



Hybrid nanoflower-incorporated foldable paper biosensor for colorimetric detection of sodium benzoate based on inhibition of D-amino acid oxidase

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ABSTRACT

Sodium benzoate (SB), a commonly used food preservative, has raised increasing health concerns, prompting the need for its rapid, portable, and reliable on-site detection methods. Herein, we report an efficient paper-based foldable microfluidic biosensor for colorimetric SB detection based on SB's ability to inhibit D-amino acid oxidase (DAAO). SB in the sample solution inhibited DAAO action, thereby suppressing the oxidation of D-serine at a neutral pH and reducing H₂O₂ production. This reduction was monitored by observing a reduced blue color resulting from the oxidation of 3,3',5,5'-tetramethylbenzidine substrate at acidic pH, which was facilitated by a peroxidase reaction using hybrid nanoflower (NF) comprising horseradish peroxidase (HRP) and Cu₃(PO₄)₂ crystals (HRP-Cu NFs). The two-pH cascade reaction-based sensing strategy was integrated into a foldable paper-based biosensor. The paper device allowed SB-suppressed serine oxidation by DAAO at a neutral pH on the first part. After simple folding, the freshly produced H₂O₂ was transferred to the second part of the device, where the HRP-Cu NFs were immobilized at acidic pH, yielding a peroxidase-mediated catalytic reaction to generate a blue product. The paper biosensor showed excellent analytical performances with a limit of detection of 2.3 μM, which is sufficient to diagnose the cut-off level of SB in foods. Moreover, the practical applicability of the biosensor was successfully validated by determining SB levels in food samples with high accuracy and reproducibility. The foldable architecture, dual-pH compatibility, and enzyme-Cu NF synergy demonstrated here offer a generalizable strategy for developing portable, low-cost biosensors for food safety and point-of-care applications.

1. Introduction

Sodium benzoate (SB) is a common food preservative that effectively inhibits the growth of a wide range of bacteria and viruses [1]. It is widely used in soft drinks, fruit juices, pickles, condiments, and a range of baked foods [2,3]. Despite being generally regarded as safe for human consumption, excessive SB intake has been linked to serious health concerns, such as allergic reactions, asthma, and hyperactivity in children [4,5]. Furthermore, SB can react with ascorbic acid (vitamin C) in acidic environments to produce benzene, a recognized carcinogen [6,7]. These risks highlight the importance of accurately detecting and controlling SB levels in food to ensure consumer safety. According to the Food and Drug Administration, the maximum allowable concentration of SB in foods is 0.1 wt% [8,9]; making reliable, selective, and sensitive monitoring of SB essential for public health.

Conventional analytical methods such as mass spectrometry, gas

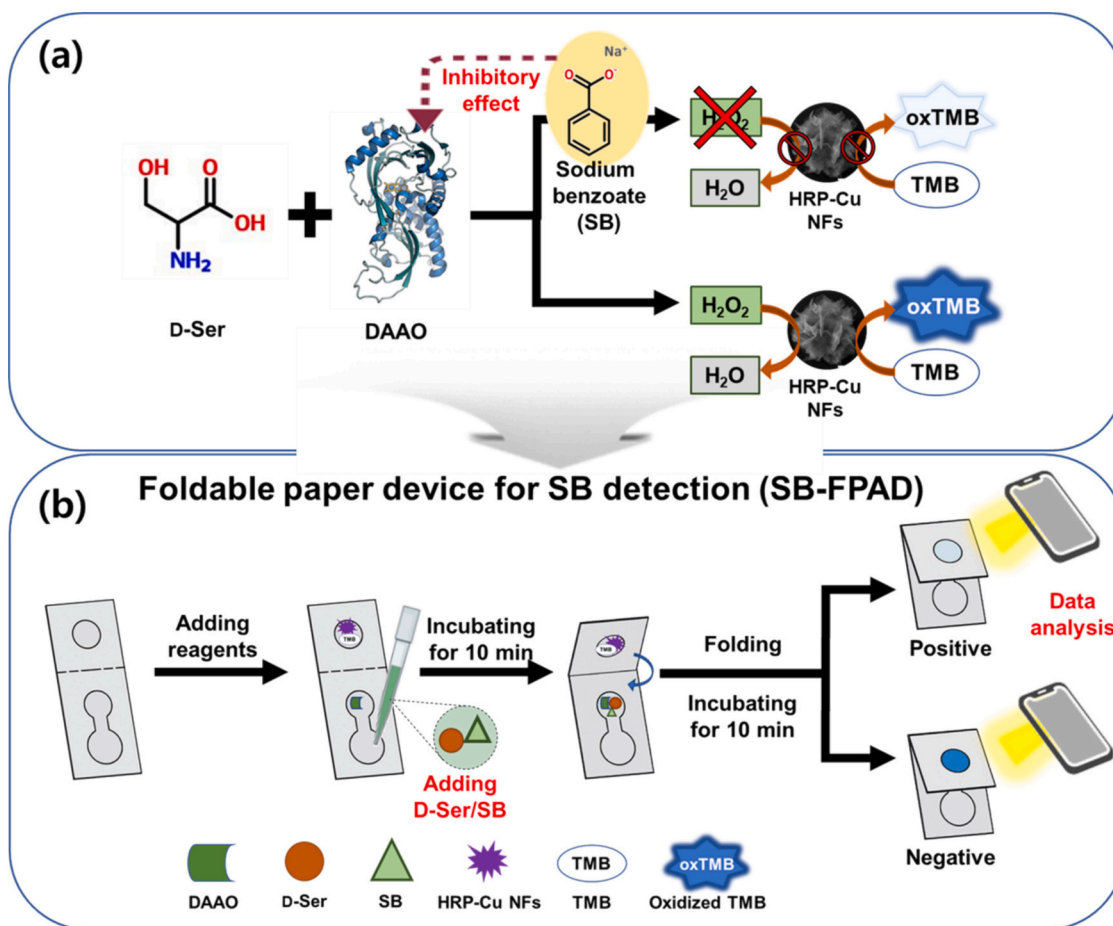
chromatography, and high-performance liquid chromatography offer high sensitivity and precision for SB determination. However, they are often expensive, time-consuming, and require complex sample pre-treatment as well as skilled personnel [10–12]. These constraints limit their suitability for real-time food analysis, particularly in point-of-care testing (POCT) scenarios where rapid, low-cost, and portable solutions are required [13]. Consequently, there is a strong demand for simple, affordable, and field-deployable SB detection platforms that retain analytical accuracy while enabling on-site operation.

A key issue in realizing a convenient method for SB detection that is applicable in the field is the selection of a proper signal mode that can be easily identified without the need for complex detection equipment. Colorimetric biosensors meet this requirement because they provide visual output that is detected with the naked eye. To construct colorimetric biosensors, enzyme catalysis-based strategies, including a cascade reaction system comprising oxidases and peroxidases, have

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Scheme 1. Schematic illustration for SB detection. (a) Colorimetric detection of SB based on its inhibitory effect on DAAO and (b) construction and operation of SB-FPAD for on-site SB detection.

been widely employed based on the versatility and high turnover rate of enzymatic catalysis. However, enzyme-based methods have several limitations, including the intrinsic instability of proteins during long-term operation or storage [14,15]. In recent years, flower-shaped hybrid enzyme-inorganic nanostructures, known as nanoflowers (NFs), have emerged as an effective strategy to address this issue. Owing to their large surface area, mesoporous structure, and strong enzyme-matrix interactions, NFs offer high enzyme loading capacity, activity retention, and operational stability [16,17]. Several colorimetric biosensors based on hybrid NF-entrapping enzymes have been reported for the detection of H₂O₂, glucose, and cholesterol via enzymatic catalysis [18–20]. Even though hybrid NFs have shown promise in developing colorimetric biosensors, more research is necessary to fully implement their use in real-world settings. This can be achieved by applying hybrid NFs to construct biosensing devices to detect important target molecules, such as SB, without the use of sophisticated assay equipment.

In this study, we developed a paper-based colorimetric SB biosensing device based on SB's inhibitory effects on D-amino acid oxidase (DAAO). Horseradish peroxidase-copper nanoflowers (HRP-Cu NFs) were employed instead of native HRP because the hybrid nanostructures exhibited higher catalytic efficiency, greater pH and thermal stability, and superior storage durability, owing to the synergistic effect between HRP and the Cu₃(PO₄)₂ matrix [21,22]. These properties make HRP-Cu NFs particularly suitable for integration into portable biosensing devices requiring long-term stability. SB inhibits DAAO action, while DAAO mediates the oxidation of D-amino acids to produce H₂O₂. This induces the oxidation of chromogenic substrates, such as 3,3',5,5'-tetramethylbenzidine (TMB), to produce colored products by peroxidase-

mediated catalytic reactions. Consequently, the amount of SB is negatively correlated with the color intensity produced by the cascade reaction (Scheme 1a). The potential of colorimetric SB detection based on its inhibitory effect on DAAO action has been reported [23]; however, the main barrier to its utilization is the limited stability and difference in the operating pH values of DAAO and HRP-Cu NFs or HRP-like nanozymes. In fact, DAAO catalyzes the oxidation of D-amino acids at neutral pH, whereas the decomposition of H₂O₂ by HRP-Cu NFs requires acidic pH (near pH 4). Without compromising the activity of any of its constituent components, this study's designed foldable paper-based microfluidic system combined HRP-Cu NF-mediated TMB oxidation and target SB-suppressed DAAO oxidation at different working pH values into a single portable device. This resolved the aforementioned limitations and facilitated the construction of an appropriate SB-detecting biosensor in POCT environments. After 20 min of incubation at room temperature (RT), with a simple folding action to transfer H₂O₂ from the reaction zone to the detection zone, successful analytical results were achieved. These results could be seen with the naked eye and quantified using a smartphone with high selectivity, sensitivity, and detection precision (Scheme 1b). Unlike conventional enzyme or nanozyme-based colorimetric sensors, the present work integrates a DAAO/HRP-Cu NF cascade within a foldable paper device that spatially separates reactions under dual pH conditions. This design enables controlled reaction coupling, improved stability, and selective detection of SB via enzyme inhibition - a unique mechanism rarely exploited in paper-based colorimetric sensing.

2. Experimental section

2.1. Cascade reaction comprising DAAO and HRP-Cu NFs for SB detection

Experimental details for the synthesis and characterization of HRP-Cu NFs [24] were described in the Supplementary material. Catalytic activity of HRP-Cu NFs was examined by performing a reaction using TMB as a chromogenic substrate in the presence of H_2O_2 . In brief, 100 μL of HRP-Cu NFs (100 $\mu\text{g}/\text{mL}$) were added to 900 μL of sodium acetate buffer (10 mM, pH 4) including H_2O_2 (10 mM) and TMB (1 mM), followed by incubation for 10 min at RT. After the reaction, the supernatant was separated by centrifugation (10,000g for 2 min) and used to analyze the blue color output with the naked eye and a microplate reader (Synergy H1, BioTek, Winooski, VT, USA). Steady-state kinetics

of HRP-Cu NFs were evaluated using TMB and H_2O_2 as substrates in acetate buffer (10 mM, pH 4.0) at room temperature. Reactions were initiated by adding HRP-Cu NFs (20 $\mu\text{g}/\text{mL}$), and the absorbance change at 652 nm was recorded over time. The apparent K_m and V_{max} values were determined by varying one substrate concentration while keeping the other constant at a saturating level: TMB (0.1–2.5 mM) at fixed H_2O_2 (10 mM), and H_2O_2 (0.1–12 mM) at fixed TMB (1 mM). The kinetic parameters were calculated using the Michaelis–Menten equation: $\nu = V_{max} \times [S]/(K_m + [S])$, where ν is the initial velocity and $[S]$ is the concentration of substrate.

The cascade reaction comprising the DAAO-mediated oxidation of the employed D-serine (D-Ser) and HRP-Cu NF-mediated reduction for producing H_2O_2 was carried out as follows. Briefly, 100 μL of DAAO (6 U/mL) was added to 100 μL of phosphate-buffered saline (PBS; 10 mM, pH 7) containing different concentrations of D-Ser, followed by

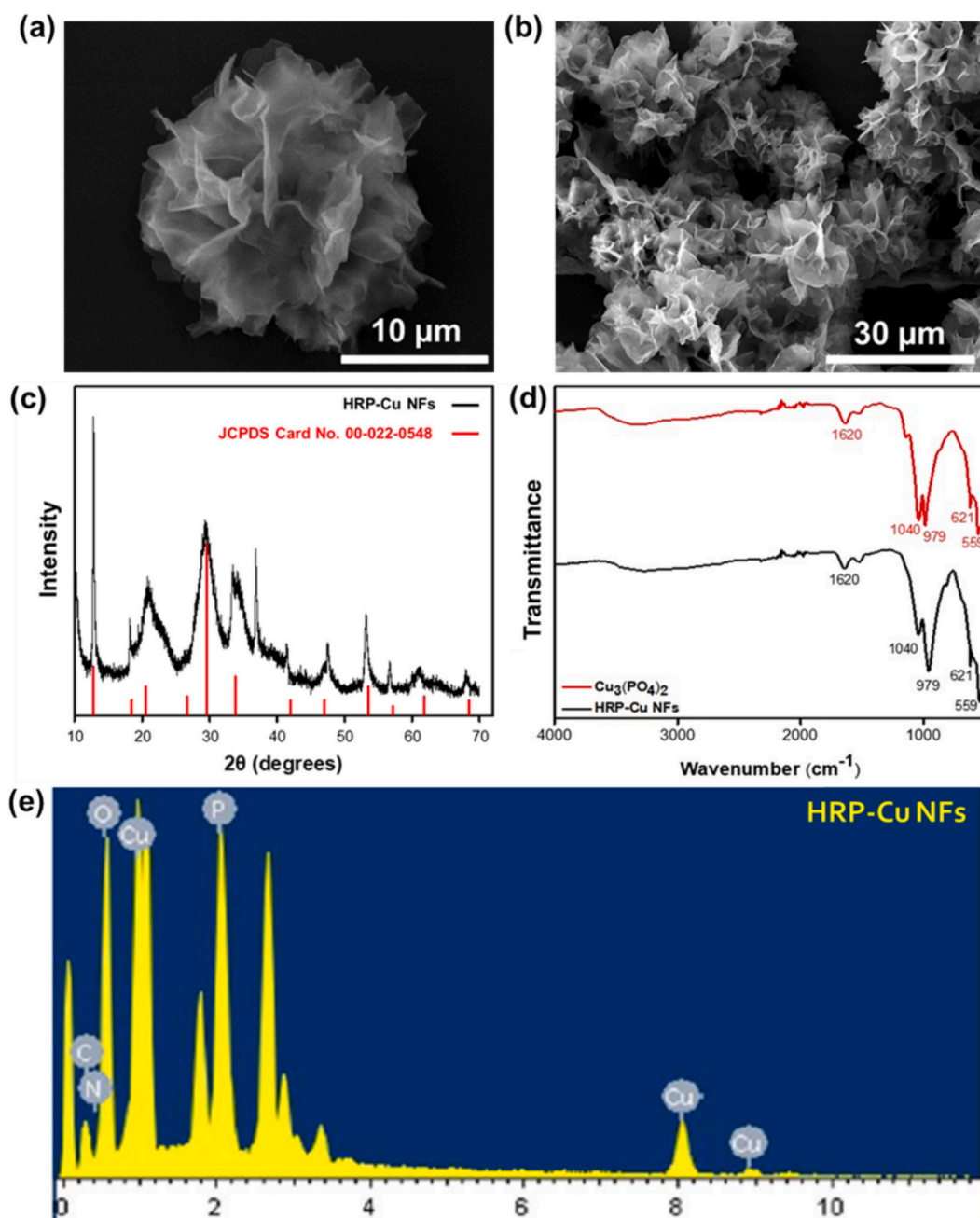


Fig. 1. Characterization of HRP-Cu NFs. (a, b) SEM images of HRP-Cu NFs, (c) XRD spectra of HRP-Cu NFs with standard peaks corresponding to crystalline $\text{Cu}_3(\text{PO}_4)_2$ (JCPDS card No. 00–022–0548), (d) FT-IR spectra of HRP-Cu NFs and crystalline $\text{Cu}_3(\text{PO}_4)_2$, and (e) EDS analysis of HRP-Cu NFs.

incubation for 15 min at RT. Subsequently, the mixture was supplied with 800 μL of sodium acetate buffer (10 mM, pH 4), HRP-Cu NFs (20 $\mu\text{g}/\text{mL}$), and TMB (1 mM) and was incubated for an additional 10 min at RT. After the reaction, the absorbance of the supernatant was monitored in the scanning mode or at 652 nm using a microplate reader.

SB was detected colorimetrically based on its inhibitory effect on DAAO activity. Briefly, 100 μL of sample solution containing SB at various concentrations was added to 100 μL of PBS (10 mM, pH 7.0) containing DAAO (6 U/mL) and D-Ser (30 mM), followed by incubation for 15 min at RT. Subsequently, the mixture was supplied with 800 μL of sodium acetate buffer (10 mM, pH 4), HRP-Cu NFs (20 $\mu\text{g}/\text{mL}$), and TMB (1 mM), and was then analyzed using the same method as described above. The calibration curve was constructed using the logarithm of SB concentration ($\log[\text{SB}]$) versus the colorimetric signal. The limit of detection (LOD) was first obtained in log units using $\text{LOD}_{\log[\text{SB}]} = 3 \times \sigma/S$, where σ is the standard deviation of the blank and S is the slope of the calibration curve. The final LOD in concentration units was then calculated as $\text{LOD}_{[\text{SB}]} = 10^{\text{LOD}_{\log[\text{SB}]}}$. The blank sample used for LOD determination contained all assay components except SB to represent the uninhibited system.

2.2. Foldable paper-based microfluidic device for SB detection

A wax-printing technique was used to construct a foldable paper-based microfluidic device for SB detection (SB-FPAD). The device was created with AutoCAD 2018 and printed using a wax printer (ColorQube 8570DN; Xerox, Japan) on Whatman chromatography paper (Grade 1). The printed SB-FPADs were baked in a hot oven at 120 $^{\circ}\text{C}$ for 90 s to create the hydrophobic barriers with a 1-mm in-line width, which allowed the wax to melt and permeate through the paper. Previously, device characteristics like baking time and temperature were optimized [25].

The SB-FPAD had a rectangular shape (20 mm $\times$ 50 mm) and was

divided into two parts separated by a folding line. The first part of the device contained two circular zones (sample zone and reaction zone), connected to each other by a 4-mm channel, in which D-Ser and DAAO were pre-immobilized, respectively. The second part of the device contained an 8-mm diameter circular zone at the center, where HRP-Cu NFs and TMB were pre-immobilized, as illustrated in Fig. S1. To construct the SB-FPAD, D-Ser (150 mM dissolved in 10 μL deionized (DI) water) and DAAO (8 U/mL dissolved in 10 μL PBS [10 mM, pH 7.0]) were added to the sample and reaction zone, respectively, followed by air drying. On the upper pad, HRP-Cu NFs (1 mg/mL, 6 μL), TMB (10 mM, 3 μL), and sodium acetate buffer (10 mM, 6 μL , pH 4) were consecutively dropped on the detection zone, thereby allowing them to air dry.

2.3. Colorimetric SB detection using SB-FPAD

The solutions containing SB at different concentrations (10 μL) were introduced to the sample zone in order to colorimetrically detect SB in a sample solution. This transferred all reactants in the sample zone to the reaction zone via capillary force and induced the SB-suppressed oxidation process for 10 min between D-Ser and pre-immobilized DAAO, producing H_2O_2 . The freshly generated H_2O_2 solution was then transferred to the upper part's detection zone by folding the paper device through the dashed line, which allowed contact between the two portions. After 10 min, the transferred H_2O_2 induced a catalytic reaction with the HRP-Cu NFs to oxidize TMB into a blue-colored product. The color output was easily observed with the naked eye and quantified using images acquired with a smartphone (Galaxy S20 Ultra, Samsung, Korea), followed by image processing using ImageJ software (NIH) with the optional mode "RGB-to-CMYK".

Orange juice and pickle samples were obtained from a local market to evaluate the applicability of SB detection in food samples. The orange juice was first centrifuged (8000 $\times g$ for 5 min) to remove pulp and larger particles and was then filtered through a 0.22 μm syringe filter to

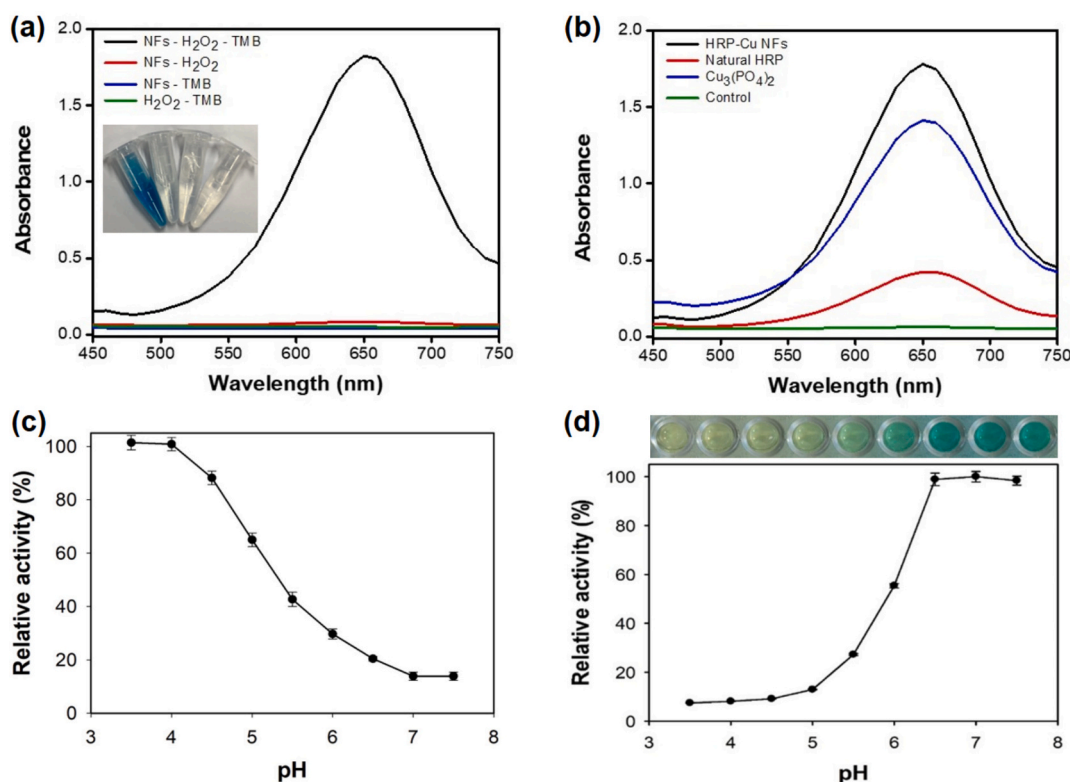


Fig. 2. Catalytic activity of HRP-Cu NFs. (a) Absorption spectra of HRP-Cu NFs-mediated color-generating reactions by oxidizing TMB with H_2O_2 and their corresponding images. (b) Comparison of peroxidase activity of HRP-Cu NFs with those of controls (natural HRP and $\text{Cu}_3(\text{PO}_4)_2$ precipitates). Effects of pH on the (c) peroxidase activity of HRP-Cu NFs and (d) DAAO activity to oxidize D-Ser.

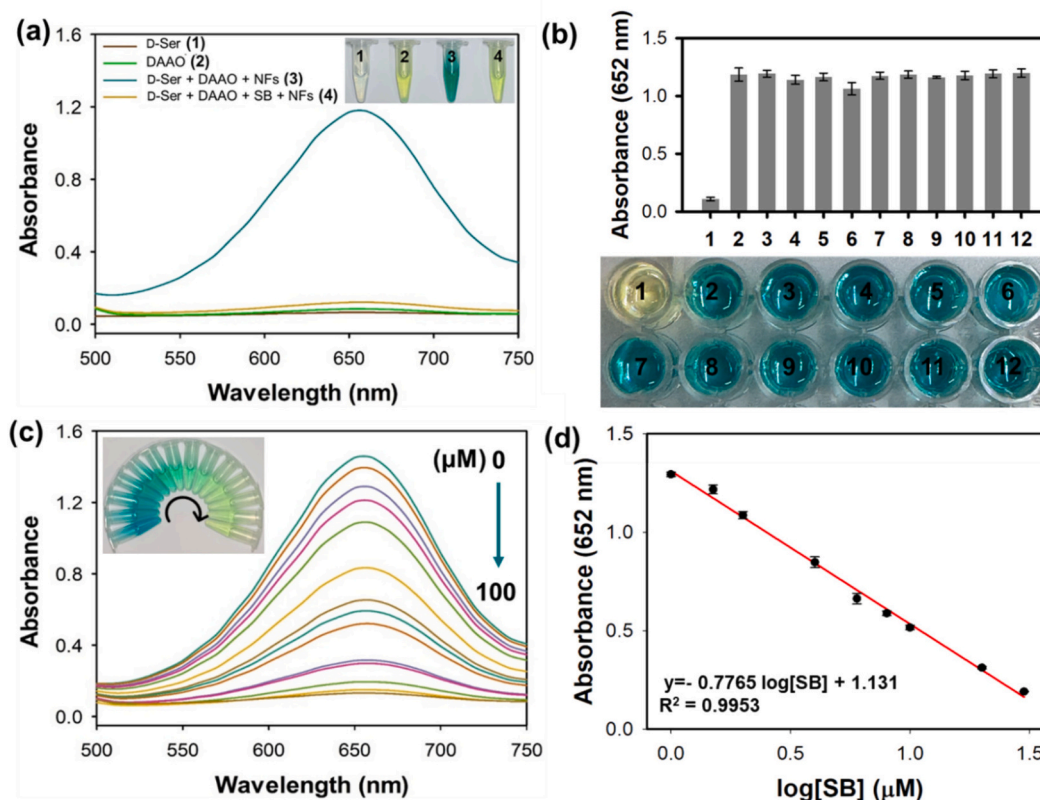


Fig. 3. Colorimetric detection of SB via cascade reaction. (a) Absorption spectra of the cascade reaction comprising DAAO and HRP-Cu NFs with or without SB. (b) Selectivity of solution-based colorimetric detection of SB using a cascade reaction composed of DAAO and HRP-Cu NFs. The labeled numbers (1–12) correspond to SB, sodium chloride, sodium propionate, sodium salicylate, sodium saccharin, potassium sorbate, methylparaben, glucose, Ca^{2+} , Mg^{2+} , NO_3^- , and blank, respectively. In the assay, the concentration of SB is 100 μM whereas that of all other interfering species is 1 mM each. (c) Photographs, absorption spectra, and (d) linear calibration plots for the solution-based colorimetric detection of SB.

eliminate any remaining solids. The resulting filtrate was diluted 100-fold in PBS (10 mM, pH 7). The pickle was initially homogenized using a blender, followed by centrifugation (8000 $\times g$ for 5 min) and filtration through a 0.22 μm filter to collect the clear supernatant. The supernatant was diluted 100-fold in PBS (10 mM, pH 7). The original SB concentrations in both samples were determined using the SB-FPAD system. The samples were then spiked with SB at pre-determined concentrations of 5, 15, and 30 μM . The same conditions as in the previously described techniques were used for further SB detections employing the paper device. To confirm the accuracy and reproducibility of SB detection in food samples using both paper devices and solution-based assays, the recovery rate (recovery [%] = measured amount / predicted amount $\times$ 100) and coefficient of variation (CV [%] = standard deviation (SD) / average $\times$ 100) were calculated.

3. Results and discussion

3.1. Synthesis, characterization, and catalytic activity of HRP-Cu NFs

SB-FPAD is composed of two catalytic units, DAAO and HRP-Cu NFs; the former catalyzes the SB-suppressed oxidation of D-Ser to produce H_2O_2 at neutral pH conditions, and the latter catalyzes the oxidation of TMB to produce a blue color proportional to the amount of transferred H_2O_2 via folding action at acidic pH conditions. As an important catalytic component of SB-FPAD, the HRP-Cu NFs were prepared by incubating CuSO_4 in PBS (pH 7.4) containing HRP. After incubation for three days at RT, the HRP molecules were efficiently entrapped within the NFs, with an encapsulation efficiency of $\sim 90\%$, as determined by the bicinchoninic acid assay. Porous, flower-like morphologies comprising

many $\text{Cu}_3(\text{PO}_4)_2$ nanopetals were observed with a diameter of $\sim 30\ \mu\text{m}$ (Fig. 1a, b). The XRD spectra of the HRP-Cu NFs showed that their crystalline structures matched well with those of $\text{Cu}_3(\text{PO}_4)_2$ [JCPDS no. 22–0548] (Fig. 1c) [26]. It confirmed the formation of crystalline $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets that create a porous, high-surface-area framework. The FT-IR spectra of the HRP-Cu NFs and $\text{Cu}_3(\text{PO}_4)_2$ precipitates revealed strong peaks at 979 and 1040 cm^{-1} , which correspond to P–O and P=O vibrations, respectively, thereby suggesting the presence of phosphate groups. The peaks at 559 and 621 cm^{-1} observed in both samples demonstrate metal-oxygen bonding, indicating the existence of Cu–O bonds (Fig. 1d) [27]. The slight peak broadening of the HRP-Cu NFs compared with $\text{Cu}_3(\text{PO}_4)_2$ precipitates indicates the coordination between Cu^{2+} ion and HRP's functional groups. EDS analysis showed that C, O, P, Cu, and N were present on the NFs with calculated atomic percentages of 21.0, 28.6, 3.1, 45.0, and 2.3 %, respectively, presumably from the $\text{Cu}_3(\text{PO}_4)_2$ crystals and HRP molecules within the NFs (Fig. 1e, Fig. S2a). XPS analysis further verified the presence of C, O, N, P, and Cu. The survey spectrum of HRP-Cu NFs exhibited a distinct N 1 s signal at $\sim 400\ \text{eV}$ - absent in the $\text{Cu}_3(\text{PO}_4)_2$ control - verifying the successful incorporation of HRP within the hybrid structure (Fig. S2b). High-resolution N 1 s spectra showed two peaks at 398.8 and 401.2 eV, corresponding to free amine groups and Cu–N coordination, respectively, indicating chemical bonding between HRP and Cu ions [28,29]. The Cu 2p spectrum displayed features characteristic of mixed $\text{Cu}^+/\text{Cu}^{2+}$ states (Fig. S2c, d) [30], suggesting a heterogeneous coordination environment that enhances electron transfer during catalysis. These characterizations confirmed the effective integration of HRP with crystalline $\text{Cu}_3(\text{PO}_4)_2$ within the HRP-Cu NFs.

The HRP-Cu NFs showed the activity to catalyze the oxidation of

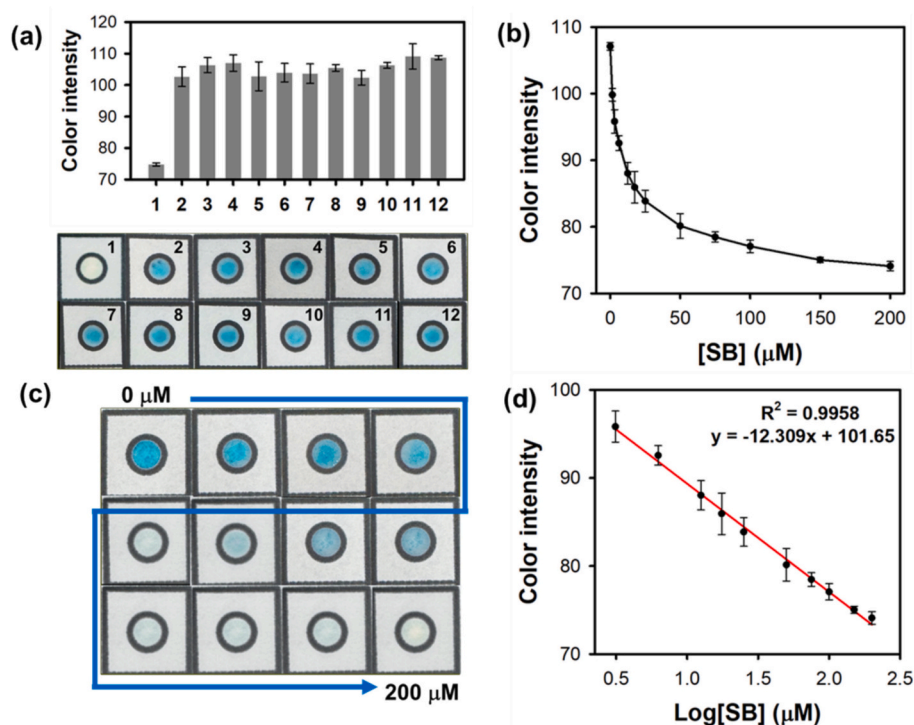


Fig. 4. Colorimetric SB detection using SB-FPAD. (a) Selectivity of colorimetric detection of SB using SB-FPAD. The labeled numbers (1–12) correspond to SB, sodium chloride, sodium propionate, sodium salicylate, sodium saccharin, potassium sorbate, methylparaben, glucose, Ca^{2+} , Mg^{2+} , NO_3^- , and blank, respectively. In the assay, the concentration of SB is 100 μM whereas that of all other interfering species is 1 mM each. (b) Dose-response curves, (c) photographs, and (d) linear calibration plots for the SB-FPAD-based colorimetric detection of SB.

TMB only in the presence of H_2O_2 , resulting in a blue color with a maximum absorption peak at approximately 652 nm (Fig. 2a). As expected, the reaction systems that included either NFs with TMB or NFs with H_2O_2 produced a faint blue color. The catalytic activity of the HRP-Cu NFs was higher than those of the individual components, including the HRP and $\text{Cu}_3(\text{PO}_4)_2$ precipitates (Fig. 2b). This improvement is likely a result of the synergistic effects of the $\text{Cu}_3(\text{PO}_4)_2$ petals, which may serve as peroxidase mimics [27]. Moreover, the entrapped HRP within the NFs, along with their large surface area and porous structure, facilitate efficient substrate transfer during the peroxidase reaction.

To further elucidate the catalytic mechanism of HRP-Cu NFs, steady-state kinetic studies were performed using TMB and H_2O_2 as substrates. The Michaelis–Menten plots (Fig. S3) yielded K_m values of 0.41 mM (TMB) and 1.03 mM (H_2O_2), and $V_{\max}$ values of 1.65×10^{-7} and $2.33 \times 10^{-7} \text{ M}\cdot\text{s}^{-1}$, respectively. The K_m for TMB is comparable to that of natural HRP (0.43 mM), while the much lower K_m for H_2O_2 indicates enhanced affinity toward the oxidant. These results suggest that the $\text{Cu}_3(\text{PO}_4)_2$ nanosheet framework within the hybrid nanoflowers facilitates substrate diffusion and redox cycling between Cu species and HRP active centers. The obtained kinetic parameters are consistent with or superior to those of other hybrid nanoflowers and peroxidase-mimicking nanozymes, confirming the high catalytic efficiency of HRP-Cu NFs (Table S1). In addition, the HRP-Cu NFs exhibited markedly enhanced stability compared with that in the non-encapsulated free HRP in the tested pH and temperature ranges, which is advantageous for their application in real environments (Fig. S4).

Although the HRP-Cu NFs were active and stable peroxidase units, their ability to oxidize TMB was observed only under acidic conditions near pH 4 (Fig. 2c). When the reaction pH increased, especially under neutral pH conditions, the activity was almost lost, possibly because of the much higher negative charges at the surface of HRP (isoelectric point: 5.5) [31]. In addition, the DAAO activity to oxidize D-Ser was present under neutral and basic pH conditions, and under acidic pH conditions near pH 4, its activity was negligible (Fig. 2d). Thus, in a

cascade reaction system composed of DAAO and HRP-Cu NFs to detect the target SB, efficiently overcoming the mismatched operational pH conditions is key.

3.2. Solution-based colorimetric detection of SB using a cascade reaction comprising DAAO and HRP-Cu NFs

DAAO is an enzyme located in peroxisomes that facilitates the oxidative deamination of several neutral and basic D-amino acids, resulting in the formation of their corresponding α -keto acids and H_2O_2 production [32]. Considering that SB inhibits DAAO activity by competing with the employed D-Ser substrate [21], the target SB was colorimetrically detected via a two-step reaction. Using DAAO deployment, D-Ser was oxidized in the presence of SB in PBS (pH 7.0) in the first reaction. The produced H_2O_2 was then reduced by HRP-Cu NF with TMB oxidation in acetate buffer (pH 4.0) to produce a blue output that was inversely proportional to the amount of SB. The results showed distinct color differences among the experimental samples (Fig. 3a). The sample containing both D-Ser and DAAO displayed a vivid blue color. However, the addition of SB to the above mixture led to a noticeable reduction in color intensity, indicating reduced production of H_2O_2 and an inhibitory effect of SB on DAAO action. Prior to SB quantification, assay parameters, including DAAO concentration (6 U/mL) and reaction time (15 min), were optimized (Fig. S5). The cascade reaction intensity slightly decreased after 15 min, probably due to the unwanted reaction between the generated H_2O_2 and α -keto acids [33].

Under the optimized conditions, SB was selectively detected and indicated by a pronounced reduction in blue color intensity, whereas other substances, such as sodium chloride, sodium propionate, sodium salicylate, sodium saccharin, potassium sorbate, methylparaben, glucose, Ca^{2+} , Mg^{2+} , and NO_3^- , produced a pronounced blue color, even though they were used at 10-fold higher concentrations (Fig. 3b). By varying the SB concentrations (0.5–100 μM), the color intensities correspondingly decreased and were inversely proportional to target SB

levels (Fig. 3c). A linear calibration plot between the logarithm of SB concentration and the absorption intensity measured at 652 nm revealed that the LOD value was as low as 0.5 μM with a dynamic linear range of 1.5–30 μM ($R^2 = 0.9953$) (Fig. 3d). These values rank among the best outcomes documented for colorimetric SB detection [21,34,35] (Table S2).

3.3. Colorimetric SB detection using SB-FPAD

We devised and built an SB-FPAD in order to demonstrate a useful implementation of the cascade reaction-based system for colorimetric SB detection, specifically in POCT situations. In order to guarantee ideal reaction circumstances and the easy transfer of H_2O_2 via folding, this device allowed two sequential reactions to occur under different pH conditions. Hydrophobic wax was printed onto chromatography paper and then melted on a hot plate to create the SB-FPAD. Both the baking temperature (120 $^\circ\text{C}$) and time (90 s) were optimized to prevent leakage of the aqueous solution (Fig. S6, S7). As depicted in Scheme 1b, the lower part of the device is intended to oxidize D-Ser via SB-suppressed DAAO action at a neutral pH to produce H_2O_2 . The H_2O_2 is the primary substrate for HRP-Cu NFs to oxidize TMB to produce a blue color at an acidic pH in the upper part of the device's detection zone. The SB-suppressed oxidation of D-Ser by immobilized DAAO continued when the target SB was present in the sample solutions, thereby producing an H_2O_2 solution within the reaction zone of the lower part. The H_2O_2 solution was then transferred to the hydrophilic detection zone of the device via folding. In less than 10 min, the transferred solution spread to the detecting zone and interacted with the immobilized HRP-Cu NFs and TMB to produce a blue color. Color development in the detection zone was readily observed with the naked eye and quantified by capturing an image, followed by image processing using a smartphone.

The optimized conditions for SB detection using SB-FPAD are shown in Fig. S8. To optimize the colorimetric signal from the cascade reaction on the paper device, the concentrations of the immobilized DAAO and D-Ser were adjusted to 8 U/mL and 150 mM, respectively. The ideal incubation time for both the DAAO-mediated oxidation and HRP-Cu NF-induced reduction was 10 min. Under the optimized conditions, high selectivity toward SB was observed by detecting only a pale greenish color, which originated from the native color of the immobilized HRP-Cu NFs in the SB-containing solution, whereas several interfering species (sodium chloride, sodium propionate, sodium salicylate, sodium saccharin, potassium sorbate, methylparaben, glucose, Ca^{2+} , Mg^{2+} , and NO_3^-) displayed an unsuppressed intense blue color, similar to that of the control buffer (Fig. 4a). The paper devices' detection zones progressively transitioned from intense blue to weak greenish as the concentration of SB rose, which corresponded to the evolution of SB from 0 to 200 μM (Fig. 4b, c). SB was detected with good linearity ($R^2 = 0.9958$) at a concentration gradient of 3.0–200 μM (Fig. 4d). Additionally, the LOD of SB-FPAD (2.3 μM) is among the lowest recorded LODs in colorimetric SB sensing investigations (Table S2). This work represents the first on-device colorimetric system that successfully overcomes the dual-pH incompatibility between DAAO- and HRP-based reactions through a foldable dual-zone configuration. Furthermore, its simple, instrument-free visual readout and foldable design make it particularly suitable for rapid on-site screening of food preservatives. Importantly, the generated color intensity has no significant change after prolonging the reaction time (Fig. S9).

3.4. Practical application of SB-FPAD to detect SB in food samples

A crucial feature of the POCT paper device is its long-term storage durability. According to the experimental findings, the SB-FPAD was able to sustain its initial SB-detecting activity over 80 % for a month under all storage conditions ($-20\text{ }^\circ\text{C}$ and $4\text{ }^\circ\text{C}$) (Fig. S10). The relative color intensity of paper devices stored at $-20\text{ }^\circ\text{C}$ almost showed no notable loss. This may have been a result of the favorable storage

Table 1

Detection accuracy and precision of the solution-based assay and SB-FPAD for detecting SB in spiked food samples. Notes: (a) Mean of five independent measurements ($n = 5$) performed under identical assay conditions. (b) Standard deviation (SD) calculated from five measurements to assess reproducibility. (c) Recovery (%) indicating the accuracy of the method in real-sample matrices. (d) Coefficient of variation (CV, %) representing the relative precision of the method.

Sample	Added (μM)	SB detected ^a $\pm$ SD ^b (μM)	Recovery ^c (%)	CV ^d (%)	
Orange juice	5	Solution-based assay	5.22 $\pm$ 0.09	104.40	1.72
		SB-FPAD	4.88 $\pm$ 0.24	97.60	4.92
	15	Solution-based assay	15.09 $\pm$ 0.36	100.60	2.39
		SB-FPAD	14.95 $\pm$ 0.67	99.67	4.48
	30	Solution-based assay	30.17 $\pm$ 0.08	100.57	0.27
		SB-FPAD	31.11 $\pm$ 0.23	103.70	0.74
Pickle	5	Solution-based assay	5.16 $\pm$ 0.14	103.20	2.71
		SB-FPAD	4.93 $\pm$ 0.27	98.60	5.48
	15	Solution-based assay	15.07 $\pm$ 0.44	100.47	2.92
		SB-FPAD	14.82 $\pm$ 0.63	98.80	4.25
	30	Solution-based assay	29.65 $\pm$ 0.49	98.83	1.65
		SB-FPAD	30.68 $\pm$ 0.36	102.27	1.17

^a Average value of five independent measurements.

^b Standard deviation of five independent measurements.

^c Recovery = (detected value/spiked value) $\times$ 100.

^d Coefficient of variation = (SD/average) $\times$ 100.

circumstances that maintained the DAAO molecules' reactivity. The paper device's marginally reduced performance at $4\text{ }^\circ\text{C}$ might be due to the loss of DAAO enzymatic activity. Overall, SB-FPAD demonstrated excellent stability under all storage conditions; the device is best kept at $-20\text{ }^\circ\text{C}$ to preserve its biosensing activity for roughly a month without suffering any appreciable loss.

Finally, we assessed the practical applicability of SB-FPAD for quantitatively determining diverse SB concentrations in actual food samples. First, we used our method to determine the baseline SB levels in diluted pickle and orange juice samples and found that immaterial amounts were present. Subsequently, pre-determined SB concentrations (5, 15, and 30 μM) were added to produce spiked samples that covered a broad spectrum of SB values. Both the solution-based assay and SB-FPAD results showed that all SB concentrations in various food matrices were measured with high precision, with CVs below 5.5 % (Table 1). Additionally, the recovery rates ranged from 97.6 % to 104.4 %, thereby confirming the remarkable reproducibility and reliability of this method. The accuracy of this SB detection strategy was further validated by analyzing unspiked orange juice and pickle samples and comparing them with results from conventional spectrophotometric method ($\lambda_{\text{max}} = 225\text{ nm}$) (Fig. S11, Table S3). The SB concentrations obtained from both methods were in close agreement, with relative differences $<3.72\%$, confirming the reliability of the proposed device for real-sample analysis.

4. Conclusions

We developed a colorimetric detection method and paper device for SB detection by utilizing HRP-Cu NFs as highly active and stable peroxidase units. The cascade reaction system selectively and sensitively detected SB based on SB's inhibitory effect on the catalytic activity of

DAAO. To effectively connect the neutral-pH-mediated DAAO and the acidic-pH-mediated HRP-Cu NFs, the two were integrated into a foldable paper microfluidic system. The foldable design of the paper device enables colorimetric detection of SB in POCT situations by utilizing two pH systems: a neutral condition for SB-suppressed DAAO oxidation and an acidic condition for H₂O₂ reduction and TMB oxidation. The device also precisely detected SB concentrations in food samples with high reproducibility and reliability. This simple, affordable, and portable device has great potential for broad use in food-safety monitoring, where accurate and timely detection is crucial.

CRedit authorship contribution statement

Thinh Viet Dang: Writing – original draft, Investigation, Conceptualization. **Kyung Mi Kim:** Validation, Investigation. **Quynh Huong Nguyen:** Investigation, Conceptualization. **Moon Il Kim:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2025.115979>.

Data availability

Data will be made available on request.

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