prepared from a 25% poly(acrylic acid) solution.

2.2. Sample preparation of food products

All food products were purchased from local supermarkets at Okayama in Japan. Food products used in this study included dashi soup powder, a miso soup pack, consommé, Japanese rice cookies, soy sauce, and tomato juice. The samples were prepared by mixing the solid or liquid food products with water followed by filtration with a syringe filter and dilution with DI water to adjust concentrations for the measurement.

2.3. Design and fabrication of the PADs

The PADs were designed using PowerPoint 2010 software, as shown in Fig. 1A. The size of each PAD is 25 × 25 mm² with a sample introduction zone (10 mm in height and 25 mm in wide at the bottom of the PAD), three detection zones (diameter of 3.5 mm), and flow channels connecting them that are surrounded by hydrophobic black wax. Three detection zones provided triplicate measurements per each sample.

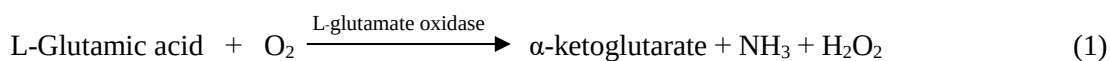
The PADs were fabricated on filter paper (200 mm × 200 mm, Chromatography Paper 1CHR, Whatman™, GE Healthcare Lifescience, UK) using a wax printer (ColorQube 8580N, Xerox, CT, USA). The printed sheet was heated in an oven (ONW-300S, AS ONE, Tokyo, Japan) at 120 °C for 2 min to allow the wax ink to penetrate the backside of the paper sheet. With this design, wax ink helps to create a hydrophobic barrier in the PAD [37]. The black face of the PAD was covered by clear tape to prevent leakage of the solution.

We found that 0.3 μL of a solution is sufficient to occupy each of the detection zones. Therefore, 0.3 μL of a phosphate buffer, horseradish peroxidase, L-glutamate oxidase, N-benzoyl leucomethylene blue, and poly(acrylic acid) were deposited in the detection zones one-by-one to prepare the PADs.

We also used a 3D printer to construct a 3D-holder to support the PADs. The 3D printer facilitated the design and fabrication of a 3D-holder that could support a perpendicular immersion of the PAD into a sample solution because we were able to modify and print the 3D-holder within several hours. The PAD setting on the 3D-holder is shown in Fig.1B. The 3D-holder was printed using polylactic acid (PLA), which is a biodegradable plastic. SketchUp software was used to design and print the 3D-holder using a XYZ 3D printer, model pro X (XYZ Printing, Taiwan).

2.4 Enzymatic reaction for glutamate determination

In this research, L-glutamic acid, or MSG, was determined by the enzymatic reaction of L-glutamate oxidase and the oxidation reaction of N-benzoyl leucomethylene blue in the presence of horseradish peroxidase [36]. When glutamate reacts with oxygen, L-glutamate oxidase catalyzes the reaction with glutamate to produce α-ketoglutarate, ammonia, and hydrogen peroxide. Then, hydrogen peroxide oxidizes BLMB, which is subsequently catalyzed by horseradish peroxidase to produce methylene blue, and this results in a color change from pale blue to deep blue. The reaction is expressed in equations 1 and 2.



After the colorimetric reactions to produce methylene blue, the images of the PADs were captured by a desktop scanner (OKI MC361dn) to analyze the color intensity by ImageJ software from the

National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin) [38] using a histogram function to obtain the red values. A desktop scanner is superior to a smartphone for capturing reproducible images without the help of a photo studio because ambient light does not influence the process. The red color intensities were plotted against the logarithm of the glutamate concentrations without subtracting the blank in the measurements.

3. Results and discussion

3.1. Design of the PAD

In the design of the PAD, the channel size was initially optimized to obtain an appropriate flow rate, which determines the sample transport and dispersion in the detection zones. The width of the channel size was varied from 0.6 to 1.0 and 1.5 mm. The 0.6-mm channel needed roughly 7 min to transport the sample solution to the detection zones while the 1.5-mm channel reduced the time to 1.30 min. Obviously, the wider channel shortened the time to transport the sample solution to the detection zones. Therefore, 1.5-mm channels were fabricated on the PADs to reduce the time to transport the sample solution.

One problem in the sample introduction was the inhomogeneous flow rate in each channel. When the PAD was placed perpendicular to the sample solution, the sample solution reached the detection zones at different times, and this led to the introduction of different volumes in the three detection zones, which resulted in different color intensities. Therefore, we bent the PADs at a right angle using a 3D-holder fabricated by a 3D printer, as shown in Fig. 1B. After bending the PAD with the 3D-holder, the sample solution flowed homogeneously and reached the three detection zones at the same time.

3.2. Effect of anionic polymer

Although a homogeneous flow was successfully achieved by bending the PADs with the 3D-holder, an inhomogeneous color persisted because of the methylene blue that had accumulated at the entrance to the detection zones. To solve the problem of inhomogeneous color intensity, a poly(acrylic acid) solution was added to the detection zones. Poly(acrylic acid) is an anionic polymer that is expected to retain cationic methylene blue in the detection zones by forming ion pairs [39]. We compared the PADs with (Fig. 2 A1) and without (Fig. 2 A2) the anionic polymer solution. The results show that the poly(acrylic acid) suppressed the dispersion of the blue color and generated a homogeneous color in the detection zones.

It should be noted that poly(acrylic acid) increases the analysis time because it increases the viscosity and retards the penetration of a solution into the detection zones. However, the times needed to occupy the detection zones with a sample solution were 1.25 and 2.14 min without and with the polymer, respectively. Therefore, an increase in the analysis time of less than 1 min would be insignificant and acceptable for rapid analysis.

The dipping time also possibly influenced the color intensity in the detection zones because the sample is continuously supplied during immersion of the PAD in the sample solution. Therefore, the dipping time was varied from 0 to 60 s after the solution reached the detection zones. The relationship between the dipping time and the red color intensity is represented in Fig. 2B. In Fig. 2B, the yield of the product correlates with the intensity of the red color, because the product is colored blue color yet it absorbs red light, which decreases the intensity of the red color intensity in the detection zones. The intensity of the color was more stable in the presence of poly(acrylic acid) at different dipping times, and a reproducible and stable intensity of the color was produced. Conversely, the intensity of the red color decreased as the product with its blue color increased for up to 50 s, although the change in intensity was small. Therefore, we adjusted the dipping time to 50 s for the analysis of glutamate.

3.3. Optimization of reaction conditions

Two enzymes, glutamate oxidase and horseradish peroxidase, were used in this detection scheme. L-Glutamate oxidase produced hydrogen peroxide in the oxidation reaction with glutamate, and horseradish peroxidase catalyzed the oxidation reaction of N-benzoyl leucomethylene blue in the hydrogen peroxide. Thus, the deposited amounts of enzymes were varied in a range of from 20 to 100 units mL⁻¹. The results obtained by changing the amounts of the enzymes are shown in Fig. 3A. For enzyme amounts larger than 20 units mL⁻¹, no significant difference in the color intensity was observed, i.e., 20 units mL⁻¹ would be much higher than that needed to obtain the maximum reaction rate. Therefore, any concentrations larger than 20 units mL⁻¹ could be added to the detection zones using this method.

The pH is a significant parameter that influences enzymatic reactions. In this study, the pH was varied using phosphate buffer solutions in a range of from 6 to 8. The results appear in Fig. 3B and show that pH 6.5 was the most appropriate for the enzymatic reaction of glutamate because the intensity of the red color was the lowest. The optimum pH is consistent with that of our previous report for horseradish peroxidase [36], which is supported by the fact that glutamate oxidase produces its maximum activity in the pH range of 6 to 8 [40]. Although the presence of poly(acrylic acid)

reduced the pH of the phosphate buffer solution from 6.5 to 6.0, this remains within the range of the optimum pH reported in the literature.

In this study, N-benzoyl leucomethylene blue was employed as the substrate for the enzymatic reaction of horseradish peroxidase. When we varied the concentration of N-benzoyl leucomethylene blue within a range of from 0.001 to 0.02 mol L⁻¹, the blue color in the detection zones was intensified with increases in the concentration of the substrate. However, the blue color of the blank also increased with increases in the concentration of N-benzoyl leucomethylene blue. Thus, 0.01 mol L⁻¹ of N-benzoyl leucomethylene blue showed the most significant difference in red color intensity between the blank and the sample.

3.4. Analytical performance

A calibration curve of glutamate was constructed under the optimum conditions. The linear equation was $\text{Intensity} = (-33.93 \pm 1.21) \log [\text{glutamate, mol L}^{-1}] + (44.319 \pm 4.536)$ with a correlation coefficient $r^2 = 0.994$. The logarithm of the glutamate concentration was plotted against the red color intensity because the calibration curve provided good linearity. The linear relationship between the logarithm of the glutamate concentration and the intensity ranged from 5×10^{-6} to 10^{-2} mol L⁻¹. The repeatability of the method in terms of the relative standard deviation (%RSD) was 4.66% for 10^{-4} mol L⁻¹ standard glutamate (n = 10). The limits of detection and quantification were 10^{-6} mol L⁻¹ and 5×10^{-6} mol L⁻¹, respectively. We estimated the sample throughput to be 40 determinations h⁻¹, which included the time for the preparation of the PAD and the sample introduction, but excluded the time needed for flowing and drying the sample solution.

3.5. Stability test

For the stability test, we prepared 100 PADs in a day and stored them at 4 °C in a refrigerator. The stored PADs were used to measure a standard solution of 10^{-3} mol L⁻¹ glutamate daily for more than two months. No degradation of the PADs was observed for more than two months when they were stored in a refrigerator. Therefore, no degradation in detection was observed for either the enzymes or the substrate when deposited on PADs that had been stored in a refrigerator.

3.6. Food analysis

The PADs were applied to the determination of glutamate in actual food samples. The samples were dissolved in, and/or diluted by, water to adjust the appropriate concentration for determination using the PADs. The amounts of glutamate determined by the PADs were validated using a commercially available Glutamate Assay Kit-WST for microtiter plate assay. The supplier guarantees that the kit can detect the lowest concentration of glutamate at 5 $\mu\text{mol L}^{-1}$.

Table 1 shows the results obtained by the PADs and the microtiter plate assay. The values obtained by the PADs showed no significant difference from those of the microtiter plate assay, as shown by the statistical paired t-test for a 95% confidence level. The calculated absolute t-value (1.95) was lower than the critical t-value (2.57) for all food samples. The PADs and the microtiter plate assays showed the same order of magnitude in the glutamate concentrations, i.e., the PADs correctly estimated the amounts of glutamate in the food samples. These results suggest that the PADs are applicable to food samples containing different matrices.

The colorimetric reactions in the PADs are enzymatic reactions that would be sensitive to temperature. Therefore, a hot sample should influence the colorimetric reaction. However, because all samples employed in this study are either powder or liquid kept at room temperature, the samples and standards were measured at room temperature. If the need arises to measure a hot liquid sample such as a cooked soup, this limitation could be overcome by using a small volume letting it sit and/or diluting it with water to cool it before the measurements.

4. Conclusions

PADs for determining glutamate in food samples were successfully designed, fabricated, and prepared using wax printing and enzymatic reactions. A 3D-holder was also designed and fabricated by 3D printing to control the flow rate of the PADs. Glutamate determination involved the enzymatic reactions of glutamate oxidase and horseradish peroxidase for which N-benzoyl leucomethylene blue was the substrate. The blue color of the methylene blue appeared when the food sample contained glutamate. We applied the proposed PADs to the determination of glutamate in several food samples and found good agreement with the results of a microtiter plate assay. The PADs are transportable, simple, inexpensive, and provide rapid analysis. Therefore, the PADs will help determine glutamate outside a laboratory setting such as in restaurants, markets, and homes.

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380 Table 1. The results obtained from the PADs and from a Glutamate Assay Kit-WST for L-glutamic
 381 acid in food samples

Sample	Glutamate amount (mg g ⁻¹ or mg mL ⁻¹ sample)	
	PADs	Glutamate Assay Kit-WST
Dashi soup powder	140±0	310±20
Miso soup pack	340±0	410±20
Consommé	14±0	98±1
Japanese rice cookie	8.4±0.0	1.7±0.2
Soy sauce	2.2±0	7.4±0.1
Tomato juice	0.6±0.0	0.3±0.0

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Figure Legends

Fig. 1. Designs of the PAD and the 3D-holder fabricated using a 3D printer. A, The designed PAD for glutamate determination; B, The 3D-holder with the PAD — A sample is added to the tray placed at the bottom of the 3D-holder.

Fig. 2. Color development and effect of dipping time on color intensity with and without 2.5% polyacrylic acid added to the detection zones. A1, Image without polyacrylic acid; A2, Image with polyacrylic acid. B, Effect of dipping time with and without 2.5% polyacrylic acid. Detection zones contained 0.3 μL of 0.01 mol L^{-1} N-benzoyl leucomethylene blue, 60 units mL^{-1} glutamate oxidase, 60 units mL^{-1} horseradish peroxidase, and phosphate buffer (pH 6.5). Sample, 10^{-4} mol L^{-1} glutamate.

Fig.3. Optimization of the reagent amounts deposited in the detection zones. A. Enzyme concentrations: blue, L-glutamate oxidase; red, horseradish peroxidase. B. Effect of pH. C. Effect of substrate (N-benzoyl leucomethylene blue) concentration. 2.5% polyacrylic acid was added to the detection zones. The images in C represent the data points for the PADs.

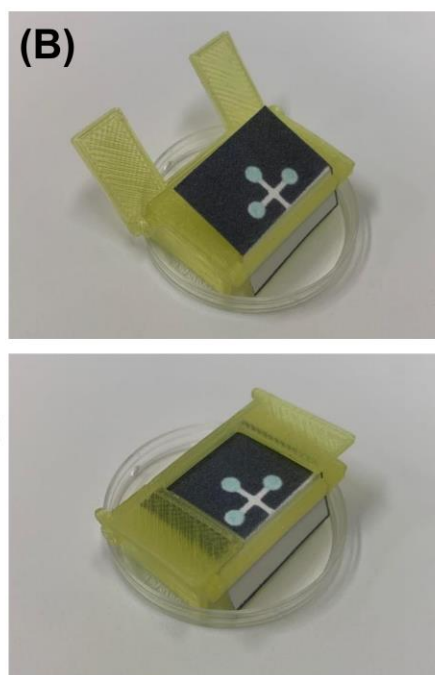
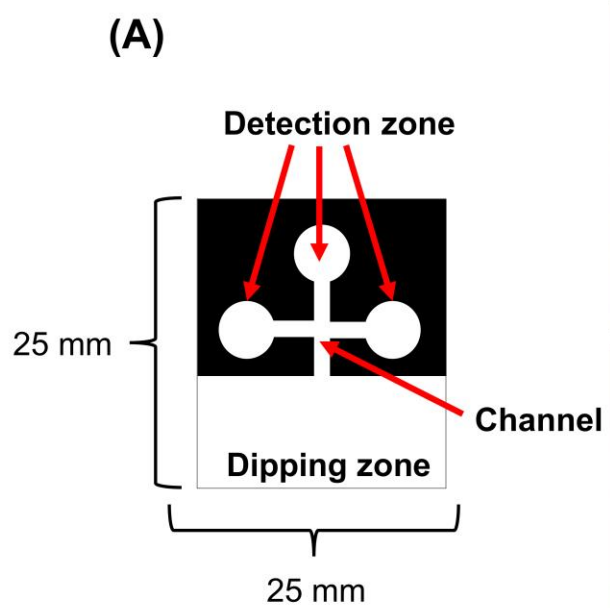


Figure 1

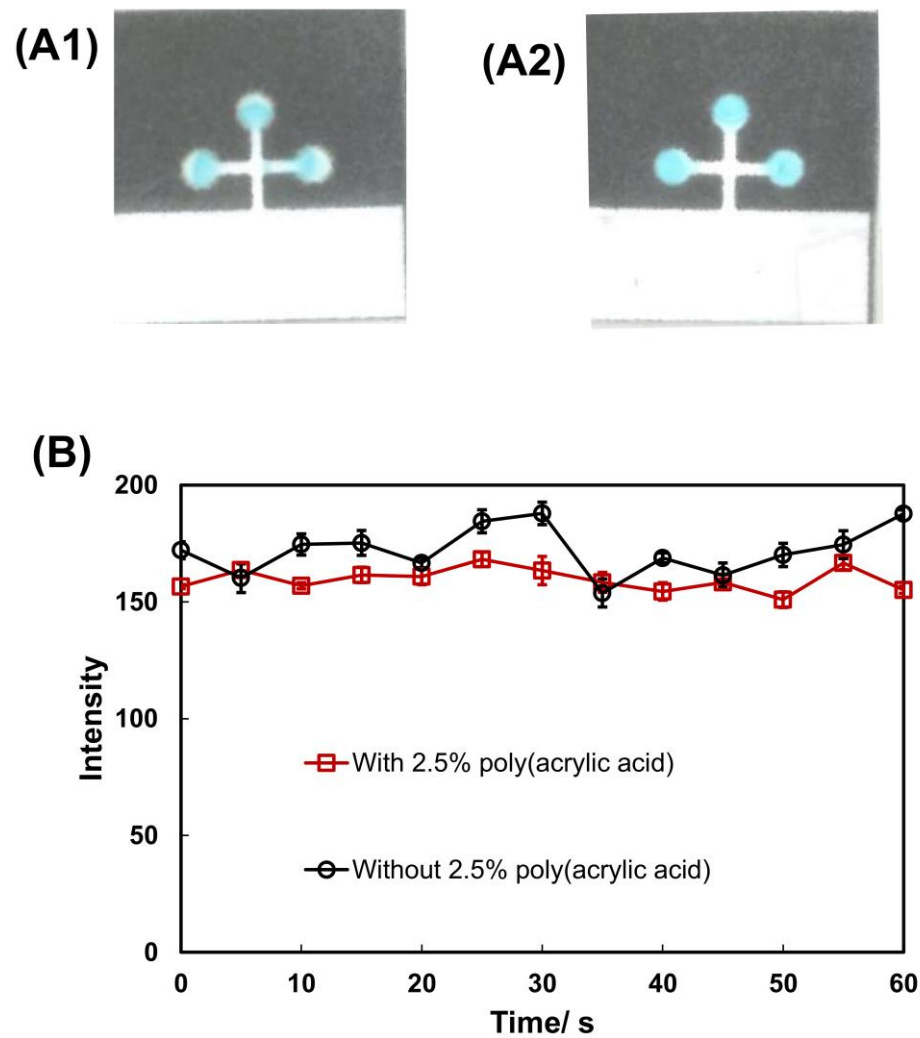


Figure 2

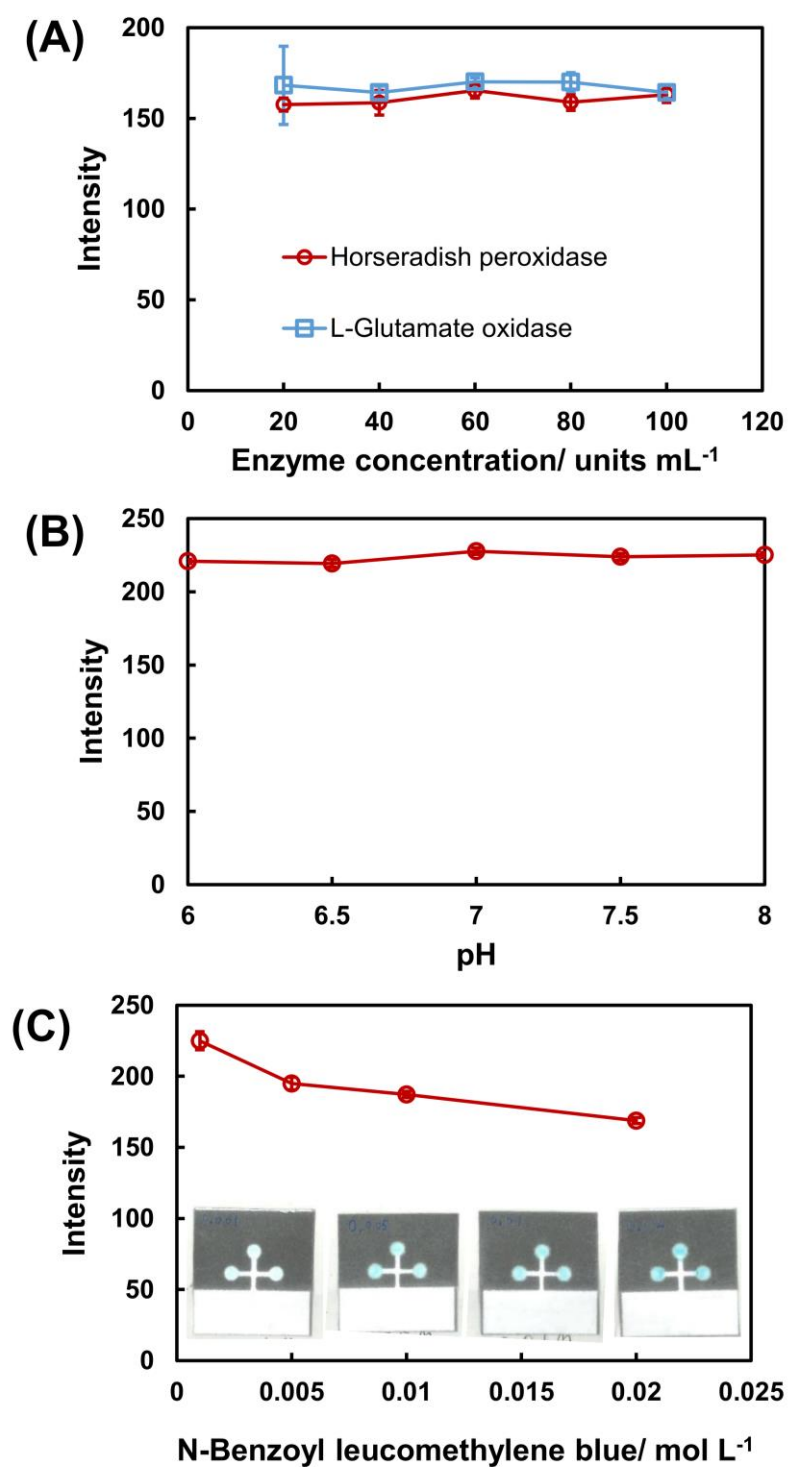


Figure 3